UNIVERSIDADE ESTADUAL PAULISTA “JULIO DE MESQUITA FILHO”
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

EFEITOS DA SUBSTITUIÇÃO DE SFB POR IGF-I SOBRE OS ASPECTOS CELULARES E MOLECULARES DA PRODUÇÃO *IN VITRO* DE EMBRIÕES BOVINOS

ELENA CAROLINA SERRANO RECALDE

Botucatu – SP
Maio/2018

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Tese apresentada ao Programa de Pós-Graduação em Biotecnologia Animal da Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista "Julho de Mesquita Filho" para a obtenção do título de Doutora em Biotecnologia Animal, área de Reprodução Animal.

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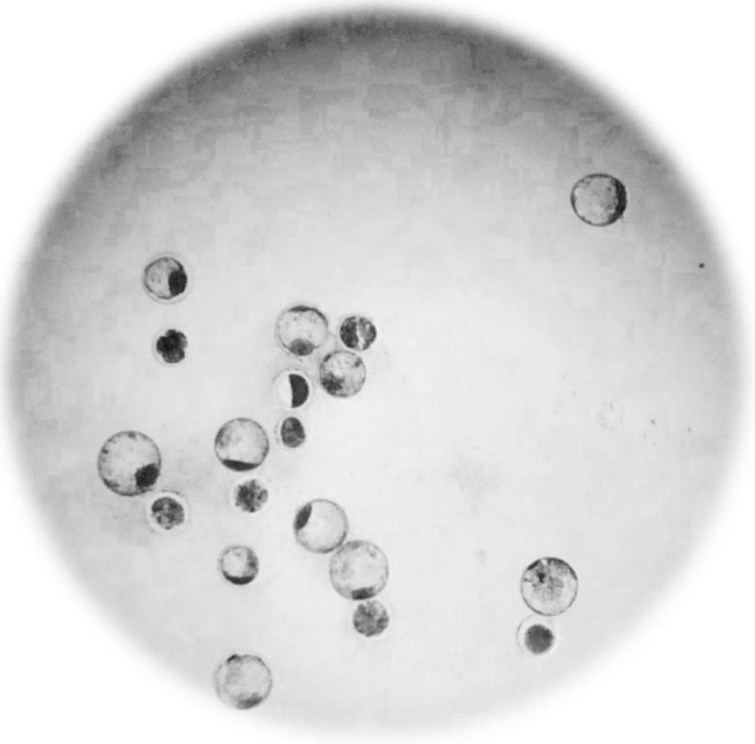

Dedico a Deus, por sempre me dar forças para continuar.

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LISTA DE ABREVIAÇÕES E SIGLAS

ANOVA	Análise de variância
AQP	Aquaporinas
BAD	Proteína promotora de morte associada à Blc-2
BME	Basal medium Eagles
CIV	Cultivo *in vitro*
COCs	Complexos cúmulus-oócito/Cumulus-oocyte complexes
FBS	Fetal bovine serum
FIV	Fertilização *in vitro*
FOXO	Fatores de transcrição "Forkhead"
GSK-3β	Glicogênio sintase quinase 3 β
ICM	Inner cell mass
IETS	Sociedade Internacional de Transferência de Embriões
IFN-τ	Interferon-tau
IGF-I	Fator de crescimento semelhante à insulina tipo I/ insulin-like growth factor I
IGF-IR	Receptor para fator de crescimento semelhante à insulina tipo I
IVC	*In vitro* culture
IVM	*In vitro* maturation
IVP	*In vitro* production
LH	Hormônio luteinizante
LOS	Large Offspring Syndrome
MAPK	Proteína quinase ativada por mitógenos/ mitogen activated protein kinase
MCI	Massa celular interna
MEM	Minimum essential medium
MIV	Maturação *in vitro*
mRNA	RNA mensageiro
mTOR	Proteína alvo da rapamicina em mamíferos
OPU	Aspiração folicular guiada por ultrassonografia
PI3K/AKT	Fosfatidilinositol-3-quinase/serina-treonina quinase/ phosphatidyl inositol-3 Kinase/serine-threonine protein kinase B
PIV	Produção *in vitro*

PVA	Polivinil álcool/ polyvinyl alcohol
RAF	Serina-treonina quinase/serine-threonine kinase
SFB	Soro fetal bovino
SOF	Fluido sintético de oviduto
TALP	Tyrode's albumin-lactate pyruvate
TCM	Tissue culture medium
TE	Trofoectoderma/ trophectoderm
TUNEL	Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling

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RESUMO

SERRANO-RECALDE, E. C. **Efeitos da substituição de SFB por IGF-I sobre os aspectos celulares e moleculares da produção *in vitro* de embriões bovinos.** Botucatu 2018, 121p. Tese (Doutorado em Biotecnologia Animal) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista "Júlio de Mesquita Filho" – UNESP.

O objetivo do presente estudo foi avaliar o benefício da substituição do soro fetal bovino (SFB) pelo fator de crescimento semelhante à insulina tipo I (IGF-1) durante a maturação *in vitro* (MIV) ou cultivo *in vitro* (CIV), sobre qualidade embrionária e expressão de genes em embriões pré- e pós-compactação. Delineamento experimental foi fatorial 3 x 3 (três suplementos de MIV e três de CIV), com 9 grupos experimentais. Foram realizadas 20 réplicas (oócitos ≈ 400/grupo). Complexos cúmulus-oócito (COCs) graus I e II foram maturados *in vitro* com a adição de 10% de SFB (SFB), ou 3 mg/mL polivinil-álcool (PVA), ou PVA + 100 ng/mL de IGF-1 (IGF) a 38,5 ºC em 5% de CO_2 em ar por 22 a 24 horas. Foram fertilizados e incubados durante 18 horas. Os zigotos foram cultivados com a adição de: 2,5% de SFB (SFB), ou 3 mg/mL de PVA (PVA), ou PVA + 100 ng/mL de IGF-1 (IGF) por sete dias a 38,5ºC em 5% de CO_2 em ar. As taxas de clivagem e blastocisto foram avaliadas após 48 e 168 horas de cultivo, respectivamente. A técnica simplificada de coloração diferencial de células da massa celular interna (MCI) e trofoectoderma (TE) foi utilizada para avaliar a distribuição celular de blastocistos (n = 155) e a técnica de TUNEL para avaliação do índice de apoptose (n = 207). Concentrações de glicose e lactato obtidas do meio MIV utilizaram-se para analisar o metabolismo de glicose. mRNA foi extraído de embriões de 6 – 8 células colhidos após 66 horas post-inseminação (4 pools de 15 embriões por grupo) e de blastocistos expandidos de 7 dias (4 pools de 5 embriões por grupo). A expressão gênica foi realizada no sistema BioMark HD® de microfluídica, pelo arranjo *96.96 Dynamic Array.* Os dados foram analisados ANOVA do PROC GLIMMIX do SAS. Foi utilizado o teste Tukey para comparação de médias. Valores de $p \leq 0.05$ foram considerados significativos. A clivagem foi maior ($p < 0,05$) nos grupos maturados em SFB. MIV e CIV com SFB presentou maior ($p < 0.05$) taxa de produção de blastocisto e maior quantidade de blastocistos expandidos que PVA e IGF. MIV com IGF-I aumentou o consumo de glicose e síntese de lactato dos COCs e levou à produção de

blastocistos com maior ($p < 0.05$) número total de células. Embriões cultivados em IGF-I tiveram maior ($p < 0.05$) quantidade de células na MCI e embriões cultivados em PVA tiveram maior ($p < 0.05$) apoptose do que SFB. Os genes *NANOG, OTX2, POU5F1* e *IFNT2* foram mais expressos em blastocistos cultivados em PVA e IGF do que em SFB. O IGF-I regula *TP53, BAX, CASP3, CASP9, HSPA1A* e *IGF1R* para prevenir apoptose. IGF-I durante a MIV em meio semi-definido estimula o metabolismo de glicose de COCs, melhora a qualidade embrionária, aumentando o número total de células de blastocistos. IGF-I durante a CIV aumenta o numero de células na MCI, melhora a expressão de biomarcadores importantes de qualidade embrionária com a possibilidade de melhorar o desenvolvimento embrionário.

Palavras-chave: fertilização *in vitro*, blastocisto, fator de crescimento, expressão gênica, desenvolvimento embrionário.

ABSTRACT

SERRANO-RECALDE, E. C. **Effects of the replacement of FBS with IGF-1 on cellular and molecular aspects of bovine *in vitro* embryo production.** Botucatu 2018, 121p. Tese (Doutorado em Biotecnologia Animal) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, São Paulo State University "Júlio de Mesquita Filho" – UNESP.

The aim of the present study was to analyze the benefits of fetal bovine serum (FBS) replacement by insulin-like growth factor I (IGF-I) during *in vitro* maturation (IVM) and *in vitro* culture (IVC), on embryo quality and temporal gene expression in embryos pre- and post-compaction. A 3 x 3 factorial design was performed (three supplements for IVM and three for IVC), with a total of 9 experimental groups. A total of 20 replicates (oocytes ≈ 400/group) were performed. Grade I and II cumulus-oocyte complexes (COCs) matured *in vitro* with the addition of 10% of FBS (FBS), or 3 mg/mL of polyvinyl-alcohol (PVA), or PVA + 100 ng/mL IGF-1 (IGF) at 38.5 ºC in an atmosphere of 5% CO_2 for 22 to 24 hours. After IVM, oocytes were fertilized and incubated for 18 hours. Possible zygotes were cultured with the respective addition of: 2.5% of FBS (FBS), or 3 mg/mL of PVA (PVA), or PVA + 100 ng/mL of IGF-1 (IGF) for seven days at 38.5 ºC in an atmosphere of 5% CO_2. Cleavage and blastocyst rates were analyzed at 48 and 168 hours of culture respectively. Simplified technique for differential staining of inner cell mass (ICM) and trophectoderm (TE) cells was performed to analyze cell allocation of blastocysts (n = 155) and TUNEL assay for apoptosis rate analysis (n = 207). Glucose and lactate concentrations were measured in IVM spent media to analyze glucose metabolism. mRNA was extracted from 6 – 8 cells embryos collected after 66 hours post insemination (4 pools of 15 embryos per group) and 7 day expanded blastocysts (4 pools of 5 embryos per group). Gene expression analysis was performed with BioMark HD® system with microfluidic chip *96.96 Dynamic Array*. Data were analyzed by ANOVA from PROC GLIMMIX model from SAS. Tuckey test was used to compare means. P value ≤ 0.05 was considered to be significant. Cleavage rate was higher ($p < 0.05$) for groups matured in FBS. IVM and IVC in FBS presented higher ($p < 0.05$) total blastocyst yield and greater quantity of expanded blastocysts than PVA and IGF. IVM with IGF-I increased glucose uptake and lactate synthesis of COCs and produced blastocysts with increased ($p < 0.05$) total cell

number. Embryos cultured in IGF-I had greater ($p < 0.05$) amount of cells in the ICM and embryos cultured in PVA had higher ($p < 0.05$) apoptosis rate than FBS. *NANOG*, *OTX2*, *POU5F1* and *IFNT2* genes were more expressed in blastocysts cultured in PVA and IGF than in FBS. IGF-I regulates *TP53*, *BAX*, *CASP3*, *CASP9*, *HSPA1A* and *IGF1R* genes to prevent apoptosis. The addition of IGF-I during IVM in chemically semi-defined media stimulates glucose metabolism of COCs and improves embryo quality, increasing total cell number of blastocysts. The addition of IGF-I during IVC increases the amount of cells of the ICM, improves expression of important embryo quality biomarkers with the possibility to enhance embryo development.

Key words: *in vitro* fertilization, blastocyst, growth factor, gene expression, embryo development.

CAPÍTULO 1

1. INTRODUÇÃO

Os avanços das biotecnologias aplicadas à reprodução animal tem permitido aumentar a produção de embriões bovinos e consequentemente de rebanhos de maior qualidade. As biotécnicas de aspiração folicular guiada por ultrassonografia (OPU) e a produção *in vitro* (PIV) de embriões são amplamente utilizadas, com o intuito de obter uma maior quantidade de indivíduos de alto valor genético. De acordo com a Sociedade Internacional de Transferência de Embriões (IETS), no ano de 2015, foram produzidos um total de 612.709 embriões bovinos a partir de OPU – PIV no mundo, e aparentemente essa produção aumenta a cada ano.

Apesar dos avanços nos procedimentos principais da PIV de embriões, a média das taxas de obtenção de blastocistos dificilmente ultrapassa 50% (HUSSEIN et al., 2006; SUDANO et al., 2014). As taxas de prenhez obtidas de embriões transferidos varia entre 30 e 60% (BLOCK et al., 2011; LEIVAS et al., 2011; VELOSO NETO et al,. 2014; SOUSA et al., 2017) em *Bos taurus taurus* e *Bos taurus indicus* de acordo com cada sistema de produção. O nascimento de bezerros de PIV, considerando-se o número embriões transferidos, é de 35 % (THOMPSON; PETERSON, 2000; BLOCK et al., 2011), porém, considerando o número de folículos aspirados é menor que 10% (VAN WAGTENDONK – DE LEEUW, 2006). Além disso, há um alto índice de perdas embrionárias variando entre 12 e 22% (BLOCK et al., 2011; FARIN et al., 2001; SOUSA et al., 2017).

O soro fetal bovino (SFB) contém uma grande quantidade de fatores de crescimento, hormônios, proteínas, aminoácidos, vitaminas, lipídeos entre outros elementos (GSTRAUNTHALER, 2003) e é o suplemento mais utilizado nos processos envolvidos na PIV, levando evidentemente à obtenção de maior quantidade de blastocistos produzidos. No entanto, a suplementação com SFB pode causar alterações na ultraestrutura dos embriões, defeitos na formação da blastocele, alterações na expressão gênica e aumento na incidência de natimortos (ABE et al., 1999). Além disso, é um risco constante de disseminação de doenças infecciosas devido à proteína animal no meio de cultivo embrionário (STRINGFELLOW; GIVENS, 2000).

Sendo assim, o desenvolvimento de um meio quimicamente definido, contendo moléculas sintéticas ou proteínas recombinantes é a melhor alternativa para a eliminação dos riscos e variações advindas da utilização dos sistemas de cultivo com SFB. Entre as vantagens de produzir um meio quimicamente definido estão: padronizar

os sistemas de cultivo *in vitro* (CIV) permitindo a análise de outros processos no sistema de produção de embriões, ter consistência entre laboratórios, eliminar o uso de produtos de origem animal da PIV e facilitar estudos e análises das necessidades exatas do desenvolvimento embrionário (CAROLAN et al., 1995).

A maioria dos meios de cultivo desprovidos de soro têm demostrado menor taxa de desenvolvimento embrionário (HOLM et al., 1999b; SIRISATHIEN et al., 2003). No entanto, a adição de alguns fatores de crescimento e citocinas ao meio de cultivo têm se mostrado benéfica no desenvolvimento embrionário previamente à implantação (NEIRA et al., 2010), devido a que os embriões no período de pré-implantação expressam fatores de crescimento, bem como receptores para esses fatores, considerados essenciais para o seu desenvolvimento inicial (HARDY; SPANOS, 2002).

A utilização do fator de crescimento semelhante à insulina tipo I (IGF-I) tem sido relacionada a um melhor desenvolvimento folicular (DEMEESTERE et al., 2004) e maturação oocitária (WALTERS et al., 2006; RODRIGUES et al., 2016) em bovinos. Da mesma forma, devido a que a produção local de IGF-I na tuba uterina é fundamental para o desenvolvimento embrionário previamente à implantação (LIGHTEN et al., 1998), tem sido associado ao cultivo *in vitro* de embriões de várias espécies, incluindo bovinos (PALMA et al., 1997; BLOCK, 2007; NEIRA et al., 2010), suínos (SIROTKIN et al., 2011), coelhos (HERRLER, et al., 1998), camundongos (LIN et al., 2003; DEMEESTERE et al., 2004) e humanos (LIGHTEN et al., 1998).

Desta forma, o aprimoramento da PIV, o desenvolvimento de meios de cultivo mais eficientes e a pesquisa de marcadores de qualidade embrionária e da transcrição de genes que estejam envolvidos com o aumento das taxas de produção, são constantes. Frente ao exposto, o presente estudo objetivou testar o efeito da substituição do SFB por IGF-I, tanto durante a maturação como durante o cultivo *in vitro*, avaliando a qualidade embrionária, diferenciação celular, apoptose, metabolismo embrionário e expressão temporal de genes indicadores da qualidade embrionária em diversos momentos do desenvolvimento *in vitro*.

2. REVISÃO DE LITERATURA

2.1. A PRODUÇÃO *IN VITRO* DE EMBRIÕES

A produção *in vitro* (PIV) de embriões é uma biotécnica utilizada para acelerar a produção de animais com alto mérito genético, e pela qual é possível a utilização de fêmeas geneticamente privilegiadas com alterações no trato reprodutivo que impedem a reprodução por inseminação artificial ou pela transferência de embriões (BRACKETT et al., 1982). A PIV de embriões envolve um conjunto de procedimentos complexos incluindo a colheita de oócitos imaturos, maturação *in vitro* (MIV), fertilização *in vitro* (FIV) e cultivo *in vitro* (CIV) de zigotos e estruturas embrionárias. Estes procedimentos foram progressivamente desenvolvidos durante décadas, previamente ao nascimento do primeiro mamífero produzido *in vitro* (CHANG, 1959), após a obtenção do primeiro bezerro nascido de FIV (BRACKETT et al., 1982), e estão em constante aperfeiçoamento.

Atualmente a PIV de embriões é utilizada comercialmente, mas a eficiência dos processos de maturação, fertilização de oócitos e desenvolvimento de embriões ainda é relativamente baixa. Embora 80% dos oócitos maturados *in vitro* sejam fertilizados com sucesso, apenas 30 a 50% se desenvolvem até a fase de blastocisto (SIRARD et al., 2006; VELOSO NETO et al,. 2014; SOUSA et al., 2017). Acredita-se que estes resultados se devem principalmente à baixa competência oocitária e falta do conhecimento de mecanismos que a melhoram (GILCHRIST; RICHANI, 2013), bem como às deficiências nos sistemas de cultivo embrionário (HIGHET et al, 2017).

No Brasil, a indústria da produção *in vitro* de embriões tem evoluído rapidamente na última década (BARUSELLI et al., 2016). Diferenças entre as raças bovinas interferem na eficiência da técnica, sendo que *Bos indicus* tem melhores resultados do que *Bos taurus* (GIMENES et al., 2015). Um dos fatores envolvidos parece ser que *B. indicus* tem maiores níveis de insulina circulante no sangue, os quais estimulam diretamente a produção de IGF-I e aumentam a sua biodisponibilidade (SARTORI et al., 2013; SARTORI et al., 2016). No ovário, o IGF-I estimula a proliferação e diferenciação celular, além de atuar em conjunto com o FSH na esteroidogênese (ECHTERNKAMP et al., 1994).

2.1.1. Maturação *in vitro* (MIV) e a utilização de soro fetal bovino (SFB)

O sucesso da PIV de embriões está estritamente relacionado com a viabilidade e competência oocitária (VIANA et al., 2012). Os oócitos devem ter a capacidade de retomar a meiose, clivar após a fertilização, desenvolver-se até a fase de blastocisto, induzir a prenhez e finalmente gerar um produto saudável. Estes eventos estão diretamente associados aos três tipos de processos de maturação que ocorrem nos oócitos: nuclear, citoplasmática e molecular (SIRARD et al., 2006).

Entre o oócito e as células do cúmulus que o circundam, que compõem o complexo cúmulus-oócito (COC), existe uma comunicação bidirecional parácrina e de junções gap fundamental para manutenção da viabilidade oocitária. As células da granulosa proveem nutrientes e sinais moleculares que controlam o desenvolvimento do oócito e regulam a maturação nuclear (EPPIG, 1991). O oócito, por sua vez, secreta fatores de crescimento solúveis de forma parácrina, que atuam nas células da granulosa, estimulando o seu próprio crescimento, ou então, regulando o seu microambiente e a expansão do cúmulus. Esta capacidade oocitária de secreção e regulação diminui imediatamente após o inicio da retomada na meiose devido a dissolução das junções entre as células (GILCHRIST et al., 2004).

No folículo imaturo, o oócito se mantém em parada meiótica, e posteriormente, com o pico de hormônio luteinizante (LH), fatores intrafoliculares estimulam a retomada da meiose pelas cascatas moleculares intracelulares (PARK et al., 2004). Próximo do momento da ovulação, o ambiente folicular se torna mais específico, e influencia diretamente a maturação do oócito, com comandos essenciais para promover as cascatas moleculares adequadas para a ativação do genoma embrionário, desenvolvimento até blastocisto e evolução subsequente (SIRARD et al., 2006).

Porém, diferente do que ocorre fisiologicamente *in vivo*, em COCs removidos de folículos imaturos de forma mecânica, a retomada da meiose ocorre espontaneamente independente da competência citoplasmática (EDWARDS, 1965). Este processo é rápido e pode reduzir a produção ou haver perda de metabólitos benéficos como íons, nucleotídeos e aminoácidos provindos das células do cúmulus (GILCHRIST; THOMPSON, 2007).

O SFB no meio de maturação é comumente utilizado (YOSHIOKA et al., 1997; LEIVAS et al., 2011; MISHRA et al., 2016), já que a sua presença geralmente leva à

obtenção de maiores taxas de produção de blastocisto. Contudo, tem se demonstrado que a ausência de soro na MIV não influência a maturação nuclear, consequentemente, não interfere na retomada da meiose e no processo de fertilização. Porém, a MIV de oócitos em meio sem soro leva a uma queda do índice de embriões clivados após a fertilização e formação de blastocistos, sendo que a exposição desses oócitos por 2 minutos ao soro antes da fertilização, aumenta os índices de clivagem e blastocistos (HOLM et al., 1999a).

A simples exposição de oócitos ao soro previamente ao período de maturação pode induzir ou facilitar alterações posteriores. Tanto protocolos de superovulação *in vivo* como a aspiração folicular *in vivo* ou *in vitro*, resultam na maturação de maior quantidade de oócitos do que seria natural, e entre os oócitos recuperados, também é possível a recuperação de oócitos anormais (YOUNG, et al., 1998).

Existem evidencias da presença de IGF-I no fluido folicular (STEWART et al., 1996; WANG et al., 2006). *In vitro* foi demostrado que o IGF-I colabora com o FSH para regular a atividade da aromatase, tendo um efeito estimulatório da ação das gonadotrofinas nas células da granulosa, aumentando os níveis de estradiol ovariano em bovinos. Além de que, as gonadotrofinas, por sua vez, aumentam a sensibilidade das células da granulosa ao efeito mitótico do IGF-I (SPICER et al., 2002),

Com o intuito de aumentar a eficiência do desenvolvimento de oócitos maturados *in vitro*, diversos autores têm trabalhado com a adição de substâncias promotoras de crescimento, tentando simular de forma mais fidedigna, as condições intrafoliculares (GOTTARDI; MINGOTI, 2009), e alguns autores, já têm obtido resultados satisfatórios com a adição de IGF-I no meio de maturação (LORENZO et al., 1994; SIROTKIN et al., 2011; RODRIGUES et al., 2016).

2.1.2. O Cultivo *in vitro* (CIV) e a utilização de soro fetal bovino (SFB)

A qualidade oocitária é um fator determinante para o desenvolvimento embrionário. Entretanto, o microambiente no qual os embriões são cultivados *in vitro* influencia diretamente o desenvolvimento e a qualidade embrionária. Processos como a primeira divisão celular e o tempo na qual ela acontece, a ativação do genoma embrionário, a compactação e formação da mórula, e a formação da blastocele e sua

respectiva diferenciação celular em trofoectoderma e massa celular interna, podem ser afetados com modificações do meio de cultivo embrionário (RIZOS et al., 2003).

O soro fetal bovino é o suplemento mais utilizado no cultivo de embriões *in vitro*, uma vez que melhora o desenvolvimento dos blastocistos (CROSIER et al., 2004). Este contém uma variedade de constituintes, entre eles, os fatores de crescimento, os quais podem agir como fatores de sobrevivência em embriões (BRISON; SCHULTZ, 1997; BRISON; SCHULTZ, 1998). Por outro lado, é provável que o SFB contenha fatores que desencadeiam o processo de apoptose, como o fator de necrose tumoral (TNF) (BYRNE et al., 1999), podendo também interferir negativamente na produção e qualidade embrionária.

O SFB contém fatores que bloqueiam o desenvolvimento dos embriões no estádio das primeiras clivagens, uma vez que, aparentemente os embriões durante esta fase de desenvolvimento preferem meios menos complexos para seguir um desenvolvimento normal. Sendo assim, os embriões podem se desenvolver até a fase de mórula sem necessidade da suplementação de SFB, mas é evidente que este contém fatores que estimulam posteriormente o desenvolvimento do embrião até a fase de blastocisto, tendo por tanto, um efeito bifásico quando da sua utilização no meio de cultivo (PINYOPUMMINTR; BAVISTER, 1994).

Embora a utilização de soro no cultivo embrionário leve a uma maior porcentagem de embriões atingindo a fase de blastocisto, ele causa alterações na ultraestrutura dos embriões, tais como: compactação reduzida no estádio de mórula (ABE et al., 1999), alteração do padrão de blastulação e antecipação da formação da blastocele (PINYOPUMMINTR; BAVISTER, 1994; YOSHIOKA et al., 1997).

O efeito da aceleração do desenvolvimento embrionário promovido pelo SFB ocorre durante os estádios de 9 a 16 células e de mórula, justamente após a ativação do genoma e sua atividade transcricional. A blastocele pode ser visualizada após 6 dias da fertilização, isto é, até 1 dia antes do que em meios sem a presença de soro (VAN LANDENDONCKT et al., 1997). Por outro lado, a utilização de soro no cultivo embrionário leva a uma variação no número total de blastocistos que atingem a fase de eclosão (HOLM et al., 1999b).

Além disso, existe uma diferença entre lotes individuais de produção de SFB, o que causa dificuldade na padronização do sistema de produção embrionária. Nestas condições, o ideal seria testar cada lote previamente à sua utilização na cultura embrionária (PINYOPUMMINTR; BAVISTER, 1994) o que encareceria o processo e

demandaria um grande tempo. Além disso, o risco de disseminação de doenças infecciosas por embriões produzidos *in vitro*, devido a presença de proteína animal no meio de cultivo, é uma preocupação constante (STRINGFELLOW; GIVENS, 2000).

Em bovinos, o microambiente ao qual o embrião é exposto durante os primeiros 7 dias de vida, influencia diretamente a sua morfologia, expressão gênica, e o desenvolvimento fetal e da placenta (FARIN et al., 2001). A utilização de SFB na produção *in vitro* de embriões está correlacionada com a ocorrência de alterações no fenótipo dos produtos, causando a denominada Síndrome do bezerro gigante (LOS - *Large Offspring Syndrome*) (YOUNG et al., 1998; FARIN et al., 2006; CHEN et al., 2015). Este distúrbio pode permitir que fetos e placentas apresentem diversas anormalidades, incluindo exacerbado crescimento fetal, aumento do peso fetal e placentário, desenvolvimento anormal do sistema musculoesquelético do feto e dos vasos sanguíneos placentários (LAZZARI et al., 2002; EVERTS et al., 2008).

Da mesma forma, a presença de SFB está relacionada a alterações do metabolismo embrionário, e indução de modificações na expressão de vários genes embrionários (ABE et al., 1999; RIZOS et al., 2003). Embriões produzidos *in vitro* podem ter falhas nos mecanismos fisiológicos e genéticos essenciais para o desenvolvimento fetal adequado e subsequente sobrevivência do produto após o nascimento. Estas falhas contribuem significativamente para a ocorrência de perdas gestacionais e neonatais (FARIN et al., 2006). O aumento de casos de distocia e morte perinatal, gestações prolongadas, aumento da incidência de hidroalantoide, e inadequada sinalização do momento do parto, aparentemente são consequências de defeitos metabólicos devido a uma insuficiência placentária causada pela presença do SFB no início da vida embrionária (SCHMIDT, 2007)

In vivo, previamente à implantação, os embriões passam por processos de divisão celular, apoptose e diferenciação. Durante este período, os embriões são dependentes das secreções luminais produzidas pela tuba uterina e o útero para se nutrir. A sua atividade e divisão celular, expressão gênica e metabolismo são diretamente influenciados pelo ambiente e fatores, incluindo fatores de crescimento produzidos pelas células do trato reprodutivo (HARDY; SPANOS, 2002)

O cultivo embrionário em fluido sintético de oviduto (SOF) sem a adição de soro é capaz de promover o desenvolvimento de embriões bovinos até a fase de blastocisto com resultados similares ao cultivo com a adição do mesmo (CAROLAN et al., 1995). Além disso, o nascimento de bezerros provenientes da transferência de embriões

produzidos em meio de cultivo definido (39 – 50%) confirma a viabilidade desses embriões (HOLM et al., 1999b; TRICOIRE et al., 1999).

Entre as moléculas encontradas no trato reprodutivo feminino estão os fatores de crescimento (BLOCK et al., 2011). Na tentativa de minimizar os efeitos deletérios do SFB várias citocinas e fatores de crescimento têm sido acrescentados aos meios de cultivo embrionários *in vitro*, com a intenção de mimetizar as condições de cultivo *in vivo* (SIRISATHIEN et al., 2003; WATSON et al., 1999; NEIRA et al., 2010).

Os embriões expressam receptores para vários fatores de crescimento, assim essas moléculas são capazes de exercer funções parácrina e autócrina logo no inicio do desenvolvimento (YASEEN et al. 2001; HARDY; SPANOS, 2002), podendo ser capazes de melhorar o desenvolvimento de embriões e a sua sobrevivência após a transferência embrionária (BLOCK et al., 2008).

O IGF-I exerce múltiplas funções no metabolismo, proliferação, crescimento e diferenciação celular (SIRISATHIEN et al., 2003; SHARMA et al., 2010; MEIYU et al., 2015). Na tuba uterina a produção local de IGF-I é fundamental para o desenvolvimento embrionário previamente à implantação (LIGHTEN et al., 1998; WATHES et al., 1998), indicando que a adição deste fator durante o CIV, seria capaz de aprimorar a produção *in vitro* de embriões.

De fato, estudos têm demonstrado que a utilização de IGF-I no meio de cultivo embrionário aumenta a produção de blastocisto (MAKAREVICH; MARKKULA, 2002; MOREIRA et al., 2002b; NEIRA et al., 2010) e as taxas de prenhez e de partos normais (BLOCK, 2007), demostrando a sua capacidade de modificar a fisiologia embrionária, podendo trazer grandes benefícios a PIV de embriões.

Os efeitos dos fatores de crescimento no meio de cultivo podem ser analisados de varias formas, incluindo porcentagem de produção de blastocistos, avaliação do desenvolvimento, número de células dos blastocistos, incidência da divisão e morte celular, metabolismo (HARDY; SPANOS, 2002) e expressão gênica (WANG et al., 2009).

2.1.3. A massa celular interna (MCI) e o trofoectoderma (TE)

Após a ativação do genoma embrionário e o período de compactação dos blastômeros com a formação da mórula compacta, se inicia o desenvolvimento da blastocele no estádio de blastocisto inicial. A formação do blastocisto ocorre após 5 a 6 divisões celulares, e resulta na formação das duas primeiras linhagens celulares, a massa celular interna (MCI) e o trofoectoderma (TE) (WATSON; BARCROFT, 2001). As células da MCI irão se diferenciar em todos os tecidos do feto em desenvolvimento. Já as células epiteliais do TE serão responsáveis pelo transporte embrionário na tuba uterina durante o desenvolvimento do blastocisto e posteriormente pela fixação do embrião ao endométrio uterino e formação da placenta (GILES; FOOTE, 1995).

O TE é o primeiro epitélio a ser formado, e as suas células se localizam mais externamente circundando a blastocele (GOPICHANDRAN; LEESE, 2003). A adesão célula – célula mediada pela caderina-E controla a polaridade celular e a maturação das junções de oclusão (BARCROFT et al., 2003), fazendo com que os blastômeros do TE possuam microvilosidades na superfície apical enquanto a superfície basolateral, a qual está em direto contato com o fluido da blastocele, é relativamente lisa (DUCIBELLA; ANDERSON, 1975).

O TE atua como barreira seletiva de solutos para dentro e fora da blastocele. A enzima Na+/K-ATPase atua diretamente na formação do blastocisto pois transporta Na+ para o interior da blastocele, favorecendo assim a entrada de água por meio do balanço osmótico (WATSON; BARCROFT, 2001). Além disso, as aquaporinas (AQP), proteínas de canal, também podem atuar como mediadores do movimento de fluidos através do TE para dentro da blastocele durante o processo de cavitação (BARCROFT et al., 2003). A concentração de substratos na blastocele deve ser ótima para o adequado desenvolvimento da MCI (BRISON et al ., 1993).

Fatores de crescimento podem regular a morte celular na MCI. Fatores presentes no meio de cultivo ativam diretamente receptores na MCI através da blastocele durante o processo de cavitação, ou os mesmos blastocistos são responsáveis por secretar fatores de crescimento de forma endógena na blastocele e atuar como fatores de sobrevivência para a MCI (BRISON; SCHULTZ, 1997).

O número de células na MCI está diretamente correlacionado com o desenvolvimento fetal adequado (LANE; GARDNER, 1997), e a proporção total de células localizadas na MCI e no TE é importante para o desenvolvimento embrionário

subsequente (FLEMING, 1987). Blastocistos com maior número de células na MCI têm maior capacidade de implantação e desenvolvimento (VAN SOOM et al., 1997). A formação do blastocisto está diretamente relacionada com a ocorrência de anomalias cromossômicas como aneuploidia. Embriões com maior número de células na MCI e com epitélio do TE mais uniforme têm menor probabilidade de sofrer aneuploidia (ALFARAWATI et al., 2011).

O número total de células dos blastocistos e o número de células da MCI são menores em embriões produzidos *in vitro* do que embriões produzidos *in vivo* (GILES; FOOTE, 1995). Blastocistos cultivados em grupos têm maior número de células tanto na MCI como no TE e possuem um maior índice mitótico quando comparado com blastocistos cultivados isoladamente (BRISON; SCHULTZ, 1997).

Foi observado que blastocistos bovinos com pequena MCI também têm capacidade de eclodir (AVERY et al., 1995), porém, embora estes embriões tenham essa capacidade, não necessariamente significa que são capazes de se implantar, já que é preciso uma quantidade suficiente de células da MCI para garantir o seu completo desenvolvimento (VAN SOOM et al., 1997). A relação MCI:TE aceitável em embriões de boa qualidade é de 1: 3, porém, blastocistos expandidos em D7 podem apresentar valores de 1:2,12 sendo considerados aceitáveis (MAYLEM et al., 2017).

O meio de cultivo com suplementação de soro leva à produção de embriões na fase de blastocisto mais rapidamente, os quais apresentam uma blastocele bem clara e uma MCI mais evidente (HOLM et al., 1999b), porém, o número total de células dos blastocistos cultivados na presença de SFB, é menor do que embriões desenvolvidos em meio de cultivo sem a suplementação do mesmo (TRICOIRE et al., 1999: BYRNE et al., 1999).

Embriões produzidos *in vitro* na presença de SFB têm placentas mais pesadas, porém, com a área de contato materno-fetal e densidade de vilosidades diminuída (FARIN et al., 2001). Adicionalmente, acredita-se que a maioria das complicações que ocorrem durante o parto de produtos advindos de PIV de embriões, podem ser explicadas pela inadequada sinalização do parto entre mãe e feto devido a uma deficiência placentária por alterações na sua vascularização (SCHMIDT, 2007).

2.1.4. O processo de apoptose durante o desenvolvimento embrionário

A morte celular pode ocorrer devido a múltiplas razoes, como por exemplo, a necessidade de eliminar células com função transiente ou durante a morfogênese dos órgãos. No entanto, na maioria dos casos ela é considerada como uma forma de eliminar linhagens de células com potencial de desenvolvimento indesejado (HANDYSIDE; HUNTER, 1986).

A presença de células em apoptose é uma característica comumente encontrada em embriões no estádio de blastocisto. Geralmente ocorre em uma ou mais células na massa celular interna e no trofoectoderma, e se apresenta morfologicamente como células com fragmentação nuclear e citoplasmática além de alterações bioquímicas e moleculares de membrana (HARDY, 1999).

Existe evidencia da presença de células em apoptose em blastocistos produzidos *in vivo* em várias espécies, incluindo coelhos (GILES; FOOTE, 1995), camundongos, ratos (HANDYSIDE; HUNTER, 1986), humanos (HARDY, 1997), suínos (PAPAIOANNOU; EBERT, 1988), ovinos, caprinos e bovinos (RUBIO POMAR, et al., 2005), o que sugere que a apoptose é um processo que ocorre normal e fisiologicamente durante a embriogênese.

No entanto, a apoptose também é considerada uma consequência do retardo do desenvolvimento embrionário prévio à implantação (HARDY, 1999), podendo ser um indicador de má qualidade embrionária. Na maioria dos casos a apoptose aumenta a partir da fase de blastocisto inicial, e é encontrada principalmente na MCI. Além disso, o índice de apoptose é correlacionado negativamente ao número total de células em um blastocistos, consequentemente diminuindo o seu potencial de desenvolvimento (BYRNE et al., 1999). Aparentemente a morte celular está mais relacionada com a diferenciação celular do estádio de blastocisto, e a maior incidência de apoptose na MCI pode estar associada a uma maior susceptibilidade das células devido a sua pluripotência quando comparadas com as células diferenciadas do TE (BRISON; SCHULTZ, 1997).

Tem sido demonstrado que há maior quantidade de células em apoptose em embriões produzidos *in vitro* do que em embriões produzidos *in vivo* (BRISON; SCHULTZ, 1998; RUBIO POMAR, et al., 2005). Além disso, a densidade de embriões na cultura, a composição do meio de cultivo e a suplementação de diversos fatores de crescimento em diferentes concentrações influenciam na incidência de morte celular em

embriões produzidos *in vitro* (BRISON; SCHULTZ, 1997; SPANOS et al., 2000; ONO et al., 2010), o que indica que fatores de crescimento produzidos pelo trato reprodutivo materno e pelo próprio embrião, têm um papel fundamental na regulação da apoptose durante o desenvolvimento embrionário prévio a implantação (HARDY, 1997; HARDY; SPANOS, 2002).

Embriões que têm uma clivagem rápida após a fertilização apresentam menor índice de apoptose na fase de blastocisto do que embriões de clivagem tardia (VAN SOOM et al., 1997; BYRNE et al., 1999). Este fator pode estar associado ao fato de que embriões que clivam rapidamente possuem melhor qualidade intrínseca, ou o agrupamento destes embriões de melhor qualidade leva à prevenção de apoptose por uma interação cooperativa entre os próprios embriões (VANDAELE et al., 2006).

Desta forma, a presença no SFB, de fatores que bloqueiam as primeiras clivagens (PINYOPUMMINTR; BAVISTER, 1994) pode diminuir a qualidade embrionária, já que em embriões cultivados *in vitro* na presença de 10% de SFB, o número total de células de blastocistos diminui, e o índice de apoptose é maior do que embriões desenvolvidos em meio de cultivo sem a suplementação do mesmo (BYRNE et al., 1999).

Blastocistos produzidos na presença de SFB no meio de cultivo, têm um aumento da expressão do gene regulador de apoptose box-α (Bax), diferente de blastocistos produzidos sem essa suplementação. O gene *BAX* é um marcador que pode ser utilizado para indicar qualidade embrionária em relação a apoptose (RIZOS et al., 2003).

2.1.5. O metabolismo de glicose na MIV e CIV

Os COCs utilizam preferencialmente glicose como substrato, a qual é convertida em piruvato e lactato pela glicólise. O consumo de glicose pelos COCs durante a maturação oocitária depende da composição do meio e da densidade de oócitos que são maturados (SUTTON-MCDOWALL et al., 2004).

As células do cumulus têm um papel importante na utilização de substratos para a produção de energia durante a MIV (revisto por SUTTON et al., 2003a). Aparentemente o oócito por si só, não tem capacidade suficiente para consumir glicose ou lactato como substrato sendo, portanto, dependente das células do cúmulus para metabolizar a glicose em piruvato, que é o principal substrato do oócito (BIGGERS et al., 1967; XIE et al.,

2016). Além disso, foi demonstrado que o metabolismo da glicose, piruvato e lactato das células do cúmulus não é alterado por fatores oocitários (SUTTON et al., 2003b)

O metabolismo da glicose durante a maturação oocitária está envolvido na regulação da progressão meiótica através da produção de substratos para a síntese de nucleotídeos (DOWNS; HUDSON, 2000), na maturação do ooplasma principalmente pelo fornecimento de energia e redução do estresse oxidativo (XIE et al., 2016), e está diretamente relacionado com a expansão do cúmulus após estímulo pelo FSH (SUTTON-MCDOWALL et al., 2004).

As necessidades de energia do embrião variam de acordo com o estádio de desenvolvimento embrionário previamente à implantação. A disponibilidade de substratos no ambiente no qual o embrião se desenvolve é fundamental para que o crescimento e as funções subsequentes ocorram adequadamente (THOMPSON, 1996). As vias de geração de energia para o embrião tanto para a MCI e o TE envolvem o metabolismo oxidativo de piruvato e a conversão de glicose em lactato (RIEGER et al., 1992; DONNAY; LEESE, 1999).

Embriões bovinos são capazes de se desenvolver *in vitro* na presença de lactato como único substrato no meio de cultivo, porém, o desenvolvimento adequado só ocorre quando existem piruvato e lactato associados (TAKAHASHI; FIRST, 1992). As concentrações de lactato no meio de cultivo afetam significativamente o consumo e metabolismo de piruvato, mas não afetam o consumo de glicose (LANE; GARDNER, 2000).

No inicio do desenvolvimento até o começo da formação da mórula compacta, embriões bovinos têm menor capacidade de utilizar a glicose como substrato. Assim, durante a fase inicial é predominantemente utilizado o piruvato e aminoácidos para o fornecimento de energia (THOMPSON et al., 1996) e são caracterizados por ter um índice ATP:ADP alto (LEESE et al., 1984).

Com o proceder do desenvolvimento e a ativação do genoma embrionário, ocorre um aumento da demanda de ATP (GARDNER e HARVEY, 2015). Portanto, no momento da compactação, ocorre um aumento no consumo de glicose e de produção de lactato, junto com o aumento do consumo de oxigênio (THOMPSON et al., 1996). O blastocisto é dependente da fosforilação oxidativa (86%) e da glicólise (14%) para a geração de energia e conseguir suportar o processo de cavitação (RIEGER et al., 1992; DONNAY; LEESE, 1999), proliferação celular (GARDNER e HARVEY, 2015) e

aumentar a síntese proteica (THOMPSON et al., 1998) levando a uma redução do índice ATP:ADP (LEESE et al., 1984).

Além disso, tem sido determinado que, as células do TE produzem proporões iguas de lactato e piruvato através da glicólise, mas que as células da MCI produzem principalmente lactato (HEWITSON e LEESE, 1993). Assim, a blastocele tende a manter altas concentrações de lactato, provavelmente devido à atividade metabólica de ambos os tipos celulares (BRISON et al. 1993).

Os transportadores de glicose SLC2A2 e SLC2A3 são expressos pela primeira vez em um embrião no estádio de oito células e permanecem presentes pelo resto do período de pré-implantação (CARAYANNOPOULOS et al., 2000). Posteriormente, o consumo de glicose na fase de transição de mórula para blastocisto é mediada pelos transportadores de glicose SLC2A1, SLC2A3 e o SLC2A8, os mesmos que também continuam a ser expressos em embriões previamente à implantação (BLOCK et al., 2008; PURCELL; MOLEY, 2009).

2.1.6. Expressão gênica em embriões produzidos *in vitro*

A expressão de RNA mensageiro (mRNA) de vários genes importantes para o desenvolvimento embrionário e fetal é alterada pelo meio de cultivo no qual os embriões se desenvolvem (FARIN et al., 2001). Os produtos destes genes estão envolvidos em vários processos biológicos incluindo controle do metabolismo celular, adaptação ao stress, regulação epigenética, apoptose, compactação e formação do blastocisto e da placenta (CORCORAN et al., 2007).

Diversos estudos têm comparado a expressão de mRNA em embriões produzidos *in vivo* e *in vitro* (CORCORAN et al., 2007; SUDANO et al., 2014). No entanto, a maioria dos estudos se limita a estudar o padrão de expressão gênica em blastocistos, por ser este o momento de análise final da produção embrionária *in vitro*. Poucos são os relatos que avaliam a abundância de transcritos nos estádios mais recentes de desenvolvimento embrionário (LONERGAN et al., 2003).

Entretanto, existem fortes indícios de que as diferenças encontradas na expressão gênica em blastocistos produzidos *in vitro*, se originam em estádios bem anteriores do desenvolvimento, em muitos casos, assim que os embriões são colocados em cultivo (EL-SAYED et al., 2006). Adicionalmente as informações sobre a expressão temporal

de genes envolvidos no desenvolvimento embrionário *in vitro* em diferentes sistemas de cultivo são ainda escassas.

As primeiras divisões celulares são controladas pelo mRNA materno restante após a oogênese no ovário. O genoma embrionário é transcrito a partir do estádio de 8 - 16 células em bovinos (SCHULTZ et al., 1992), enquanto os transcritos maternos estão sendo permanentemente degradados. A formação de blastocistos é dependente da expressão de diversos produtos incluindo as subunidades Na/KATPase, caderina - E, e os componentes das junções de oclusão (WATSON; BARCROFT, 2001).

Alguns genes têm sido associados a um melhor desenvolvimento embrionário. Por exemplo, o *VEGF* é fundamental para o desenvolvimento angiogênico fetal e placentário. A não expressão de *VEGF* no embrião, leva à morte intrauterina do mesmo, devido à falha na formação vascular (FERRARA, 2004). Por outro lado, o aumento da expressão do gene *IFITM3* na MCI em embriões, pode reduzir a taxa de proliferação celular na própria MCI e eventualmente alterar a relação MCI:TE (SMITH et al., 2007).

A alta expressão do gene *G6PD*, mesmo relacionado com metabolismo, estimula o desenvolvimento e proliferação celular (TIAN et al., 1998). Genes relacionados à pluripotência, assim como *POU5F1, SOX2*, e *NANOG*, são mais expressos nas células da MCI do que nas células do TE. Em contrapartida, os genes *GATA3, ELF5* e *CDX2* são mais expressos no TE, e a expressão do gene *CDX2* por sua vez, inibe a expressão de *POU5F1* permitindo a diferenciação celular (CHEN et al., 2010; OZAWA et al., 2012).

Alguns dos genes considerados bons indicadores de qualidade embrionária, podem ser relacionados a apoptose (*BAX, BCL2, HSPA1A, CASP3, CASP9*) (FEAR; HANSEN, 2011; LEIDENFROST et al., 2011), à adesão celular (*PKP2, DSC2* e *3*)(WRENZYCKI et al., 1998; STINSHOFF et al., 2011), ao metabolismo de glicose (*SLC2A1, 3, 5, G6PD*) (DOVOLOU et al., 2011; STINSHOFF et al., 2011), ao metabolismo lipídico (*ACSL3, 6, DGAT1, SREBF1*) (GHANEM et al., 2014; SUDANO et al., 2014), ao desenvolvimento embrionário (*OTX2, PA2G4, VEGF*) (HARVEY et al., 2007; SMITH et al., 2007; GAD et al., 2011.) e ao reconhecimento materno da gestação e implantação (*IFN-T, BMP15, DSC2*) (STINSHOFF et al., 2011; GHANEM et al., 2014; WANG et al., 2014)

Genes necessários para implantação (*COX2* e *CDX2*), metabolismo de carboidratos (*ALOX15*), fator de crescimento (*BMP15*), transdução de sinal (*PLAU* e *RGS2*), ligação de proteínas (*KRT8* e *ELOVL1*), ligação ao DNA (*H3F3B* e *H2FA*), constituição

estrutural do ribossomo (*RPLP0*), e específicos da placenta 8 (*PLAC8*) são altamente expressos em biopsias de embriões que resultaram em bezerros nascidos (EL-SAYED et al., 2006; GHANEM et al., 2011).

Transcritos envolvidos na fosforilação da proteína (*KRT8*), membrana plasmática (*OCLN*) e metabolismo da glicose (*PGK1* e *AKR1B1*) estão presentes em embriões que resultam em reabsorção (EL-SAYED et al., 2006). Embriões que não resultam em prenhez, apresentam alta expressão de genes envolvidos nas citocinas inflamatórias como fatores de necrose tumoral (*TNF*s), ligação de aminoácidos proteicos (*EEF1A1*), fatores de transcrição (*MSX1, PTTG1*), ligação de proteínas (*HSPD1*), ligação ao RNA (*PA2G4*), ligação a ions cálcio (*S100A10* e *S100A14*), metabolismo da glicose e atividade oxido-redutase (*ND1, FL405, PGK1* e *AKR1B1*) e *CD9*, que é um inibidor da implantação (EL-SAYED et al., 2006; GHANEM et al., 2011).

O sistema de IGF também está relacionado com a qualidade embrionária (SATRAPA et al., 2013b). A expressão de *IGF-II*, por exemplo, favorece o crescimento fetal (SWALES; SPEARS, 2005). O *IGF-IIR* é um gene imprinting em bovinos, e é considerado um regulador negativo de crescimento de células somáticas. O aumento da expressão de *IGF-IIR* está relacionado com o LOS - Large Offspring Syndrome (CHEN et al., 2015).

Os IGFs interferem na regulação do crescimento e desenvolvimento embrionário e estão envolvidos no reconhecimento materno da gestação (KO et al., 1991). A falha na expressão gênica normal destes fatores pode ser de grande impacto na viabilidade fetal. Assim, a adição de IGF-I no meio de cultivo pode alterar a expressão de diversos transcritos importantes que podem estar envolvidos no desenvolvimento e sobrevivência após a transferência embrionária (BLOCK et al., 2008).

2.2. O FATOR DE CRESCIMENTO SEMELHANTE À INSULINA TIPO I (IGF-I)

O IGF-I é um peptídeo formado por 70 aminoácidos com peso molecular de 7649 Da e a sua estrutura apresenta homologia com a pró-insulina (RINDERKNECHT; HUMBEL, 1978). O sistema dos fatores de crescimento semelhantes á insulina (IGFs) consiste dos ligantes IGF-I e IGF-II, dos receptores de superfície IGF-IR, IGF-IIR, receptor de insulina (IR) e receptor hibrido (IR/IGF-IR), e de seis proteínas de ligação (IGFBP 1 - 6) (FOULSTONE et al., 2005) .

O IGF-I se liga ao IGF-IR e ativa a via de sinalização da fosfatidilinositol-3-quinase/serina-treonina quinase (PI3K/AKT). Consequentemente são ativados substratos derivados que controlam a transcrição (Fatores de transcrição "Forkhead" – FOXO), o metabolismo (glicogênio sintase quinase 3 β -GSK-3β), a apoptose (proteína promotora de morte associada a Blc-2 – BAD), o crescimento e migração celular (proteína alvo da rapamicina em mamíferos – mTOR). Simultaneamente, também é ativada a via de sinalização da proteína serina-treonina quinase RAF, levando à proliferação celular (proteína quinase ativada por mitógenos – MAPK) (FOULSTONE et al., 2005; PAVELIC et al., 2007).

Na espécie bovina, o IGF-I endócrino está associado a vários eventos reprodutivos, os quais compreendem a taxa de concepção ao primeiro serviço (PATTON et al., 2007), ovulações duplas (ECHTERNKAMP et al., 2004), e desenvolvimento embrionário antes da implantação (VELAZQUEZ et al., 2005).

Em oócitos, a ativação de PI3K/AKT está relacionada com o processo de maturação nuclear e com a expansão do cúmulus (HOSHINO et al., 2004). A ativação de MAPK ocorre ao mesmo tempo ou pouco antes da quebra da vesícula germinativa, geralmente entre 6 e 9 horas de maturação *in vitro*, e leva à fosforilação de fosfolipases, transcrição de fatores e proteínas citoesqueléticas (QUETGLAS et al., 2010; LANDIM-ALVARENGA; MAZIERO, 2014).

O IGF-I é produzido pelas células da granulosa e, aparentemente, regula os níveis produzidos de IGF-II (VITHOULKAS et al., 2016). A produção de IGFBPs pelas células da granulosa é regulada pelo IGF-IR. Um aumento da atividade de IGF estimula a produção de IGFBPs na tentativa de reduzir a sua biodisponibilidade (WALTERS et al., 2006). Oócitos bovinos têm expressão de mRNA para IGF-I e IGF-II, seus receptores IGF-IR e IGF-IIR e as proteínas de ligação IGFBP2 e IGFBP4 (SATRAPA et al., 2013a). As células do cúmulus expressam mRNA de IGF-I, receptor IGF-IR e as proteínas de ligação IGFBP2 e IGFBP4 (NUTTINCK et al., 2004).

In vitro, a adição de IGF-I ao meio de maturação, estimula o metabolismo oxidativo e a maturação nuclear de oócitos, mas não estimula a expansão das células do cúmulus (LORENZO et al., 1994; PAWSHE et al., 1998; RIEGER et al., 1998). Mesmo assim, há um estímulo do IGF-I nas células da granulosa que leva a uma melhor maturação citoplasmática, o que pode resultar em efeitos benéficos no desenvolvimento embrionário (PAWSHE et al., 1998).

Os níveis de IGF-I no fluido folicular em humanos varia de 69 a 90 ng/mL (FARAJ et al., 2017). Já em bovinos os níveis de IGF-I no fluido folicular é maior que 60 ng/mL em novilhas e menor que 35 ng/mL em vacas *Bos taurus* (GINTHER et al., 2002; SANCHEZ et al., 2014). E os níveis de IGF-I no fluido folicular de vacas mestiças de *Bos taurus* e *Bos indicus* varia de 83 a 148 ng/mL (CARVALHO et al., 2014).

Um estudo em gado leiteiro demostrou que, folículos maiores que 8,5mm têm concentrações de IGF-I mais altas (≈ 120 ng/ml) e a quantidade de receptores para IGF-I nas células da teca é de duas vezes maior bem como ocorre em folículos dominantes em comparação com folículos pequenos (STEWART et al., 1996). A quantidade de IGFBP2, IGFBP4 e IGFBP5 é reduzida em folículos dominantes (STEWART et al., 1996; DE LA SOTA et al., 1996).

Alguns estudos têm demonstrado que o IGF-I aumenta a taxa de maturação, favorece a competência oocitária (PAWSHE et al., 1998; SAKAGUCHI et al., 2002; KIAPEKOU et al., 2005, WALTERS et al., 2006, SIROTKIN et al., 2011; MEIYU et al., 2015) e resulta em um aumento da porcentagem de oócitos clivados (KAYA et al., 2017). Porém, outros autores demostraram que a utilização de 100 ng/mL de IGF-I no meio de maturação não altera a taxa de clivagem ou de produção de blastocisto, assim como não interfere no número total de células dos blastocistos (RIEGER et al., 1998; MAKAREVICH; MARKKULA, 2002; RODRIGUES et al., 2016;).

Concentrações de IGF-I acima da fisiológica durante a maturação de oócitos, podem não ser benéficas (PAULA-LOPES, et al., 2013) ou até interferir na competência oocitária (THOMAS et al., 2007). Aparentemente esse efeito parece ser dose dependente, sendo que doses menores a 25 ng/mL parecem mimetizar melhor as condições fisiológicas do microambiente oocitário (OBERLENDER et al., 2013; RODRIGUES et al., 2016).

Os IGFs estão presentes no trato reprodutivo da fêmea, tanto na tuba uterina como no útero (WATSON et al., 1999) e o IGF-I é fundamental para o desenvolvimento embrionário previamente à implantação (LIGHTEN et al., 1998; WATHES et al., 1998). Mas Existem controvérsias sobre a expressão de mRNA do sistema IGF em embriões bovinos. De acordo com alguns estudos há expressão de mRNA para IGF-I a partir da primeira clivagem (SCHULTZ et al., 1992; YOSHIDA et al., 1998; LONERGAN et al., 2000). Já outros autores não detectaram IGF-I em embriões cultivados *in vitro* (YASEEN et al. 2001; WANG et al., 2009; SATRAPA et al.,

2013b), portanto se acredita que as funções biológicas de IGF-I aconteçam através da sua ligação com o IGF-IR (WANG et al., 2009)

A expressão de mRNA para IGF-IR ao longo do desenvolvimento embrionário prévio à implantação tem sido considerado como um potencial marcador de boa qualidade embrionária (YOSHIDA et al., 1997; YASEEN et al. 2001; WANG et al., 2009). A expressão máxima de IGF-IR ocorre em embriões no estádio de 2 - 4 células, posteriormente diminui no estádio de 8 - 16 células e volta a aumentar até a fase de blastocisto expandido. Esta variação na expressão de IGF-IR está relacionada com a ativação do genoma embrionário (POPPICHT et al., 2014). Embriões bovinos também têm expressão para mRNA de IGF-II e IGF-IIR, a qual aumenta continuamente ao longo do desenvolvimento até blastocisto. (YASEEN et al., 2001), e para IGFBP2 e IGFBP4 (SATRAPA et al., 2013b).

Em embriões o IGF-I tem um papel fundamental na regulação da proliferação, diferenciação, apoptose celular, síntese de proteínas e gliconeogênese (WATSON et al., 1994; PAVELIC et al., 2007), promove o desenvolvimento de blastocistos e a formação da blastocele estimulando o consumo de glicose (CARAYANNOPOULOS et al., 2000). Além disso, controla a expressão gênica e regulação epigenética que podem ser alteradas pelo estresse térmico e outros tipos de estresse (BONILLA et al., 2011a).

A adição de IGF-I ao meio de cultivo melhora a resposta mitogênica do embrião (BYRNE et al. 2002). Esta suplementação leva consequentemente a um aumento do número de embriões que se desenvolvem até o estádio de blastocisto (PALMA et al.,1997; MAKAREVICH; MARKKULA, 2002; BLOCK et al., 2003; LIN et al., 2003; SIRISATHIEN et al. 2003; NEIRA et al., 2010). O efeito positivo do IGF-I no desenvolvimento embrionário é mediado pela ativação da via MAPK, ocorrendo principalmente entre 4 a 8 dias após a fertilização (BONILLA et al., 2011b)

Além disso, tem sido comprovado que embriões produzidos *in vitro* na presença de IGF-I têm maior potencial para sobreviver após a transferência embrionária e também quando há estresse térmico (BLOCK et al., 2003; BLOCK, 2007). A utilização de IGF-I no meio de cultivo foi o primeiro fator de crescimento a melhorar as taxas de prenhez e partos, principalmente de animais de produção que sofrem com os efeitos climáticos de altas temperaturas (BLOCK, 2007).

O mecanismo de reconhecimento materno da gestação por parte embriões produzidos *in vitro* é mais tardio, consequentemente, leva a menores taxas de prenhez do que embriões produzidos *in vivo* (FARIN et al., 2001). O aumento das taxas de

prenhez pela utilização de IGF-I pode estar relacionado com a qualidade e potencial embrionário em produzir interferon-tau (IFN-τ), já que estudos *in vitro* têm demonstrado que o IGF-I e o IGF-II estimulam a produção de IFN-τ pelo embrião (KO et al., 1991).

Este efeito pode ser benéfico sobre o desenvolvimento embrionário, tornando-o mais capacitado para posteriormente interferir na resposta uterina durante o início da gestação bloqueando a luteólise e evitando morte embrionária precoce (WATHES et al., 1998; MOREIRA et al., 2002a). Mas, outro estudo verificou que, a utilização de IGF-I no CIV aumenta a sobrevivência embrionária após a transferência, porém, não altera a produção de IFN-τ pelo embrião (BLOCK et al., 2007).

2.2.1. A ação de IGF-I na MCI e TE de blastocistos produzidos *in vitro*

O microambiente no qual o oócito é maturado e fertilizado interfere na competência oocitária, mas também é capaz de afetar a qualidade de blastocistos. Oócitos sob ação de IGF-I durante a maturação *in vivo*, se desenvolvem em blastocistos com maior número de células na MCI (DEMEESTERE et al., 2004; VELAZQUEZ et al., 2012). Porém, esse efeito do IGF-I não foi observado quando utilizado no meio de maturação de oócitos *in vitro* (MOREIRA et al., 2002b).

A divisão, diferenciação e morte celular em embriões é regulada por fatores de crescimento (BRISON; SCHULTZ, 1998). A adição de fatores de crescimento e citocinas ao meio de cultivo embrionário levam ao aumento do número de células da MCI e do TE (NEIRA et al., 2010). Neste sentido o IGF-I atua como um mitógeno em embriões (LIGHTEN et al., 1998; SIRISATHIEN et al. 2003). A adição de IGF-I ao meio de cultivo embrionário, leva ao aumento do número total de células de blastocistos (LIN et al., 2003), e esse estímulo proliferativo ocorre predominantemente na MCI (SMITH et al., 1993; LIGHTEN et al., 1998; SIRISATHIEN et al. 2003).

O IGF-I é capaz de aumentar a formação de blastocistos através do incremento da quantidade de blastocistos que atingem o número crítico de células para se diferenciar. O efeito proliferativo de IGF-I em embriões na fase de pré-implantação envolve principalmente a via de ativação da MAPK. E como consequência do aumento da proliferação de celular, ocorre a melhora do desenvolvimento embrionário (BONILLA et al., 2011b).

IGF – II é capaz de se ligar ao receptor IGF-IR (FOULSTONE et al., 2005), e estimular a migração celular do trofoectoderma de forma parácrina através da ativação das cascatas de sinalização da PI3K-AKT e a MAPK, assim, da mesma forma o IGF-I também deve ser capaz de estimular essa migração celular (KIM et al., 2008)

2.2.2. A ação anti-apoptótica de IGF-I em embriões produzidos *in vitro*

Em animais de produção, o estresse térmico tem demonstrado ter efeitos deletérios no crescimento folicular (WOLFENSON et al., 1995), na competência oocitária (AL-KATANANI et al., 2002), e consequentemente no desenvolvimento embrionário previamente à implantação (EALY et al., 1993; PAULA-LOPES; HANSEN, 2002). Da mesma forma, estudos *in vitro* mostram que oócitos expostos a temperaturas maiores do que as fisiológicas têm menor capacidade oocitária de se desenvolver até blastocisto (ROTH; HANSEN, 2004). O IGF-I diminui os efeitos deletérios de altas temperaturas em oócitos, reduz o número de oócitos com apoptose (RODRIGUES et al., 2016) e aumenta a quantidade de oócitos que clivam após a fertilização (MEIYU et al., 2015).

A suplementação do meio de cultivo embrionário com IGF-I tem se mostrado benéfica por aumentar a proliferação celular e reduzir o índice de apoptose (HERRLER et al., 1998; BYRNE, 2002; MAKAREVICH; MARKKULA, 2002; LIN et al., 2003). O IGF-I tem uma ação anti-apoptótica no desenvolvimento de embriões *in vitro* que pode ser observada principalmente na MCI. Aparentemente o IGF-I aumenta o número de células na MCI através de uma redução em seu índice de apoptose (BYRNE et al., 2002; MAKAREVICH; MARKKULA, 2002).

Apesar disso, em outros estudos, não foi observada influência de IGF-I no número total de células, porcentagem de apoptose, ou diferença na porcentagem de células da MCI e TE. Isto pode ter ocorrido na falta de um sinal pro-apoptótico, não sendo possível observar efeitos do IGF-I na proporção das células em blastocistos. Porém, o IGF-I evidentemente altera a expressão de diversos genes considerados importantes para o desenvolvimento embrionário e consequente sobrevivência após a transferência embrionária (BLOCK, 2007; BLOCK et al., 2008).

Além disso, o IGF-I também é capaz de aumentar o número total de células de blastocistos expostos a altas temperaturas, evitando que blastômeros sofram apoptose (BRISON; SCHULTZ, 1997; JOUSAN; HANSEN, 2004). O efeito termo protetor do

IGF-I depende do estádio de desenvolvimento embrionário. Embriões com mais de 16 células que passam por estresse térmico demostram se beneficiar do efeito do IGF-I, mas embriões de 2 células não obtém os mesmos efeitos positivos (BONILLA et al., 2011a).

O efeito anti-apoptótico em embriões na fase de pré-implantação exercido pelo IGF-I utiliza a via de ativação da PI3K, já o efeito proliferativo do IGF-I é mediado pela ativação da via da MAPK. Aparentemente, a melhora do desenvolvimento de blastocistos expostos a altas temperaturas parece ser independente da ação anti-apoptótica de IGF-I (JOUSAN; HANSEN, 2007; BONILLA et al., 2011b).

2.2.3. A ação de IGF-I no metabolismo de glicose de embriões produzidos *in vitro*

O consumo de glicose e a produção de lactato são aumentados em embriões bovinos produzidos *in vitro* na presença de SFB (KRISHER et al., 1999). Baixas concentrações de IGF estimulam o consumo de glicose e a glicólise, consequentemente, a formação de lactato em diferentes tecidos (FROESCH et al 1985).

Quando da adição de IGF-I ao meio de maturação, COCs atingem os mesmos níveis de metabolismo de piruvato que quando é utilizado SFB (RIEGER et al., 1998). Oócitos maturados na presença de IGF-I, levam a produção de embriões com maior expressão de mRNA de transportadores de glicose, o que indica que posteriormente resultaria em um aumento do consumo de glicose nos blastocistos, levando ao maior desenvolvimento da massa celular interna (MCI) (VELAZQUEZ et al., 2012).

O IGF-1 estimula a atividade do SLC2A1, através do IGF1R em blastocistos cultivados *in vitro*. Esta ação de IGF-I requer pelo menos 80 minutos para atingir o máximo da estimulação do transporte de glicose no embrião, a mesma que pode chegar a ser maior que 50%. Porém, este efeito aparentemente não está envolvido na regulação aguda do metabolismo da glicose, mas na coordenação de longo prazo do metabolismo com o ciclo celular e o desenvolvimento embrionário (PANTALEON e KAYE, 1996).

Por outro lado, um estudo posterior demonstrou que o estímulo do transporte de glicose no embrião pelo IGF-1 não é através do SLC2A1, e que o transportador responsável por este efeito é o SLC2A8. Além disso, verificaram que tanto células murais como polares do trofoectoderma e até células primitivas do endoderma

apresentam transportadores SLC2A8 na superfície celular ou na membrana plasmática (CARAYANNOPOULOS et al., 2000).

O controle da proteína mTOR sobre a proliferação celular e a translação proteica é também mediada pela disponibilidade de nutrientes, incluindo glicose, aminoácidos e níveis energéticos intracelulares (GARDNER e HARVEY, 2015). Em resposta a flutuações na disponibilidade de AMP, ADP e ATP, a proteína quinase AMP-ativada (AMPK) inibe a mTORC1 via fosforilação do complexo tuberous sclerosis (TSC) ou Raptor (INOKI et al. 2012), além de modular a captação de glicose e glicólise, síntese de ácidos graxos e oxidação e biogênese mitocondrial para manter a homeostase da energia celular (HARDIE et al. 1998). A adição de IGF-I, por sua vez, pode continuar a estimular a atividade da mTOR através da via de sinalização da PI3K/AKT (PAVELIC et al., 2007).

2.2.4. A ação de IGF-I na expressão gênica de embriões produzidos *in vitro*

A adição de IGF-I ao meio de cultivo evidentemente altera a expressão de vários genes considerados importantes para o desenvolvimento embrionário. Por exemplo, a presença de IGF-1 leva a uma diminuição da expressão do gene *IFITM3* (BONILLA et al., 2011a), gene que tem como função inibir a proliferação celular da MCI (SMITH et al., 2007).

O gene *DSCII* (Desmocolina II – *DC II*) está relacionado com a formação dos desmossomos, os mesmos que participam da estabilização do TE durante a expansão do blastocisto (FLEMING et al., 1991). O gene *Na/K ATPase*, por sua vez, regula o acúmulo de fluido na blastocele (WATSON; BARCROFT, 2001). A presença de IGF-I no meio de cultivo leva ao aumento da expressão dos genes *Na/K ATPase* e *DSC II*, o que indica que embriões tratados com IGF-I poderiam se encontrar em uma fase mais avançada de expansão do blastocisto (BLOCK et al., 2008).

O gene interferon-tau (*IFNT2*) é considerado como o agente principal responsável pelo reconhecimento materno da prenhez em bovinos (WRENZYCKI et al., 1999). Já, o *DNMT3A* tem função de nova metiltranferase, fundamental para o estabelecimento dos *imprints* maternos e paternos (KANEDA et al., 2004) e a presença de IGF-I no meio de cultivo leva ao aumento significativo da expressão de *IFNT2* e de *DNMT3A* em blastocistos podendo melhorar a qualidade embrionária (KAYA et al., 2017).

Tem sido demonstrado que, a adição de IGF-I ao meio de cultivo leva ao aumento da expressão do gene anti-apoptótico *BCL* e à diminuição da expressão gene pro-apoptótico *BAX* em blastocistos (KIM et al., 2006). Em contraste, outro estudo mostra que a adição de IGF-I ao meio de cultivo aumenta a expressão de *BAX* e de *IGFBP-3*, diminui a expressão de *IGF-IR*, mas não altera o *BCL* em blastocistos expandidos e genes como o *IGF-I* e *IGF-IR* estão envolvidos no processo de apoptose celular (BLOCK et al., 2008) .

Outros genes anti-apoptóticos (*IL6ST, DYRK3, NFATC3, ANP32 e EIF3A*) e pro-apoptóticos (*IER3IP1* e *RNASEL*) também têm apresentado aumento de expressão na presença de IGF-I, ao contrario dos genes pro-apoptóticos (*DPYSL4*, *MST1*, *NFRSF11A*, *NODAL* e *ARHGEF10L*) e anti-apoptótico (*NT5E*) que têm apresentado diminuição de expressão (BONILLA et al., 2011a). Por outro lado, o IGF-I leva à diminuição da expressão de *HSP70* (proteína de choque térmico 70 - *HSPA1A*) (BLOCK et al., 2008) cuja função é bloquear o processo de apoptose (GARRIDO et al., 2003).

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3. OBJETIVOS

Investigar os efeitos da substituição de SFB pelo PVA durante a maturação *in vitro* de oócitos e o cultivo *in vitro* de embriões bovinos sobre a produção embrionária, número de células da massa celular interna e trofoectoderma, incidência de apoptose, metabolismo de glicose e expressão de genes relacionados à qualidade embrionária nos estádios iniciais e mais avançados.

Investigar os efeitos da substituição de SFB pelo PVA com a suplementação de IGF-I durante a maturação *in vitro* de oócitos e o cultivo *in vitro* de embriões bovinos sobre a produção embrionária, número de células da massa celular interna e trofoectoderma, incidência de apoptose, metabolismo de glicose e expressão de genes relacionados à qualidade embrionária nos estádios iniciais e mais avançados.

4. HIPÓTESES

A substituição de SFB pelo PVA na maturação *in vitro* de oócitos e o cultivo *in vitro* de embriões bovinos prejudica a taxa de desenvolvimento de blastocistos, o número de células da massa celular interna e trofoectoderma, aumenta a incidência de apoptose, diminui o metabolismo de glicose e diminui a expressão de genes relacionados à qualidade embrionária.

A substituição de SFB pelo PVA com a suplementação de IGF-I na maturação *in vitro* de oócitos e o cultivo *in vitro* de embriões bovinos proporciona melhor taxa de desenvolvimento de blastocistos, aumenta o número de células da massa celular interna e trofoectoderma, diminui a incidência de apoptose, melhora o metabolismo de glicose e aumenta a expressão de genes relacionados à qualidade embrionária.

CAPÍTULO 2

Artigo I

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Insulin-like Growth Factor-1: the anti-apoptotic effect explained through gene expression of bovine *in vitro* produced embryos

Insulin-like Growth Factor-1: the anti-apoptotic effect explained through gene expression of bovine *in vitro* produced embryos

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ABSTRACT

To better understand the effects of insulin-like growth factor 1 (IGF1) on apoptosis, cell proliferation, and transcription in embryos, the aim of this study was to analyze embryo production with the addition of IGF1 to *in vitro* maturation (IVM) or *in vitro* culture (IVC) media and compare embryo production with fetal bovine serum (FBS) and polyvinyl alcohol (PVA), analyzing apoptosis rate and gene expression of genes related to apoptosis and stress-response, epigenetic regulation and DNA repair, and to lipid metabolism in 6-8 cell stage embryos and expanded blastocysts. A 3 x 3 factorial design was performed, which consisted of three supplements of IVM (FBS: 10% FBS, or PVA: 3 mg/mL of PVA, or IGF: PVA + 100 ng/mL IGF1) and three supplements of IVC (FBS: 2,5% of FBS, or PVA: 3 mg/mL of PVA, or IGF: PVA + 100 ng/mL of IGF1). Maturation in FBS presented higher ($p < 0.05$) total blastocyst yield and greater number of expanded blastocysts than PVA and IGF. Embryo culture in FBS had a greater ($p < 0.05$) total blastocyst yield than PVA and IGF culture. Cell death detection assessed by TUNEL assay demonstrated that IVC medium influenced ($p < 0.05$) the number of apoptotic cells of embryos. Higher apoptosis rate was observed in blastocysts cultured in PVA than in FBS. Total cell number of embryos was greater ($p < 0.05$) in blastocysts resulting from IVM and IVC with IGF1. Real-time polymerase chain reaction (qRT-PCR) analyzed by Fluidigm Biomark™ HD system revealed that the addition of IGF1 to embryo culture is capable to regulate genes known for inducing apoptosis, tumor protein p53 (*TP53*), *BCL2*-associated X protein (*BAX*), caspase 3, apoptosis-related cysteine peptidase (*CASP3*) and caspase 9 (*CASP9*), preventing apoptosis, heat shock 70kDa protein 1A (*HSPA1A*; previously known as *HSP70*), B-cell CLL/lymphoma 2 (*BCL2*) and IGF1 receptor (*IGF1R*) and involved in lipid metabolism, fatty acid desaturase 2 (*FADS2*), fatty acid synthase (*FASN*) and Acetyl-CoA acetyltransferase 1 (*ACAT1*) during different stages of embryo development with the purpose of preventing apoptosis. We have demonstrated that oocyte maturation or embryo culture with IGF1 in the absence of FBS does not improve the number of embryos that reach blastocyst stage. However, the addition of IGF1 to *in vitro* culture regulates gene expression to prevent cell death in embryos and might reduce apoptosis rate of blastocysts improving embryo quality.

Keywords: Blastocyst, Apoptosis, IGF1, Embryo culture, Lipid metabolism

1. Introduction

Embryo quality is correlated with the incidence of cell death [1] and with total cell number of embryos [2]. Embryos of normal development have no evidence of apoptosis before compaction. However, embryos at morula and blastocyst stages usually present scattered cells with characteristic features of apoptosis including cytoplasmic, nuclear, and DNA fragmentation in different species [3,4], suggesting that apoptosis is a process that occurs normally and physiologically during embryogenesis.

Embryos that suffer arrest present higher amount of apoptotic cells [2]. There are more apoptotic cells in embryos produced *in vitro* than *in vivo* [4,5]. Moreover, the number of embryos cultured, composition of culture media, and supplementation of several growth factors at different concentrations influence the incidence of cell death in *in vitro* produced embryos [5,6]. Growth factors produced by the maternal reproductive tract, for instance IGF1, play a fundamental role in the regulation of apoptosis during embryonic development prior to implantation [2,7,8].

Research has demonstrated that IGF1 increases maturation rate, promotes oocyte competence [9,10], and has an slight anti-apoptotic effect on heat-shock exposed oocytes [11,12]. Furthermore, IGF1 has a role in the regulation of apoptosis, proliferation, differentiation, protein synthesis, and gluconeogenesis in embryos [13,14] and its anti-apoptotic effect is particularly observed in heat-shock exposed embryos [15,16]. Moreover, treatment of embryos during culture with IGF1 increases post-transfer survival when transferred into heat-stressed, lactating dairy cows [17,18].

The effects of IGF1on gene expression are more likely to be responsible for better subsequent embryo survival after transfer [18,19], but there are inconsistent amounts of transcripts obtained with the addition of IGF1 to culture media. Authors have obtained an increased expression of the anti-apoptotic *BCL* gene and decreased expression of the pro-apoptotic *BAX* gene in blastocysts [20]. In contrast, another study shows that the addition of IGF1 to culture medium increases the expression of *BAX* and *IGFBP-3* while decreasing *IGF1R* and *HSPA1A* expression, but does not alter *BCL* abundance of transcripts in expanded blastocysts [19]. Genes such as *IGF1* and *IGF1R* are involved in the apoptosis process [19] and, according to other research, none of these genes were differentially regulated by the addition of IGF1 to culture media [21].

It is known that *in vitro* produced embryos have different expression of genes related to lipid metabolism than *in vivo* produced embryos [22]. *ACACA*, *ACAT1*,

FADS2, and *FASN* are genes involved in embryo fat metabolism and different culture media has been shown to affect their mRNA expression [23,24] and the presence or absence of serum in the culture medium can influence the morphology and fatty acid composition of the bovine oocyte and embryos [25]. However, lipid metabolism in embryos is not completely understood [25] or the effects of IGF1on lipid metabolism without the presence of FBS.

Therefore, the objective of this study was to determine cellular and molecular effects of IGF1 on bovine *in vitro* produced embryos through the assessment of apoptosis rate and gene expression analysis of genes related to apoptosis, stress-response, epigenetic regulation, DNA repair, and lipid metabolism and to compare with embryos produced with FBS and PVA

2. Materials and methods

2.1 Experimental design

A 3 x 3 factorial experimental design was used to test three supplements of IVM (FBS, PVA or IGF) and three supplements of IVC (FBS, PVA or IGF), completing a total of 9 experimental groups. A total of 20 replicates (oocytes ≈ 400/group) were performed.

2.2 In vitro production of bovine embryos

Most chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA) unless otherwise stated.

2.2.1 Oocyte recovery

Ovaries of crossbred *Bos indicus* cattle were transported from the slaughterhouse to the laboratory in sterile saline (0.9% NaCl) at 35 to 37°C during approximately 1 hour. With a 10 mL syringe and a 40 x 12 needle, cumulus–oocyte complexes (COCs) were aspirated from 2 to 8 mm diameter follicles and pooled in a tube (Falcon®, NY, USA). Grade I and II COCs [26] (n = 3,819, in 20 replicates) were selected in Dulbecco's

modified flush (DMPBS) (Nutricell, Campinas, SP, Brasil) supplemented with 3 mg/mL of polyvinyl-alcohol (PVA).

2.2.2 In vitro maturation (IVM)

Pre-IVM medium was tissue culture medium - 199 (TCM - 199 HEPES with Hanks salts) containing L-glutamine and phenol red (Gibco, Grand Island, NY, USA) supplemented with 0.2 mM pyruvate, 1 μg/mL FSH (Folltropin – V, Bioniche Animal Health Canada Inc., Bellevile, Ontario, Canada), 50 μg/mL LH (Lutropin – V, Bioniche Animal Health), 3 mg/mL of PVA, 100 μg/mL streptomycin sulfate, 100 UI/mL penicillin, and 85 μg/mL amikacin. Base IVM medium was TCM-199 sodium bicarbonate with Earle's salts (Gibco), supplemented with 0.2 mM sodium pyruvate, 1 μg/mL FSH, 50 μg/mL LH, 100 μg/mL streptomycin sulfate, 100 UI/mL penicillin, and 85 μg/mL amikacin. Selected COCs were washed in pre-IVM medium and IVM was performed in three different media: 1) FBS: IVM medium + 10% of fetal bovine serum (FBS) (Gibco); 2) PVA: IVM medium + 3 mg/mL of PVA and 3) IGF: IVM medium + 3 mg/mL of PVA + 100 ng/mL of IGF1 in drops of 90 μL overlaid with sterile mineral oil. COCs (20 per drop) were incubated at 38.5 °C in an atmosphere of 5% CO_2 in humidified air for 22 to 24 hours.

2.2.3 In vitro fertilization (IVF)

After IVM, COCs were washed and transferred to 90 μL drops of IVF-TALP [27] a modified Tyrode's albumin-lactate pyruvate (TALP) medium (6 mg/mL fatty acid–free BSA, 0.2 mM sodium pyruvate, 10 μg/mL heparin, 40 μL/mL, 18 μM penicillamine, 10 μM hipotaurine, 1,8 μM epinephrine, 100 μg/mL streptomycin sulfate, 100 UI/mL penicillin, and 85 μg/mL amikacin. For fertilization, a dose of 2×10^6 sperm/mL of a single batch of frozen semen from one Nelore (*Bos indicus*) bull (CRV Lagoa, Sertãozinho, São Paulo, Brazil) was used. Percoll® (90% and 45%) (Nutricell) was used to obtain purified spermatozoa. COCs were incubated for 18 hours at 38.5 °C and 5% CO_2 in humidified air.

2.2.4 In vitro culture (IVC)

Base embryo culture medium was synthetic oviduct fluid (SOFaaci) [28] containing 10 µL/mL basal medium Eagles (BME) essential amino acids, 10 µL/mL minimum essential medium (MEM) nonessential amino acids, 0,34 mM tri-sodium citrate, 2.7 mM myoinositol, 0.2 mM pyruvate, 5 mg/mL BSA, 100 µg/mL streptomycin, 100 UI/mL penicillin, and 85 µg/mL amikacin. Presumptive zygotes were denuded of cumulus cells by repeated pipetting. Three groups of IVC were also established: 1) FBS: SOFaaci medium + 2,5% of FBS; 2) PVA: SOFaaci medium + 3 mg/mL of PVA and 3) IGF: SOFaaci medium + 3 mg/mL of PVA + 100 ng/mL of IGF1. Embryos were cultured in 90µL drops covered with sterile mineral oil, at 38.5 °C and 5% CO_2 in humidified air.

Cleavage rate was determined at 66 hours post insemination (hpi) and cleaved embryos were transferred to 90 µL drops of new media. At 114 hpi, 50 µL of each drop was replaced with new media. Blastocyst rate and embryo quality were analyzed at Day 7.

2.3 TUNEL labelling

In situ cell detection kit fluorescein staining (Roche Diagnostics, Mannhein, BW, Germany) was used for TUNEL assay according to the technique previously described by Paschoal et al. [29]. Early, expanded and hatched blastocysts were removed from culture medium, washed in PBS (Nutricell) containing 1 mg/mL of polyvinylpyrrolidone (PBS-PVP), fixed in 100 µL drop of 4% paraformaldehyde in PBS for 1 h at room temperature (RT), and stored at 4 °C in PBS-PVP until TUNEL assay.

Embryos were transferred to a permeabilization solution (0.1% triton X-100 with 0.1% sodium citrate in PBS) at RT for 1 h and washed again with PBS-PVP. Positive and negative controls were incubated in DNase RNase-Free DNase Set (Qiagen, Toronto, Ontário, Canadá) solution at 37 °C for 1 h in a humid chamber. Embryos were then incubated with TUNEL reaction mixture (enzymatic and marking solution) as recommended by the manufacturer at 37 °C in the dark for 1 h. Negative control was incubated only with the marking solution, without the enzyme solution to verify the signal. All groups were washed in PBS-PVP and stained with 25 µg/mL Hoechst 33342

(Invitrogen, Eugene, OR, USA) in PBS at RT for 15 min in the dark. Embryos were then placed on a glass microscope slide in a 10 µL drop of glycerol gently flattened with a coverslip.

Samples were analyzed using an inverted epifluorescence microscope (Leica DMI 3000 B) at 20x magnification. Blue excitation intensity with 450 – 490 nm filter was used for TUNEL and UV with 340 – 380 nm filter for Hoechst 33342. All healthy and apoptotic cells were stained with Hoechst 33342 and nuclei with green fluorescence (FITC) were considered to be TUNEL positive (fragmented DNA). Cells were counted from digital images obtained from 207 embryos produced in 6 replicates.

2.4 RNA extraction and reverse transcription

Embryos from two stages of development of each group were stored at -80 °C with recombinant ribonuclease inhibitor (RNaseOUT®) (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from 4 pools of 15 cleaved embryos (6 – 8 cells) collected at 66 hpi and also from 4 pools of 4 expanded blastocysts of each group, using the RNA isolation kit (PicoPure®) (Arcturus, Mountain View, CA, USA). The extracted RNA was assessed for quantity and quality by a spectrophotometer (ND-2000, NanoDrop, Wilmington, DE, USA) and a 2100 Bioanalyzer apparatus (Agilent Technologies, CA, USA) with RNA Pico Chips (Agilent Technologies) respectively and later stored at -80 °C until further use. All analyzed samples had RNA integrity number (RIN) > 9. Reverse transcription of RNA isolated was performed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions.

2.5 Real-time polymerase chain reaction (qRT-PCR)

Gene expression analysis of bovine embryos was performed using the Applied Biosystems™ TaqMan® Assays, specific for *Bos taurus* species. mRNA abundance of 96 genes was analyzed per functional categories of embryos (for further details, see supplementary material 1). Before qRT-PCR thermal cycling, each sample was submitted to sequence-specific preamplification process as follows: 1.25 µL assay mix (TaqMan® Assay was pooled to a final concentration of 0.2X for each of the 96 assays), 2.5 µL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128) and 1.25

µL cDNA (5 ng/µL). The reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and amplification at 60ºC for 4 min for 14 cycles. Preamplified products were diluted 6-fold prior to qRT-PCR analysis.

Sample solution for gene expression analysis was prepared, which consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR Master Mix (2X, Applied Biosystems), and 0.25 µL of 20X GE Sample Loading Reagent (Fluidigm), and the assay solution: 2.5 µL of 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 µL of 2X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used for data collection. After priming, the chip was loaded with 5 µL of each assay solution and 5 µL of each sample solution. The qRT-PCR thermal cycling was performed in the Biomark HD System (Fluidigm, South San Francisco, CA, USA) using the protocol TaqMan GE 96x96 Standard, consisting of one stage of Thermal Mix (50ºC for 2 min, 70ºC for 20 min and 25ºC for 10 min) followed by a Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of denaturation (95ºC for 15 s), primer annealing, and extension (60ºC for 60 s).

The relative expression of target genes in each sample was calculated using the ΔΔCt method using the mean of control group as calibrator [30]. Data was normalized using the geometric mean of the most stable reference genes in embryos (*GAPDH, HMBS* and *PPIA*).

2.6 Statistical analysis

Sources of variations in the model included IVM supplements (FBS, PVA and IGF), IVC supplements (FBS, PVA and IGF), and first order interactions; all factors were considered fixed effects. Data were analyzed by analysis of variance (ANOVA) from PROC GLIMMIX model from SAS software (SAS Inst. Inc., Cary, NC, USA), if ANOVA was significant, Tuckey's test was used to compare the means. A P value ≤ 0.05 was considered to be significant.

3. Results

Embryo production from twenty replicates using three different supplements of IVM (FBS, PVA or IGF) and three supplements of IVC (FBS, PVA or IGF) was

analyzed. The additive effect of maturation and culture treatment was only observed for total blastocyst production, thus results were presented for IVM and IVC separately.

3.1 Effect of IVM treatment on embryo production

A significant effect of IVM treatment was observed on cleavage rate (Table 1). Oocytes matured in FBS resulted in a higher ($p < 0.05$) percentage of cleaved embryos than PVA and IGF groups. Maturation in FBS presented higher ($p < 0.05$) total blastocyst yield and greater quantity of expanded blastocysts than PVA and IGF. Moreover, total blastocyst production and number of expanded blastocysts of PVA group were higher than maturation in IGF group. There was no difference ($p > 0.05$) for hatched blastocysts between groups.

3.2 Effect of IVC treatment on embryo production

There was no effect ($p > 0.05$) of IVC treatment on cleavage rate (Table 2). Distribution of blastocysts according to developmental stage was different between groups. Embryo culture in FBS resulted in an increased ($p < 0.05$) percentage of early, expanded and hatched blastocysts than PVA and IGF culture. Consequently, FBS group had a greater ($p < 0.05$) total blastocyst yield than PVA and IGF groups.

3.3 Effect of IVM and IVC on Apoptosis in blastocysts

Cell death detection was assessed by TUNEL assay, where DNA fragmentation was revealed as green nuclei fluorescence. No IVM treatment effect ($p > 0.05$) or IVM x IVC interaction were observed in apoptosis rate or total cell number of embryos (Table 3). There was an IVC treatment effect ($p < 0.05$) in which embryos cultured in FBS presented lower apoptosis rate than embryos cultured in PVA (Table 4). No differences ($p > 0.05$) on total cell number of blastocysts between groups of IVC were observed. When the effect of IVM and IVC were analyzed together, total cell number was higher in blastocysts of IGF-IGF group compared to FBS-PVA group (Supplementary material 2).

3.4 Effect of treatment in Gene Expression of embryos assessed by qRT-PCR

The Applied Biosystems™ TaqMan® Assays were used to perform gene expression analysis of pools of 6-8 cell stage embryos and pools of expanded blastocysts. Twenty two genes for apoptosis and stress-response, ten for epigenetic regulation and DNA repair and twelve genes for lipid metabolism were analyzed.

3.4.1 Expression of genes related to apoptosis and stress-response

There was no effect of IVM treatment in genes for apoptosis and stress-response in 6-8 cell stage embryos, but it was observed an IVM treatment effect ($p < 0.05$) on one gene in expanded blastocysts. *IGFBP4* gene in FBS group was up-regulated in relation to PVA and IGF groups (Fig. 1). There was no IVM x IVC interaction.

IVC treatment effect in genes for apoptosis and stress-response in 6-8 cell stage embryos is presented in Fig. 2. From the 22 analyzed genes, 18 were up-regulated in PVA group in relation to FBS and IGF groups. Among these genes, *GPX1* and *IGF1R* in IGF group were down-regulated in relation to FBS group. Moreover, *HSPD1* and *SOD2* genes were less ($p < 0.05$) expressed in IGF group compared to FBS and PVA groups too. No significant differences were observed for *IGFBP2* and *IGFBP4* genes.

In expanded blastocysts, there was also an IVC treatment effect (Fig. 3). *BAX*, *CASP3* and *HSP90AA1* genes presented higher amount of transcripts in PVA group compared to FBS group. *HSPD1*, *IGFBP2* and *IGFBP4* genes were up-regulated in PVA and IGF groups in relation to FBS group. *CASP9* was more expressed in PVA than in IGF group. *TP53* in IGF group was up-regulated in relation to PVA and FBS groups and PVA had higher expression than FBS group. No differences were observed for the other 14 genes.

3.4.2 Expression of genes related to epigenetic regulation and DNA repair

No IVM treatment effect ($p > 0.05$) or IVM x IVC interaction was found in genes for epigenetic regulation and DNA repair in 6-8 cell stage embryos or expanded blastocysts.

However, an IVC treatment effect ($p < 0.05$) on 6-8 cell stage embryos genes showed that 6/10 analyzed genes had higher amount of transcripts in PVA group in

relation to FBS and IGF groups (Fig. 4). An IVC treatment effect ($p < 0.05$) was also observed in expanded blastocysts (Fig. 5). *DNMT3A* was more expressed in FBS and PVA groups compared to IGF group and *DNMT3B* was up-regulated in PVA group in relation to the other two groups.

3.4.3 Expression of genes related to lipid metabolism

IVM treatment had no influence ($p > 0.05$) on genes for lipid metabolism in 6-8 cell stage embryos, but there was an IVM treatment effect ($p < 0.05$) in 1/12 genes analyzed in expanded blastocysts (Fig. 6). *FASN* gene in IGF group was up-regulated in relation to FBS group. There was no IVM x IVC interaction.

IVC treatment effect ($p < 0.05$) on genes for lipid metabolism in 6-8 cell stage embryos is presented in figure 6. *ACACA* and *ACSL3* genes were more expressed in PVA and IGF groups different than in FBS group. *ACAT1* was more expressed in PVA group than in IGF group. *FADS2* and *FASN* in IGF group were up-regulated in relation to FBS and PVA groups.

There was also an IVC treatment effect ($p < 0.05$) on genes for lipid metabolism of expanded blastocysts (Fig. 7). *ACAT1*, *ELOVL6* and *FASN* genes in PVA and IGF groups were up-regulated in relation to FBS group. Conversely, *ACSL6* was down-regulated in PVA and IGF groups compared to FBS group and *FADS2* was down-regulated in IGF group in relation to FBS and PVA groups.

4. Discussion

This study aimed to analyze embryo production with the addition of IGF1 to IVM or IVC media and compare embryo production with FBS and PVA, analyzing apoptosis rate and gene expression of genes related to apoptosis and stress-response, epigenetic regulation and DNA repair, and to lipid metabolism in cleaved embryos and expanded blastocysts.

Oocytes matured in FBS resulted in higher quantity of cleaved embryos, consequently, FBS had a greater total blastocyst production in comparison with IVM containing PVA and IGF1. Similar results were obtained in a previous study [31], which demonstrates that the oocyte maturation process does not successfully occur with IGF1

only, but there is a slight positive effect, since the quantity of hatched blastocyst was similar to embryo production derived from maturation with FBS.

Conversely, IVC media had no effect on cleavage rate, embryo culture with IGF1 obtained similar numbers of cleaved embryos than FBS embryo culture. However, IVC with FBS still produced the highest numbers of embryos. Previous studies reported that IGF1 improves embryo development and blastocyst production in defined media [32,33]. In our study, blastocyst rate of embryo culture with IGF1 and with PVA did not differ, therefore no positive effect of IGF1 was observed on blastocyst yield.

Embryo requirements for embryo culture vary according to embryo development stage prior to implantation and the availability of substrates in the environment in which the embryo develops is critical for subsequent growth and functions to occur properly [34]. So it is possible that culture media was enough for the first embryo stages of development, but after cleavage and genome activation other molecules than IGF1 may be necessary.

Previous reports indicate that the use if IGF1 in oocyte maturation does not increase total cell number of embryos [12,15,31]. On the other hand, culture media with 100 ng/ml of IGF1 has increased total cell number of blastocysts [15,35], but not with 40 ng/ml of IGF1 [36]. When IVM and IVC treatments were analyzed separately, IGF1 did not have an effect on total cell number of blastocysts. However, total cell number was higher in blastocysts of IGF-IGF group compared to FBS-PVA group. IGF1 has a mitogenic effect by the activation of MAPK pathway through IGF1R and the increased quantity of cells in embryos may be the result of this proliferative effect [33,37].

The apoptotic rate of blastocyst was not altered by the IVM treatment and only two of all the analyzed genes had significant differences. Conversely, IVC medium influenced the number of apoptotic cells of embryos and most of the analyzed genes presented differences between treatments. Four genes related to apoptosis and stress-response (*GPX1*, *IGF1R*, *HSPD1* and *SOD2*), were down-regulated in cleaved 6-8 cells stage embryos with the addition of IGF1 to culture media. In addition, a gene related to epigenetic regulation and DNA repair was also less expressed in IGF1 cultured embryos. These results indicate that cleavage stage is an important period for gene regulation during embryo development and the use of IGF1 in culture media may prevent subsequent apoptosis process in expanded blastocysts.

The beneficial effects of IGF1 in *in vitro* culture have been previously observed, including increase of cell proliferation and apoptosis rate reduction [15,37,38]. In our

study, embryos cultured with PVA had higher amount of cells with fragmented nuclei and DNA than embryos cultured in FBS, but embryos cultured with IGF1 did not differ from both groups, presenting an intermediate value. In addition, higher amount of transcripts for *BAX*, *CASP3* and *HSP90AA1* genes were observed in expanded blastocysts cultured in PVA than in FBS, but IGF group had results between the two groups. Thus, the addition of IGF1 to embryo culture with PVA seemed to slightly reduce apoptosis rate, probably due to its anti-apoptotic effect [6,15], but this positive effect of IGF1 may require an adverse condition, such as heat stress to become evident [18].

Down-regulation of *IGF1R* gene was reported with addition of 100 ng/ml of IGF1 in bovine embryo culture [32]. A high concentration of IGF1 induced apoptosis in embryos and was associated with reduced *IGF1R* expression through the activation of the *TP53* pathway [39]. *TP53*, when increased, regulates cell cycle arrest, in case of DNA damage it allows DNA repair or triggers apoptosis activating pro-apoptotic proteins such as *BAX* and repressing anti-apoptotic *BCL2* protein, which results in induction of the caspase signaling [40]. Surprisingly, in our study, *TP53* gene was up-regulated in expanded blastocysts cultured with IGF1. In contrast, *CASP9* gene was down-regulated, which suggests that embryos were under a slight stress, but not enough to undergo apoptosis.

HSPA1A function is to block apoptosis process by the inhibition of the apoptosome, the caspase activation complex and apoptosis inducing factor [41]. In a previous report, embryos cultured in IGF1 had lower expression of *HSPA1A* [19], as it was observed in our research in 6-8 cell stage embryos in comparison with PVA group. However, this down-regulation was not observed in expanded blastocysts, similar to results obtained in a recent research in blastocysts [38]. Perhaps, there is no need of an increase in *HSPA1A* expression to be enough to suppress caspase activity. Furthermore, it has been demonstrated that poor-quality *in vitro* produced bovine blastocysts have a higher expression of *HSPA1A* compared to good quality embryos [42].

The opposite occurred in the absence of IGF1. In embryos cultured with PVA, genes related to apoptosis, stress-response, epigenetic regulation and DNA repair, including *HSPA1A*, *TP53*, *BAX*, *CASP3*, and *CASP9* genes, were up-regulated in 6-8 cell stage embryos and expanded blastocysts compared to either FBS or IGF or both groups. Thus, embryos cultured with PVA had higher apoptosis rate. Apparently, the lack of growth factors or other molecules that are involved in embryo development

caused this overexpression of genes as response and survival strategy. Nevertheless, embryo culture with IGF1 and FBS presented similar results for most genes, therefore both seem to have alike ability to regulate gene expression.

DNMT3A, a gene related to epigenetic regulation, has the role of new methyltransferase [43] and the downregulation of *DNMT3A* expression activates caspase-3 and -9 activity and increases *TP53* expression promoting apoptosis and DNA damage [44]. Our results registered that *DNMT3A* had a lower amount of transcripts in expanded blastocysts cultured in IGF1, which probably stimulated *TP53* expression and consequently increased *CASP3* expression. In contrast, a recent study demonstrated that IGF1 in culture media leads to an up-regulation of *DNMT3A* in blastocysts [38]. Inconsistency of these results can be due to the addition of FBS after day 4 of embryo culture in the previous study.

Alterations in the expression of genes related to lipid metabolism during pre-implantation period affect developmental capacity [45]. Overexpression of fatty acid synthase (*FASN*) gene stimulates cell proliferation and its inhibition reduces cell viability and triggers apoptosis [46]. Moreover, the Acetyl-CoA acetyltransferase 1 (*ACAT1*) function is to catalyze esterification of intracellular free cholesterol. Inhibition of *ACAT1* causes an excess of free cholesterol that is not adequately esterified, which increases caspase-3/7 activation and subsequent cell death [47,48].

In the present study, *FASN* and *FADS2* were up-regulated in 6-8 cell stage embryos cultured with IGF1 compared to embryos cultured in PVA and FBS, but *ACAT1* was down-regulated in IGF1 group in relation to PVA group. Nevertheless, *CASP3* or *CASP9* expressions were not increased in IGF1 group at this stage. In expanded blastocysts, however, *FASN* and *ACAT1* genes were up-regulated in IGF1 and PVA blastocysts in comparison with embryos cultured in FBS. Furthermore, IVM with IGF1 induced a higher expression of *FASN* in expanded blastocysts compared to IVM with FBS. These results suggest that embryo production without FBS and with the addition of IGF1 may improve lipid metabolism preventing cell death of embryos.

Apoptosis in preimplantation embryos does not completely compromises their future development, as there is evidence that limited apoptosis can allow embryos to recover from stress and continue to develop [49]. The present study determined apoptosis rate and gene expression pattern of embryos produced with the addition of 100 ng/mL of IGF1 during *in vitro* maturation or embryo culture in the absence of FBS

and elucidated some IGF1 actions at a molecular level that can improve embryo quality and developmental competence.

In conclusion, present results demonstrate that the addition of IGF1 to oocyte maturation or embryo culture in the absence of FBS does not promote blastocyst development. The use of IGF1 in *in vitro* embryo production does not reduce apoptosis rate of blastocysts produced in normal conditions where apoptosis rates are low. However, the addition of IGF1 to embryo culture regulates multiple genes related to apoptosis and stress-response, epigenetic regulation and DNA repair, and lipid metabolism during different stages of embryo development in order to prevent cell death in blastocysts. Therefore, *in vitro* culture of embryos with IGF1 might reduce apoptosis rates and improve embryo quality in adverse conditions and increase post-transfer survival.

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Conflict of interest

The authors declare no conflict of interest.

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Table 1

Cleavage rate and blastocyst development of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during oocyte maturation.

Treatment		Embryo Development/Oocytes				
IVM	Oocytes cultured	Cleavage (%)	BL (%)	BX (%)	BH (%)	Total Blastocyst (%)
FBS	1252	1071 (85.6) [a]	100 (8.0) [a]	185 (14.9) [a]	22 (1.8) [a]	307 (24.7) [a]
PVA	1284	923 (72.5) [b]	69 (5.3) [b]	147 (11.7) [b]	11 (0.9) [a]	227 (18.0) [b]
IGF	1283	909 (71.1) [b]	55 (4.3) [b]	103 (8.1) [c]	17 (1.3) [a]	175 (13.8) [c]

[a, b] Data with different superscripts in the same column differ significantly ($p < 0.05$).

Abbreviations: BL, blastocyst; BX, expanded blastocysts; BH, hatched blastocysts.

Table 2

Cleavage rate and blastocyst development of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during embryo culture.

Treatment		Embryo Development/Oocytes				
IVC	Oocytes cultured	Cleavage (%)	BL (%)	BX (%)	BH (%)	Total Blastocyst (%)
FBS	1250	937 (75.3) [a]	106 (8.5) [a]	261 (21.0) [a]	41 (3.3) [a]	408 (32.8) [a]
PVA	1270	964 (76.2) [a]	58 (4.6) [b]	76 (6.0) [b]	4 (0.3) [b]	138 (10.9) [b]
IGF	1299	1002 (77.8) [a]	60 (4.7) [b]	98 (7.6) [b]	5 (0.4) [b]	163 (12.8) [b]

[a, b] Data with different superscripts in the same column differ significantly ($p < 0.05$).

Abbreviations: BL, blastocyst; BX, expanded blastocysts; BH, hatched blastocysts.

Table 3

Total cell number and apoptosis rate of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA), or PVA + IGF1 (IGF) during oocyte maturation (Mean ± S. E.)

Treatment	Cell analysis	
	Apoptosis	Total cell
IVM	(%)	n
FBS	0.9 ± 0.2 [a]	80.3 ± 4.7 [a]
PVA	1.0 ± 0.2 [a]	86.2 ± 3.8 [a]
IGF	1.1 ± 0.2 [a]	92.1 ± 5.0 [a]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

Table 4

Total cell number and apoptosis rate of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA), or PVA + IGF1 (IGF) during embryo culture (Mean ± S. E.)

Treatment	Cell analysis	
	Apoptosis	Total cell
IVC	(%)	n
FBS	0.6 ± 0.1 [b]	86.3 ± 3.1 [a]
PVA	1.5 ± 0.2 [a]	82.4 ± 5.1 [a]
IGF	1.0 ± 0.2 [ab]	89.9 ± 5.1 [a]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

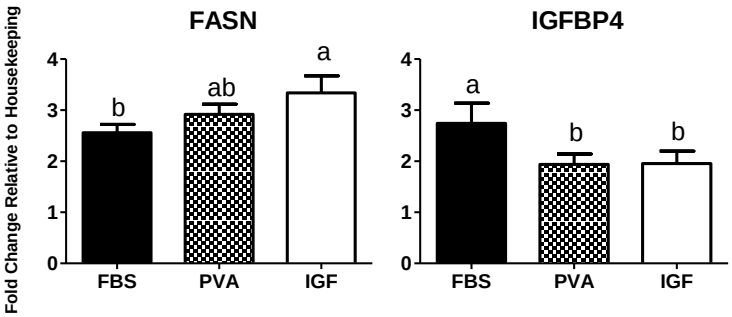


Fig. 1. Mean ± SEM of transcripts of genes for lipid metabolism and apoptosis of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVM with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

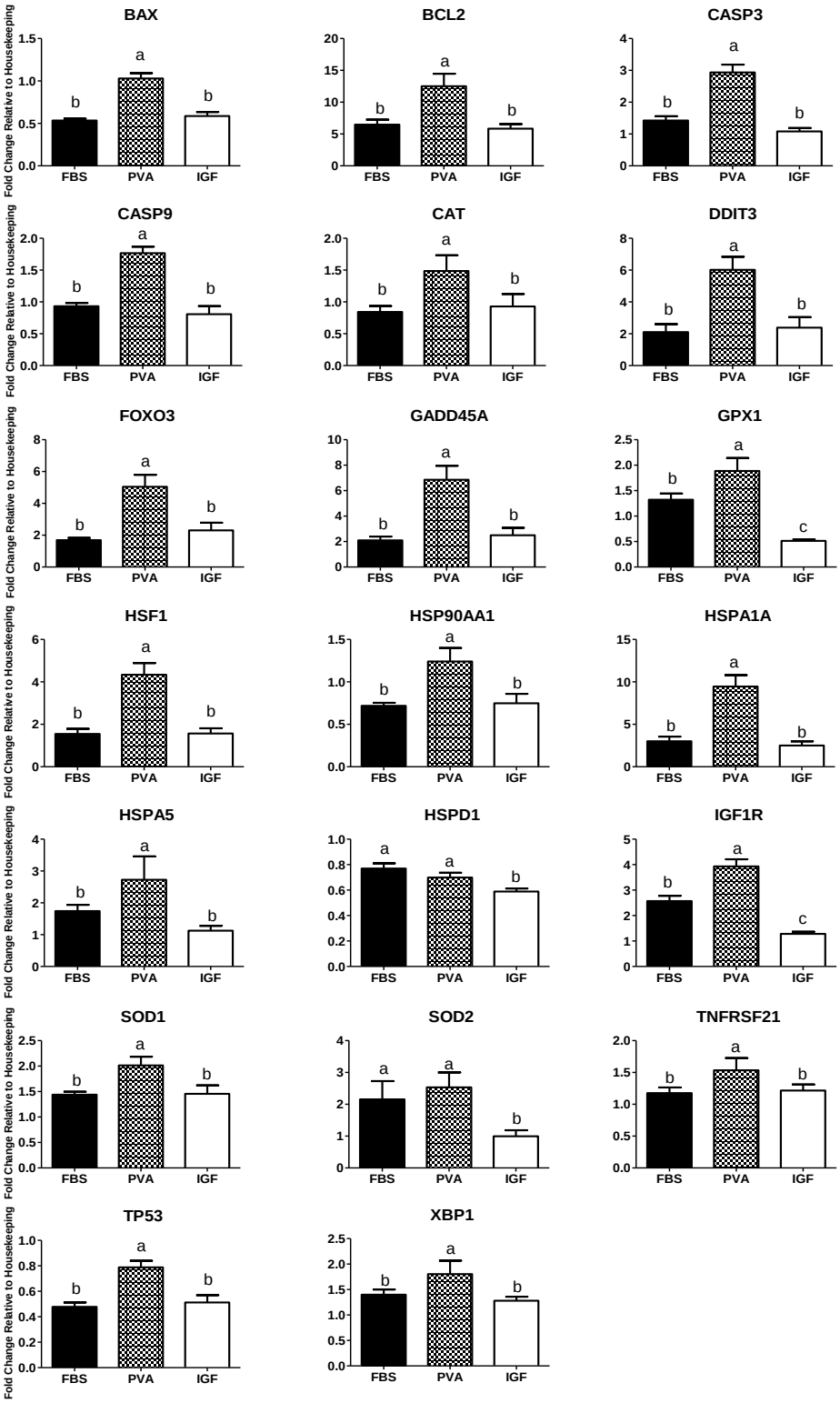


Fig. 2. Mean ± SEM of transcripts of genes for apoptosis and stress-response of 6-8 cell stage embryos. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

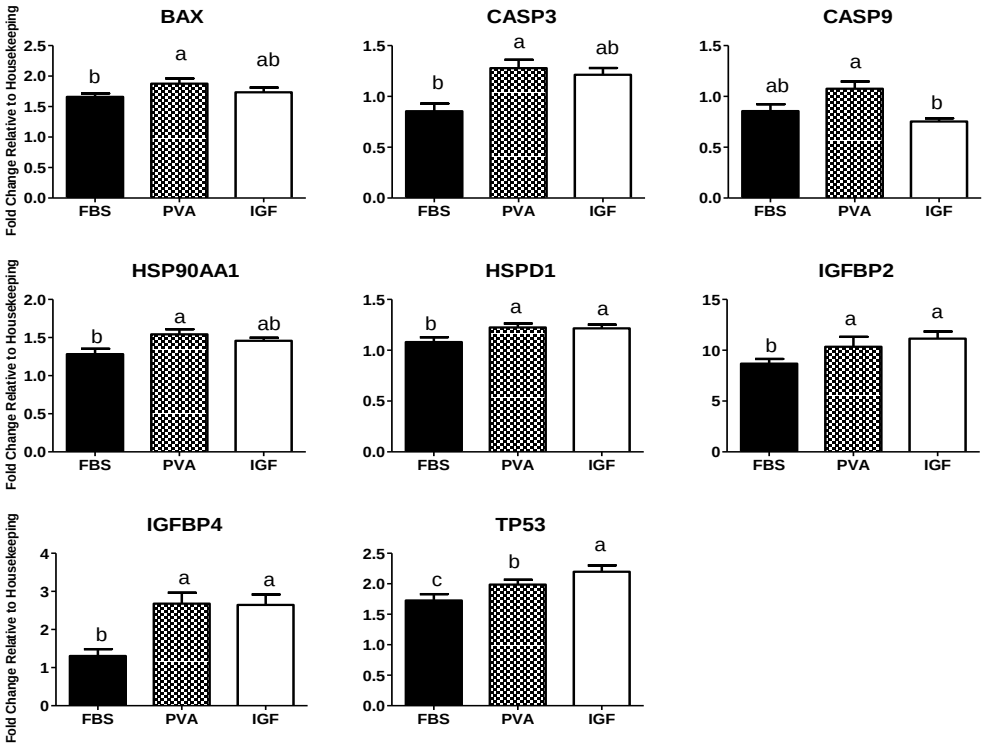


Fig. 3. Mean ± SEM of transcripts of genes for apoptosis and stress-response of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

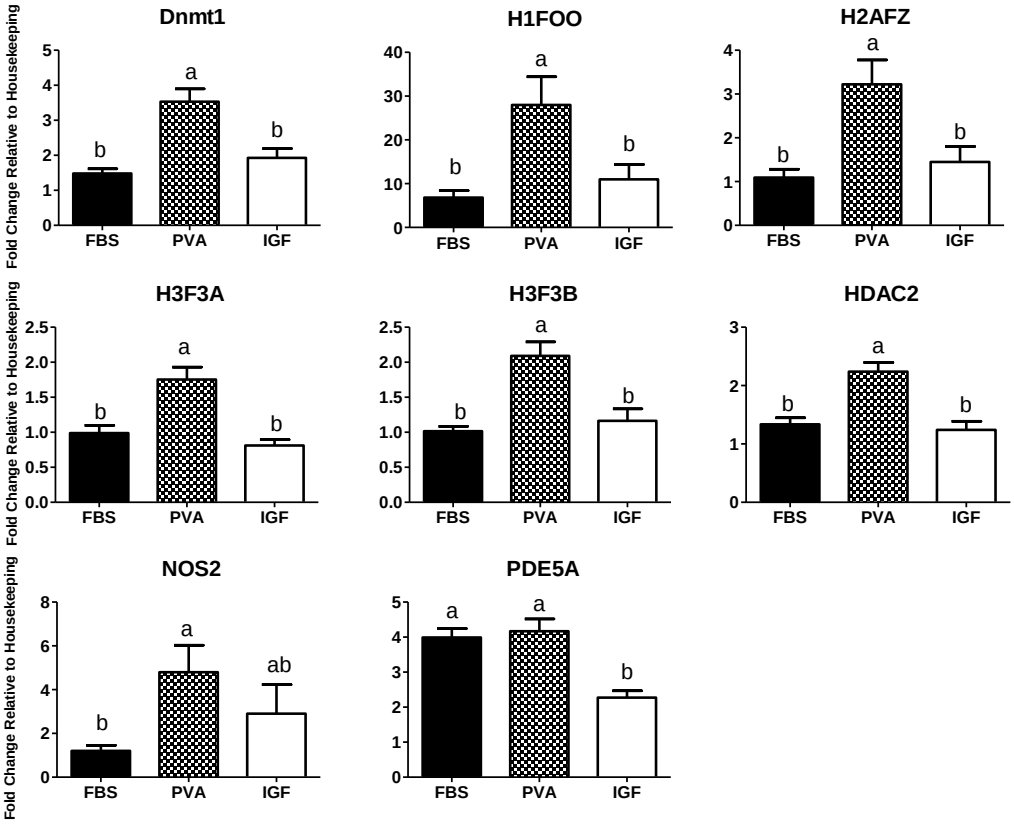


Fig. 4. Mean ± SEM of transcripts of genes for epigenetic regulation and DNA repair of 6-8 cell stage embryos. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

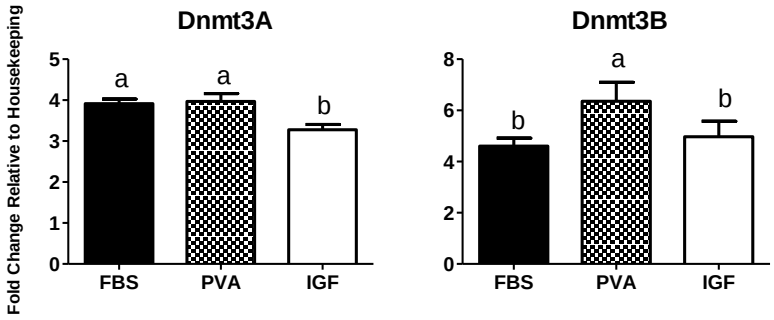


Fig. 5. Mean ± SEM of transcripts of genes for epigenetic regulation and DNA repair of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

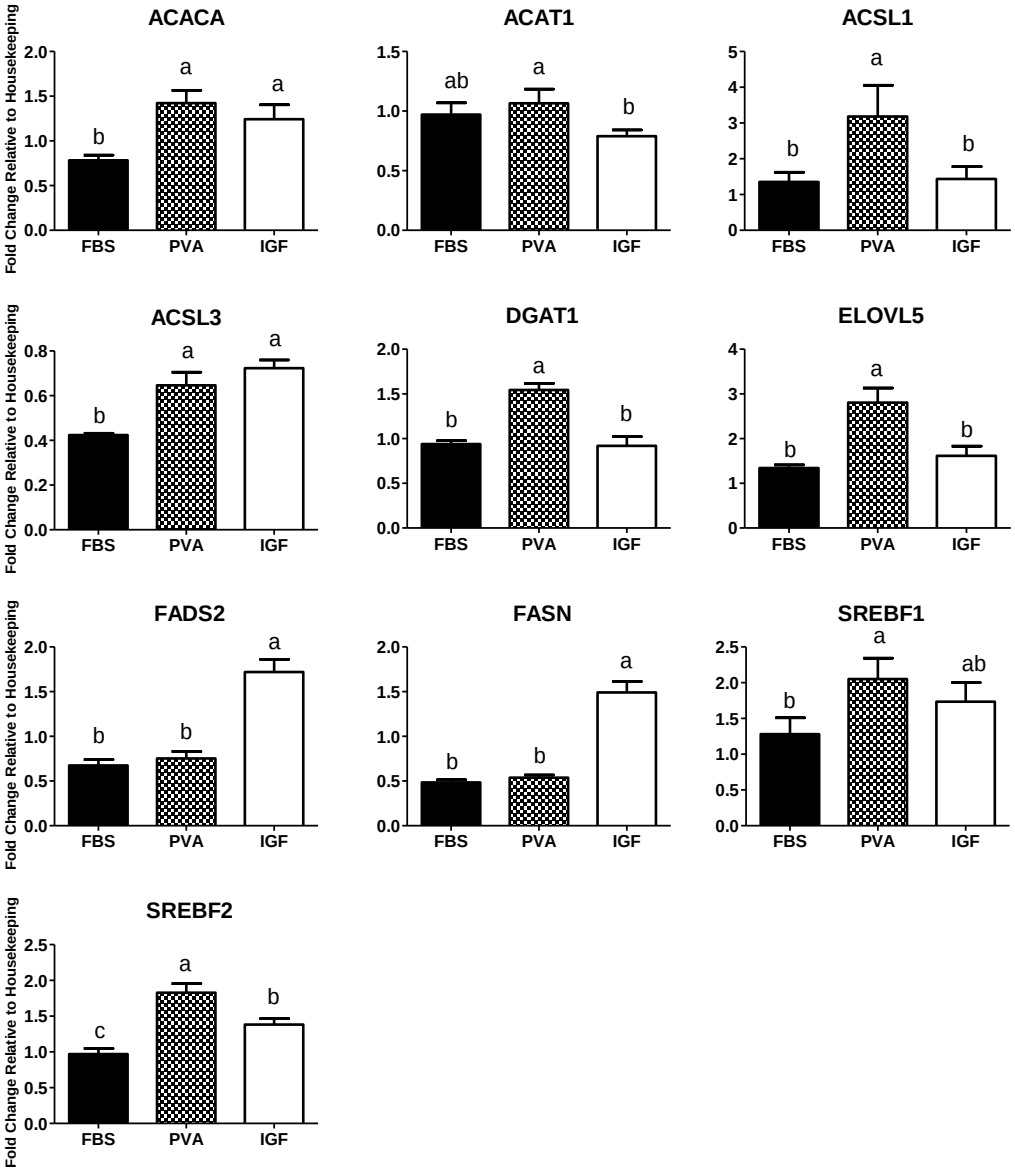


Fig. 6. Mean ± SEM of transcripts of genes for lipid metabolism of 6-8 cell stage embryos. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

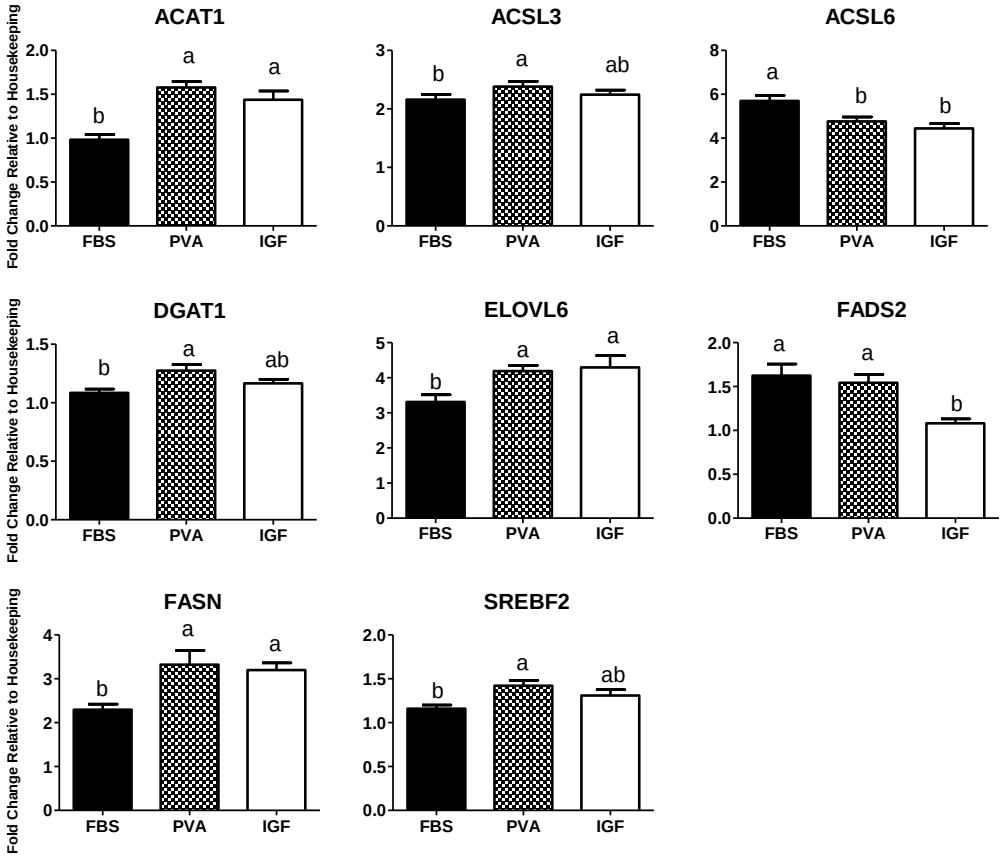


Fig. 7. Mean ± SEM of transcripts of genes for lipid metabolism of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

Supplementary File 1. Array of genes.

Symbol	Taqman ID	Fullname	Function	References
ACACA	Bt03213389_m1	Acetyl CoA carboxylase (ACC)	Lipid metabolism	Al Darwich et al. Prost & Lipid Med 2010. 93: 30-36
ACAT1	Bt03238649_g1	Acetyl-CoA acetyltransferase 1(ACAA1)	Lipid metabolism	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
ACSL1	Bt03248469_m1	Acyl-CoA synthetase long-chain family member 1	Lipid metabolism	Suzuki et al. J Reprod Dev. 2016;62(1):79-86
ACSL3	Bt04282138_m1	Acyl-CoA synthetase long-chain family member 3	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
ACSL6	Bt03231692_m1	Acyl-CoA synthetase long-chain family member 6	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
ADCY9	Bt04287024_m1	adenylate cyclase 9	Metabolism	Hacker et al. Genomics. 1998. 50: 97-104
AGTR1	Bt03213473_m1	Angiotensin II receptor type 1	Metabolism	Huang et al. J Cell Physiol. 2007. 211:816-25
AGTR2	AIY9Z3D	Angiotensin II receptor type 2	Metabolism	Pedersen-Bjergaard et al. Ame J Medic 121:3:246.e8
AKR1B1	Bt03218049_g1	Aldo-Keto Reductase Family 1, Member B1	Glucose metabolism	Arias-Alvarez et al. Theriogenology. 2011. 75(5):887-96.
AQP3	Bt03253663_m1	Aquaporin 3	Water channel	Wang et al. PLoS One. 2014. 9(4). 1-8
ATP5L	Bt03210836_g1	ATP synthase, H+ transporting, mitochondrial Fo complex subunit E	Energy production	Gad et al. Embryology. 2011. 26 (7):1693-1707
BAX	Bt03211777_g1	BCL2-Associated X Protein	Apoptosis and oxidative stress	Rizos et al. Biol Reprod. 2003.685:236-43
BCL2	Bt04298952_m1	B-cell CLL/lymphoma 2	Antiapoptotic	Fear et al. Biol Reprod.2011. 84. 43–51
BMP15	Bt03286494_u1	Bone Morphogenetic Protein 15	Growth factor activity/ fertility	Ghanem et al. Reproduction. 2011. 142(4):551-64.
CASP3	Bt03250954_g1	Caspase 3, apoptosis-related cysteine peptidase	Cell death inducers	Juriscova et al. Mol Hum Rep. 2003. 9(3):133-41.
CASP9	Bt04282453_m1	Caspase 9, apoptosis-related cysteine peptidase	Cell death inducers	Leidenfrost et al. 2011. 6(7): 1-13
CAT	Bt03228713_m1	Catalase	Cell death and survival	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
CDCA8	Bt03257041_g1	Cell division cycle associated 8	Cellular Development	Aghajanova et al. 2012. 86 (11): 1-21
CDH1	Bt03210093_g1	Cadherin 1	Embryonic compaction process	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
CDX2	Bt03649157_m1	Homeobox protein CDX-2	Differentiation and Implantation	Rayon et al. Sci Rep. 2016. 6:27139
DDIT3	Bt03251320_g1	DNA-damage-inducible transcript 3	Stress-responsive gene	Fontanier-Razzaq et al. Bio of Repro. 2001. 64: 1386–91
DGAT1	Bt03251719_g1	diacylglycerol O-acyltransferase 1	Lipid metabolism	Ghanem et al. Theriogenology. 2014. 82:238–50.
DICER1	Bt03217754_m1	Dicer 1, Ribonuclease III	Embryo development	Cui et al. Bioch & Bioph Research Com 2007. 352: 231–36
Dnmt1	Bt03224737_m1	DNA (Cytosine-5-)-Methyltransferase 1	Epigenetic regulation	Cho et al. J Vet Sci. 2014. 15(2): 225–231.
Dnmt3A	Bt01027164_m1	DNA (Cytosine-5-)-Methyltransferase 3Alpha	Epigenetic regulation	Wang et al. PLoS One. 2014. 9(4). 1-8
Dnmt3B	Bt03259810_m1	DNA (Cytosine-5-)-Methyltransferase 3Beta	Epigenetic regulation	Dobbs et al. PLoS One. 2013; 8(6): e66230.
DSC2	Bt03649202_m1	Desmocollin-II	Implantation signalling	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
DSC3	Bt04301926_m1	Desmocollin-III	Implantation signalling	Wrenzycki et al. Mol Reprod Dev. 1999. 53:8-18.
EGFR	AJT96D7	Epidermal growth factor - receptor	Embryo development	Kliem et al. Mol Reprod Dev. 1998. 51(4):402-12.
ELOVL5	Bt03235956_m1	ELOVL fatty acid elongase 5	Lipid metabolism	Warzych et al. Theriogenology. 2016

ELOVL6	Bt00907566_m1	ELOVL fatty acid elongase 6	Lipid metabolism	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
FADS2	Bt03256255_g1	Fatty acid desaturase 2	Lipid metabolism	Al Darwich et al. Prost & Lipid Med 2010. 93: 30-36
FASN	Bt03210485_m1	Fatty Acid synthase	Lipid metabolism	Stinshoff et al. Reprod Fertil Dev. 2014. 26. 502–510
FOXO3	Bt03649334_s1	Forkhead box O3	Cell signaling/Oxidative stress	Bomfim et al. 2017. Mol Reprod Dev. 84:1296–1305.
FSHR	Bt03212674_m1	Follicle-stimulating hormone receptor	Physiology	Patsoula et al. 2003. Fertil Steril. 79 (5) :1187-93
G6PD	Bt03649181_m1	Glucose 6 phosphate desidrogenase	Glucose metabolism	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
GAPDH	Bt03210912_g1	Glyceraldehyde-3-phosphate dehydrogenase	Housekeeping	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
GADD45A	Bt03225650_m1	growth arrest and DNA-damage-inducible, alpha	DNA repair/Stress response	Kaufmann et al. Gene Expr Patterns. 2011. 11: 465–70
GATM	Bt03237896_m1	Glycine amidinotransferase	Amino acid metabolism/ free radical scavenging	Bunel et al. Theriogenology. 2015. 83: 228–37
GPX1	Bt03259217_g1	Glutathione Peroxidase 1	Antioxidant defense	Harvey et al. Biol Reprod. 2007. 77:93-101.
GSK3A	Bt03273695_m1	Glycogen Synthase Kinase 3 alpha	Embryo development	Harris et al. Biol Reprod. 2013. 88(3):74.
GUCY1B3	Bt03215602_m1	Guanylate cyclase 1, soluble, beta 3	Metabolism	Saino et al. Onco Reports 2004. 12:47-52
H19	Custom TaqMan	Imprinted Maternally Expressed Transcript	Epigenetic control	Ibala-Romdhane et al. Eur J Hum Genet 2011. 19:1138-43
H1FOO	Bt03228652_g1	H1 histone family, member O, oocyte-specific	Epigenetic control	Bonilla et al. Mol Cel Endoc. 2011. 332: 170-79.
H2AFZ	Bt03216346_g1	H2A histone family, member Z	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
H3F3A	Bt03278804_g1	H3 histone, family 3A	Epigenetic control	Ghanem et al. Reproduction. 2011. 142(4):551-64.
H3F3B	Bt04319377_g1	H3 histone, family 3B (H3.3B)	Epigenetic control	Tang et al. Genesis 2013. 51:142–46.
HAND1	Bt04318733_g1	Heart and neural crest cell derivative 1	Trophoblast differentiation	Hall et al. Mol Reprod Dev. 2005. 72(1):16-24.
HDAC2	Bt03244871_m1	histone deacetylase 2	Chromatin structure	McGraw et al. Biol Reprod. 2003. 68: 383–89
HMBS	Bt03234763_m1	Hydroxymethylbilane synthase	Housekeeping	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
HSF1	Bt03249686_m1	Heat Shock Transcription Factor 1	Heat shock response	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
HSP90AA1	Bt03218068_g1	Heat Shock Protein 90kDa Alpha(cytosolic), class A member 1	Heat shock response	Bomfim et al. 2017. Mol Reprod Dev. 84:1296–1305.
HSPA1A	Bt03292670_g1	Heat shock 70kDa protein 1A	Heat shock response	Fear et al. Biol Reprod.2011. 84. 43–51
HSPA5	Bt03244880_m1	Heat shock protein family A (Hsp70) member 5	Heat shock response	Sudano et al. Reprod Fertil Dev. 2014. 26(8):1129-41
HSPD1	Bt04301470_g1	Heat Shock 60kDa protein 1A	Heat shock response	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
IFITM3	Bt03292973_g1	Interferon induced transmembrane protein 3	Germ cell competence in the epiblast	Smith et al. Reproduction. 2007. 133(1):231-42.
IFNT2	Bt03210589_g1	Interferon tau	Differentiation and Implantation	Wang et al. PLoS One. 2014. 9(4). 1-8
IGF1R	Bt03649217_m1	Insulin-Like Growth Factor 1 Receptor	IGF-I metabolism/MAPK cascade	Stinshoff et al. Reprod Fertil Dev. 2014. 26. 502–510
IGFBP2	Bt01040719_m1	Insulin-Like Growth Factor Binding Protein 2	IGF-I metabolism	Stinshoff et al. Reprod Fertil Dev. 2014. 26. 502–510
IGFBP4	Bt03259500_m1	Insulin-Like Growth Factor Binding Protein 4	IGF-I metabolism	Stinshoff et al. Reprod Fertil Dev. 2014. 26. 502–510
KRT8	Bt03225178_g1	Keratin proteins 8	Cellular assembly and organization	Ghanem et al. Reproduction. 2011. 142(4):551-64.
MAPK1	Bt03216718_g1	Mitogen-Activated Protein Kinase	Cellular Development	Bonilla et al. Mol Cel Endoc. 2011. 332: 170-79.

NANOG	Bt03220541_m1	Nanog homeobox	Pluripotency	Ozawa et al. BMC Dev Biol. 2012. 12:33.
NFkB2	Bt03272789_g1	Nuclear Factor Of Kappa Light Polypeptide Gene Enhancer In B-Cells 2	Epigenetic control	Thakur et al. Oncogene. 1994. 9 (8):2335-44
NOS2	Bt03249597_m1	Nitric oxide synthase 2, inducible	Nucleic acid metabolism	Harvey et al. Biol Reprod. 2007. 77:93-101.
NR1H3	Bt03218363_m1	nuclear receptor subfamily 1 group H member 3	Embryo development	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
NRP1	Bt04297034_g1	Neuropilin 1 (VEGF receptor)	Embryo development	Climato et al. Circualtion. 2009. 119 (16):2170-8
NRP2	Bt04316732_m1	Neuropilin 2 (VEGF receptor)	Embryo development	Fukahi et al. Clin Cancer Res. 2004. 10 (2):581-90
OTX2	Bt04316301_g1	Orthodenticle Homeobox 2	Embryo development	Smith et al. Reproduction. 2007. 133(1):231-42.
PA2G4	Bt03211241_g1	Proliferation-Associated 2G4	Embryo development	Gad et al. Embryology. 2011. 26 (7):1693-1707
PAF1	Bt03239371_g1	Platelet-activating factor	Implantação/Trofoblasto/Angiogênese	Bomfim et al. 2017. Mol Reprod Dev. 84:1296–1305.
PDE5A	Bt03214261_m1	Phosphodiesterase 5A, cGMP-specific	DNA replication/repair	Aghajanova. Biol Reprod. 2012. 86(1):11, 1–21
PFKP	Bt04316551_m1	Phosphofructokinase, platelet	Glucose metabolism	Boruszewska et al. Reprod Biol Endocrinol. 2015; 13: 44.
PGK1	Bt03225854_mH	Phosphoglycerate Kinase 1	Metabolism	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
PKP2	Bt03257632_m1	Plakophilin 2	Cell adhesion	Stanton et al. Mol Hum Reprod. 2002. 8(2):149-66.
POU5F1	Bt03223846_g1	POU class 5 homeobox 1 (OCT4)	Pluripotency	Wu and Schöler. Cell Regen (Lond). 2014; 3(1): 7.
PPIA	Bt03224617_g1	Peptidylprolyl isomerase A	Housekeeping	Bomfim et al. 2017. Mol Reprod Dev. 84:1296–1305.
PTGS2/COX2	Bt03214489_m1	Prostaglandin-endoperoxide synthase 2	Implantation	El-Sayed et al. Physiol Genomics. 2006. 28(1):84-96.
RPL15	Bt03288449_g1	Ribosomal Protein L15	Protein binding	Rigbolt et al. Sci Signal. 2011. 4(164):rs3
S100A10	Bt03215645_m1	S100 calcium binding protein A10	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
S100A14	Bt03230771_g1	S100 calcium binding protein A14	Embryo development	Ghanem et al. Reproduction. 2011. 142(4):551-64.
SLC2A1	Bt03215314_m1	solute carrier family 2 (facilitated glucose transporter), member 1	Glucose metabolism	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
SLC2A3	Bt03259514_g1	solute carrier family 2 (facilitated glucose transporter), member 3	Glucose metabolism	Stinshoff et al. Theriogenology. 2011. 76(8):1433-41
SLC2A4	Bt03215316_m1	solute carrier family 2 (facilitated glucose transporter), member 4	Glucose metabolism	Zheng et al. Mol Hum Reprod. 2007. 13(6):361-71.
SLC2A5	Bt03258296_m1	solute carrier family 2 (facilitated glucose/fructose transporter), member 5	Glucose metabolism	Dovolou et al. Reprod Domest Anim. 2011. 46(5):862-9.
SOD1	Bt03215423_g1	superoxide dismutase 1, soluble	Antioxidant defense	Harvey et al. Biol Reprod. 2007. 77:93-101.
SOD2	Bt03244551_m1	superoxide dismutase 2, mitochondrial	Antioxidant defense	Ghanem et al. Theriogenology. 2014. 82:238–50.
SREBF1	Bt03276370_m1	Sterol Regulatory Element Binding Transcription F1	Lipid metabolism	Ghanem et al. Theriogenology. 2014. 82:238–50.
SREBF2	Bt04283467_m1	Sterol Regulatory Element Binding Transcription F2	Lipid metabolism	Kivela et al. Atherosclerosis. 2012. 225(2)335-40
TFAM	Bt03260078_m1	Transcription Factor A, Mitochondrial	Mitochondrial DNA	Spikings et al. Biol Reprod 2007. 76(2)325-35
TNFRSF21	Bt03250597_m1	tumor necrosis factor receptor superfamily member 21	Promotes apoptosis	DeRosa et al. Cancer Immynol Immunother 57(6):777-87
TP53	Bt03223213_m1	tumor protein p53	Cell division/apoptosis	Bermejo-Alvarez et al. Reprod Fertil Dev. 2010. 22: 426–36
VEGFA	Bt03213282_m1	Vascular Endothelial Growth Factor A	Embryo development	Harvey et al. Biol Reprod. 2004. 71(4):1108-19.
XBP1	Bt03227621_g1	X-Box Binding Protein 1	Endoplasmic reticulum stress	Zhang et al. Biol Reprod. 2012. 86(4):128.

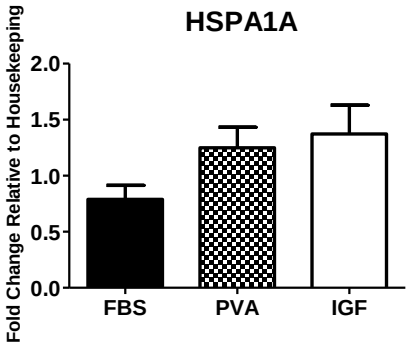
Supplementary File 2.

Total cell number and apoptosis rate of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA), or PVA + IGF1 (IGF) during oocyte maturation, embryo culture or both (Mean ± S. E.)

Treatment		Cell analysis	
IVM	IVC	Apoptosis (%)	Total cell n
FBS	FBS	0.39 ± 0.23 [a]	83.24 ± 5.18 [ab]
FBS	PVA	1.44 ± 0.38 [bc]	74.40 ± 8.57 [b]
FBS	IGF	1.01 ± 0.45 [abc]	83.18 ± 10.00 [ab]
PVA	FBS	0.46 ± 0.23 [a]	88.36 ± 5.12 [ab]
PVA	PVA	1.83 ± 0.34 [c]	85.84 ± 7.61 [ab]
PVA	IGF	0.87 ± 0.30 [ab]	84.50 ± 6.77 [ab]
IGF	FBS	1.04 ± 0.26 [abc]	87.25 ± 5.87 [ab]
IGF	PVA	1.21 ± 0.45 [abc]	86.91 ± 10.00 [ab]
IGF	IGF	1.06 ± 0.43 [abc]	102.08 ± 9.58 [a]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

Supplementary File 3.



Mean ± SEM of transcripts of gene *HSPA1A* of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

CAPÍTULO 3

Artigo II

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Effects of Insulin-like Growth Factor-1 on bovine embryo development, cellular characteristics and gene expression of blastocysts produced *in vitro*

Effects of Insulin-like Growth Factor-1 on bovine embryo development, cellular characteristics and gene expression of *in vitro* produced blastocysts

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Summary Sentence

The addition of IGF1 to i*n vitro* embryo production in the absence of FBS improves embryo quality, increasing total cell number, cells of the ICM, stimulates glucose metabolism and improves expression of embryo quality biomarkers.

Keywords: *in vitro* culture, IGF1, inner cell mass, blastocyst, glucose metabolism

Abstract

This study aimed to investigate the addition of insulin-like growth factor 1 (IGF1) during *in vitro* maturation (IVM) or *in vitro* culture (IVC) in chemically semi-defined media on embryo quality, analyzing cell allocation, total cell number, glucose metabolism and expression of genes related to embryo development, implantation, glucose metabolism, and oxidative phosphorylation. IVM was performed in three different media with the addition of 10% fetal bovine serum (FBS), or 3 mg/mL of polyvinyl alcohol (PVA), or PVA + 100 ng/mL IGF1. IVC was performed in three different media with the addition of 2,5% of FBS, or 3 mg/mL of PVA, or PVA + 100 ng/mL of IGF1. Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells demonstrated that embryos resulting from IVM with IGF1 had increased ($p < 0.05$) total cell number of blastocysts and embryos cultured with IGF1 had increased ($p < 0.05$) amount of cells of the ICM than embryos cultured in FBS. Glucose and lactate concentrations, demonstrated that IGF1 increases glucose uptake and lactate synthesis of cumulus-oocyte complexes. qRT-PCR revealed higher expression of *VEGFA* in 6-8 cell stage embryos cultured with IGF1 than cultured with PVA or FBS. In expanded blastocysts, *IFNT2, NANOG, OTX2*, and *POU5F1* were up-regulated in PVA and IGF groups in relation to FBS. The addition of IGF1 during IVM in chemically semi-defined media improves embryo quality, increases total cell number of blastocysts, stimulates glucose metabolism and during IVC increases cells of the ICM, and improves expression of embryo quality biomarkers.

Introduction

The microenvironment in which the oocyte undergoes maturation and posterior fertilization, can affect oocyte competence and embryo development [1]. In addition, culture media where the embryo develops until blastocyst stage has a direct influence on embryo quality and subsequent developmental potential [2]. In the attempt to improve potential embryo development *in vitro* and replicate *in vivo* conditions, the effects of several growth factors have been studied in culture media and in the female reproductive tract [3-6].

Research has shown that insulin-like growth factor I (IGF1) plays an important role in bovine reproduction [7,8]. It improves follicular development and steroidogenesis [9,10], and is involved in oocyte maturation[11]. It is known that IGF1 is produced by cumulus cells [11] and both cumulus cells and oocytes express IGF1R receptor [12,13]. IGF1 is also found in the maternal environment in the oviduct and the uterus secretions, and its presence is important during early embryo development [5,6,14]. IGF1 binds to its receptor IGF1R, which activates a cytoplasmic signal cascade through phosphatidyl inositol-3 kinase/serine-threonine protein kinase B (PI3K/AKT) and serine-threonine kinase (RAF) – mitogen activated protein kinase (MAPK) pathways that regulate cell proliferation, apoptosis, differentiation, migration and transcription [15,16].

The addition of IGF1 to IVM media stimulates oocyte maturation, oxidative metabolism, enhances nuclear and cytoplasmic maturation and increases protein synthesis [17-20]. A study showed that the intraovarian IGF1 injection increases the number of cells from the inner cell mas (ICM) of *in vitro* produced blastocysts derived from morulae recovered from treated cows [1]. However, the addition of 100 ng/mL IGF1 during IVM has shown no effect on cleavage rate, percentage of blastocyst or

blastocyst total cell number [19-21]. Nevertheless, IGF1 effects during IVM of bovine oocytes without the exposure to fetal bovine serum (FBS) and their developmental competence have not been well studied.

On the other hand, supplementation of IGF1 during IVC improves embryo development and leads to higher blastocyst yield [3, 21-24]. The addition of IGF1 in culture media results in greater mitogenic response [25], and the pro-developmental effects of this growth factor occur during the period from 4 to 8 days after fertilization involving actions mediated by MAPK pathway [26]. Thus, embryos produced with IGF1 during IVC have higher blastocyst cell numbers [3,23] and this proliferative effect appears predominantly in the ICM [3,6,27].

Gene expression can be altered by *in vitro* culture conditions in bovine embryos [28]. Some genes have important roles in pregnancy and embryo survival or loss in blastocysts during preimplantation period [29], for instance, *IFNT2* is responsible for maternal recognition [30], *POU5F1* and *NANOG* are related to pluripotency [31] and *VEGF* to embryo development [32]. Embryos cultured in IGF1 have higher IFNT2 and *DNMT3A* gene transcripts [33] and an increase on *Na/K ATPase* and *DSCII* expression has also been shown, which may improve blastocyst expansion and development after hatching [34].

FBS is still the most used supplement during *in vitro* embryo production (IVP) [35-37] and it contains multiple growth factors, including IGF1 [22,35]. Most studies that investigate IGF1 effects on embryo production include FBS during IVM or IVC. However, the addition of IGF1 to IVM and IVC in chemically defined media and its response during embryo development are not fully understood. Therefore, the aim of this study was to evaluate the effects of the addition of IGF1 during IVM or IVC in chemically semi-defined media on embryo quality, including cell allocation, total cell

number, glucose metabolism and gene expression analysis of genes related to embryo development, implantation, glucose metabolism, and oxidative phosphorylation.

Materials and methods

In vitro embryo production

Unless otherwise stated, all chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA). Follicles with a diameter of 2 – 8 mm were aspirated from the ovaries of slaughtered crossbred *Bos indicus* cows using a 10 mL syringe with a 40 x 12 needle. Cumulus–oocyte complexes (COCs) were collected and pooled in a conical tube (Falcon®, NY, USA). Oocytes with three or more compact layers of cumulus cells and homogeneous cytoplasm were selected for *in vitro* maturation.

Oocyte maturation medium consisted of TCM-199 sodium bicarbonate with Earle's salts (GIBCO, Grand Island, NY, USA) supplemented with 0.2 mM pyruvate, 1 μg/mL FSH (Folltropin – V, Bioniche Animal Health Canada Inc., Bellevile, Ontario, Canada), 50 μg/mL LH (Lutropin – V, Bioniche Animal Health), 100 μg/mL streptomycin sulfate, 100 UI/mL penicillin, and 85 μg/mL amikacin. Three groups of IVM were established with the respective addition of: 1) FBS: 10% of fetal bovine serum (FBS) (GIBCO), or 2) PVA: 3 mg/mL of PVA, or 3) IGF: 3 mg/mL of PVA + 100 ng/mL of IGF1. COCs (20 per drop) were matured in 90μL drops covered with sterile mineral oil at 38.5 °C and 5% CO_2 in humidified air for 22 to 24 hours.

Fertilization was performed in 90 μL drops of IVF-TALP medium [38,39]. Frozen-thawed spermatozoa of a single batch from one *Bos indicus* bull (CRV Lagoa, Sertãozinho, São Paulo, Brazil) were purified by centrifugation in Percoll® (Nutricell, Campinas, SP, Brasil) gradient (90% and 45%) at 900g for 5 minutes followed by

centrifugation in IVF-TALP at 200g for 3 minutes. Concentration was adjusted to 2×10^6 sperm/mL and oocytes and spermatozoa were incubated under the same conditions as the *in vitro* maturation procedure for 18 hours.

Presumptive zygotes were denuded of cumulus cells by repeated pipetting within the fertilization drop and synthetic oviduct fluid (SOFaaci) [40] was used for embryo culture, supplemented with 0.2 mM pyruvate, 5 mg/mL BSA, 100 µg/mL streptomycin, 100 UI/mL penicillin, and 85 µg/mL amikacin. Three groups of IVC were established with the respective addition of: 1) FBS: 2,5% of FBS, or 2) PVA: 3 mg/mL of PVA, or 3) IGF: 3 mg/mL of PVA + 100 ng/mL of IGF1. Embryos were cultured in 90 µL drops covered with sterile mineral oil, at 38.5 °C and 5% CO_2 in humidified air. At 66 hours post insemination (hpi), cleavage rate was observed and Day 7 (168 hpi) blastocyst rate and embryo quality were analyzed.

Differential staining of inner cell mass (ICM) and trophectoderm (TE) cells

Differential cell staining was carried out as described by Thouas et al. [41]. Blastocysts of different developmental stages (early, expanded and hatched blastocysts) were incubated in 50 µl of BSAfree Hepes buffered medium with 1% Triton X-100 and 100 µg/ml propidium iodide for 10 s. Blastocysts were transferred into 400 µl of fixative solution of 100% ethanol with 25 µg/ml Hoechst 33342 (Invitrogen, Eugene, OR, USA) and stored at 4°C overnight. Embryos were transferred to a glass microscope slide in a 10µL drop of glycerol gently flattened with a coverslip.

Samples were analyzed using an inverted epifluorescence microscope (Leica DMI 3000 B, Leica Microsystems Wetzlar GmbH, Germany). Green excitation intensity with 515 – 560 filter was used for PI, and UV with 340 – 380 filter for Hoechst 33342. Red

fluorescent nuclei were considered from TE and blue from ICM. Cell counting was performed from digital images obtained from 155 embryos produced in 6 replicates.

Glucose metabolism

Glucose and lactate concentrations were determined in spent media (including media without oocytes/embryos during IVM and IVC). At 22 h of IVM, 66 hpi and 168h hpi spent media were collected, snap frozen, and stored at -80°C. A Hitachi 912 chemical analyzer (F. Hoffmann-La Roche Ltd.) was used to measure glucose and lactate levels from 5 experimental replicates. To determine glucose uptake, concentration of glucose in media blanks (drops of media, cultured without cells) was taken as reference. The base medium (TCM199) did not contain lactate. Glucose uptake and lactate production were expressed as pmol/COC per h [42].

RNA extraction and Reverse transcription (RT)

To reduce individual variation and increase the amount of extracted RNA, four pools of 15 cleaved embryos with at least 6 – 8 cells collected at 66 hpi and four pools of 4 expanded blastocysts from each group were stored at -80 °C with Recombinant Ribonuclease Inhibitor (RNaseOUT®) (Invitrogen, Carlsbad, CA, USA). Total RNA was extracted from each pool using RNA Isolation Kit (PicoPure®) (Arcturus, Mountain View, CA, USA). Extracted RNA was assessed for quantity by a spectrophotometer (ND-2000, NanoDrop, Wilmington, DE, USA) and for quality by 2100 Bioanalyzer apparatus (Agilent Technologies, CA, USA) with RNA Pico Chips (Agilent Technologies). All analyzed samples had RNA integrity number (RIN) > 9. The High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City,

CA, USA) was used to perform the reverse transcription (RT) of samples according to the manufacturer's instructions.

Real-time polymerase chain reaction (qRT-PCR) by Fluidigm Biomark™ HD system

With the Applied Biosystems™ TaqMan® Assays, specific for *Bos taurus* species, gene expression analysis of bovine embryos was performed. The mRNA abundance of 96 genes was analyzed per functional categories of embryos (for further details, see supplementary material 2). Prior to qRT-PCR thermal cycling, each sample was submitted to sequence-specific preamplification process as follows: 1.25 µL assay mix (TaqMan® Assay was pooled to a final concentration of 0.2X for each of the 96 assays), 2.5 µL TaqMan PreAmp Master Mix (Applied Biosystems, #4391128) and 1.25 µL cDNA (5 ng/µL). Reactions were activated at 95°C for 10 min followed by denaturing at 95ºC for 15 s, annealing and amplification at 60ºC for 4 min for 14 cycles. The preamplified products were diluted 6-fold prior to qRT-PCR analysis.

For gene expression analysis, the sample solution prepared consisted of 2.25 µL cDNA (preamplified products), 2.5 µL of TaqMan Universal PCR Master Mix (2X, Applied Biosystems) and 0.25 µL of 20X GE Sample Loading Reagent (Fluidigm); and the assay solution: 2.5 µL of 20X TaqMan Gene Expression Assay (Applied Biosystems) and 2.5 µL of 2X Assay Loading Reagent (Fluidigm). The 96.96 Dynamic Array™ Integrated Fluidic Circuits (Fluidigm) chip was used to data collection. After priming, the chip was loaded with 5 µL of each assay solution and 5 µL of each sample solution. The qRT-PCR thermal cycling was performed in the Biomark HD System (Fluidigm, South San Francisco, CA, USA) using the protocol TaqMan GE 96x96 Standard, that consisted of one stage of Thermal Mix (50ºC for 2 min, 70ºC for 20 min

and 25ºC for 10 min) followed by a Hot Start stage (50ºC for 2 min and 95ºC for 10 min), followed by 40 cycles of denaturation (95ºC for 15 s), primer annealing and extension (60ºC for 60 s).

Relative expression of target gene in each sample was calculated using the ΔΔCt method using the mean of control group as calibrator [43]. Data subjected to statistical analysis were generated by normalization of CT values from the target genes with the geometric mean of the CT values from the reference genes in embryos (*GAPDH, HMBS* and *PPIA*).

Experimental design and statistical analysis

A 3 x 3 factorial design (three supplements of IVM and three of IVC) determined a total of 9 experimental groups. Data were analyzed by analysis of variance (ANOVA), from PROC GLIMMIX model from SAS software (SAS Inst. Inc., Cary, NC, USA). Tuckey test was used to compare the means. Sources of variations in the model included IVM supplements (FBS, PVA and IGF), IVC supplements (FBS, PVA and IGF) and first order interactions; all factors were considered fixed effects. P value ≤ 0.05 was considered to be significant.

Results

Effect of treatment on embryo development.

Groups matured in FBS had higher ($p < 0.05$) percentage of cleaved embryos (Table 1). Greater ($p < 0.05$) number of expanded blastocyst were observed in all groups cultured with FBS. Embryo production with FBS during IVM and IVC (FBS-FBS) had greater

($p < 0.05$) total blastocyst yield than other groups, followed by PVA-FBS and IGF-FBS. In contrast, IGF-PVA and IGF-IGF presented decreased ($p < 0.05$) total blastocyst rates.

Effect of treatment on ICM, TE and total cell number in blastocysts

Data was arranged by IVM and IVC treatment separately. Blastocysts obtained from oocytes matured with IGF had greater ($p < 0.05$) total cell number than embryos obtained from oocytes matured in FBS (Table 2). Furthermore, PVA and IGF groups produced embryos with higher ($p < 0.05$) percentage of ICM cells than embryos derived from IVM with FBS. There was also an IVC treatment effect, embryos cultured in IGF had an increased ($p < 0.05$) percentage of ICM cells than embryos cultured with FBS (Table 3).

Effect of treatment on glucose metabolism

Oocytes matured with PVA and IGF had higher ($p < 0.05$) glucose uptake compared to oocytes matured with FBS (Figure 1). Moreover, oocytes matured with IGF had greater lactate synthesis than oocytes of FBS group, which produced more lactate than oocytes matured with PVA. Glucose and lactate concentrations in spent media at 66 hpi and 168h hpi were not feasible to measure by the analyzer.

Effect of treatment on expression of genes related to embryo development and implantation

Gene expression was not altered ($p > 0.05$) by IVM treatment in 6-8 cell stage embryos. Expression of genes related to embryo development of expanded blastocysts that had an IVM treatment effect, are presented in figure 2. *S100A10* and *S100A14* were up-regulated in IGF group in relation to FBS and PVA groups.

IVC treatment effect on embryo development and implantation genes of 6-8 cell stage embryos are presented in figure 3. Of the 26 genes analyzed, 14 genes were up-regulated ($p < 0.05$) in PVA group in relation to FBS and IGF groups. *IFITM3* gene in IGF group was down-regulated ($p < 0.05$) in relation to FBS and PVA groups. *S100A10* in IGF group was down-regulated in relation to FBS group. In contrast, *VEGFA* had higher ($p < 0.05$) expression in IGF group in relation to PVA and FBS. No significant differences ($p > 0.05$) were observed for *AQP3, COX2, DSC2, IFNT2, KRT8, OTX2*, and *S100A14* genes.

In expanded blastocysts, *IFNT2, NANOG, OTX2*, and POU5F1 genes were up-regulated ($p < 0.05$) in PVA and IGF groups in relation to FBS (Figure 4). *CDX2, PKP2* and *S100A14* genes of embryos cultured in IGF were down-regulated ($p < 0.05$) in relation to FBS and PVA groups.

Expression of genes related to glucose metabolism and oxidative phosphorylation

Results of IVC treatment effect on glucose metabolism and oxidative phosphorylation genes of 6-8 cell stage embryos are presented in figure 5. Ten genes were analyzed, *SLC2A1, 3, 4* and *GATM* genes in PVA group were up-regulated in relation to FBS and IGF groups. *ATP5L, PFKP* and *PGK1* genes in IGF group were down-regulated in relation to FBS group. Conversely, *G6PD* gene in IGF was up-regulated in relation to the other two groups. No significant differences were observed for *SLC2A5* gene. However, in expanded blastocysts, *SLC2A5* was down-regulated in IGF group compared to the other two groups (Figure 6).

Discussion

We analyzed the effects of the addition of IGF1 during IVM or IVC in chemically semi-defined media on embryo development and compared with blastocyst production with FBS. Results suggest that the use of IGF1 in a semi-defined media during IVM or IVC or both is not able to improve embryo development in order to obtain similar blastocyst rate with FBS, but there are potential benefits of IGF1 addition in *in vitro* culture of embryos.

There was no positive effect of IGF1on oocyte competence to undergo cleavage or achieve blastocyst stage. These results are similar to previously obtained in semi-defined [19] and undefined media [20,21]. It is evident that oocyte maturation is a complex process where multiple growth factors, hormones and proteins have an additive action to promote nuclear and cytoplasmic maturation [17,19,44]. Moreover, studies have shown that association of IGF1 to other growth factors during IVM significantly enhanced nuclear and cytoplasmic maturation of oocytes, cumulus cells response [17,19] and subsequent embryo development [19].

Even there was no effect of IGF1 in blastocyst formation, hatched blastocyst rate was similar between IGF-FBS and FBS-FBS, probably as a result of a cumulative effect of IGF1 and FBS during IVM and IVC. Total cell number of blastocysts was higher in embryos resulting from oocytes matured with IGF1 compared to IVM with FBS and greater amount of cells was observed in IGF-FBS group. Different from other studies [19-21], this proliferative effect may be a result of the mitogenic effect of IGF1 by the activation of MAPK pathway through IGF1R [3,25].

There was no effect of IGF1 in IVM on the ICM or TE of blastocysts. However, FBS in IVM decreased ICM number of cells, alternatively increased TE number of

cells, consequently altered ICM:TE ratio. FBS can be responsible for these results, as it is known that blastocysts cultured with FBS have clear large blastocoels and more evident ICM [40], but they have less total cell number than blastocysts cultured without FBS [45 ,46].

It is possible that the IGF1 concentration used during oocyte maturation was not ideal, as no benefits were observed and even reduced embryo production was detected. It is believed that IGF1 concentration of 100 ng/mL might be supraphysiological [47], as IGF1 concentrations in follicular fluid of *Bos taurus* cows is lower than 35 ng/mL [48,49]. However, in crossbred cows (*Bos taurus* and *Bos indicus*) IGF1 levels in follicular fluid range between 83 and 148 ng/mL [50]. We used *Bos indicus* cows, therefore, apparently IGF1 dose used is physiological. Although IGF1 concentration used seems to be suitable, there is still evidence that IGF1 effects are more evident when lower doses are used *in vitro* [3, 20].

Total embryo production obtained from IVC in semi-defined media with or without addition of IGF1 did not reached similar results to production with the presence of FBS during embryo culture. Considering IVM and IVC in semi-defined media groups only, IVC with IGF1 increased 26 to 31 % blastocyst rate than groups cultured in PVA, but these numbers were not significant to affirm that IGF1 had an effect on blastocyst yield. In contrast, many authors have found that IGF1 improves embryo development and blastocyst production in defined [3] and undefined media [21-23].

Likewise IVM, a study demonstrated that the association of several growth factors and cytokines, including IGF1 during IVC, improved embryo development and increased percentage of hatched blastocysts. This association also promoted the increase of cell number in the ICM and the TE [24]. It is known that IGF1 is present in the oviduct and uterus fluid during embryo development [5,6], but it seems that it is

necessary the presence of other “embryotrophic factors” besides IGF1 to enhance embryo development [5].

IGF1 during IVC did not have effect on total cell number of blastocysts, different from previous studies [3,6,21,23]. In contrast, blastocysts cultured with IGF1 had higher numbers of ICM cells than blastocysts cultured in FBS, similar to results obtained by other authors [3,6,27]. Most studies have verified IGF1 effects during IVC in media containing fetal bovine serum, which contains many other growth factors and molecules, possibly interfering in the results [35].

Cleaved embryos cultured in PVA or IGF apparently were in advanced stage of cleavage, compared to embryos cultured in FBS. PVA or IGF embryos had more number of cells at 66 hpi than embryos of FBS group. This fact might be due to serum biphasic effect on bovine embryo development, which inhibits the first cell divisions of embryos during culture, but further it enhances blastocyst formation [51].

Cumulus cells surrounding zygotes during IVC usually have no effect on cleavage or blastocyst rate [52]. In this study, presumptive zygotes were not completely denuded of cumulus cells by repeated pipetting; therefore a granulosa cell monolayer from cumulus cells adhered to plastic dishes at the bottom of cleaved embryos and blastocysts of IVC containing FBS. In contrast, in IVC with PVA and IGF remaining cumulus cells stayed at the zona pellucida and embraced all the embryos. Thus, embryos were carefully separated when cleavage rate was determined, to prevent embryo arrest.

Regarding glucose metabolism during IVM, PVA and IGF groups had higher glucose uptake compared to FBS. However, FBS medium had initially lower concentration of glucose than other groups and this probably occurred due to addition of 10% of serum in TCM199 media, which causes a dilution of initial concentration of

glucose. Nevertheless, oocytes matured in IGF1 produced more lactate than oocytes of the other two groups.

Low concentrations of IGF1 stimulate glucose consumption and glycolysis, consequently, the formation of lactate in different tissues [53] and oocytes matured with IGF1 lead to embryos with greater expression of *SLC2A8* glucose transporter, which indicates that it would subsequently result in increased glucose uptake [1]. According to our results, the addition of IGF1 in oocyte maturation leads to higher efficiency to use glucose as a substrate and produce higher quantities of metabolites as lactate through the glycolytic pathway.

There are few studies of gene expression in embryos derived from oocytes matured with IGF1 [1,54]. Apparently, IVM media does not have strong effects on gene expression of 6-8 cell stage embryos and only three of the thirty six analyzed genes were altered in expanded blastocysts. In our study, *OTX2*, was down-regulated in PVA and IGF groups compared with FBS group. Furthermore, two other important genes for embryo development related to calcium ion binding, *S100A10* and *S100A14*, were more expressed in embryos derived from oocytes matured with IGF1. A research that analyzed gene expression profile of *in vivo* derived bovine embryo biopsies based on pregnancy outcomes, revealed that embryos which ended up with no pregnancy were found to be enriched with transcripts of both *S100A10* and *S100A14* genes [55]. Therefore, even though the addition of 100 ng/mL of IGF to maturation medium produced embryos with higher amount of cells, no other positive effects were obtained, even production rate was reduced, suggesting that this concentration may not be suitable for embryo production as it may reduce pregnancy rates. On the other hand, the addition of 100 ng/mL of IGF to culture medium decreased the amount of transcripts of

S100A10 gene in 6-8 cell stage embryos and of *S100A14* gene in expanded blastocysts, probably improving post-transfer embryo survival.

IGF1 in culture media decreased *IFITM3* expression [56]. *IFITM3* function is to inhibit ICM cell proliferation [57]. As expected, *IFITM3* in IGF group was down-regulated in relation to FBS and PVA groups of 6-8 cell stage embryos in the present study, which also can be related to the higher numbers of ICM cells obtained in blastocysts cultured with IGF1. However, this IVC effect was not observed anymore in expanded blastocysts transcripts, this indicates that ICM and TE differentiation stage starts before blastocyst formation and concentrations of IGF1 needed for embryo development may differ from stage to stage.

Interestingly, *VEGFA* in IGF group was up-regulated in relation to PVA and FBS groups of 6-8 cell stage embryos. *VEGF* is considered critical for fetal and placental angiogenic development. The non-expression of *VEGF* in the embryo leads to intrauterine death due to vascular formation failure [32]. Thus, embryo culture supplementation with IGF1has a positive effect on embryo development as it stimulates angiogenesis.

In addition, *IFNT2*, *NANOG*, *OTX2*, and *POU5F1* genes were more expressed in expanded blastocysts of PVA and IGF groups in relation to FBS group. These genes are considered potential embryo quality biomarkers, *POU5F1* and *NANOG* are genes related to pluripotency [31], *OTX2* to embryo development [57], and *IFNT2* to gestational embryo recognition [58]. In a recent study, significant high expression of *IFNT2* was also observed in blastocysts derived from IGF1 supplemented embryo culture conditions [33]. So, FBS removal from IVC appears to be advantageous and IGF1 addition can be favorable to *in vitro* embryo production.

POU5F1 and *NANOG* genes are more expressed in the ICM cells than in TE cells. In contrast, *CDX2* is more expressed in TE cells, and its expression inhibits *POU5F1* expression allowing cellular differentiation [31,59]. In our results, *CDX2* in IGF group was down-regulated in relation to FBS and PVA groups and *POU5F1* was more expressed in PVA and IGF groups. Apparently this gene expression matches cell allocation, as embryos cultured with IGF1 had higher percentage of ICM cells and less TE cells, opposed to embryos cultured with FBS which had higher percentage of TE cells and less ICM cells. These results reinforce the importance of embryo production without FBS and that IGF1 improves formation of the ICM.

The *DSC2* gene is involved in the desmosomes formation which has an important role in stabilizing the TE during blastocyst formation and expansion [60]. A study previously obtained an increased amount of transcripts for *DSC2* gene in embryos cultured with IGF1 [34]. However in our results, *DSC2* did not present significant difference in 6-8 cell stage embryos and in expanded blastocysts it was similar to embryos cultured in FBS. Then, FBS and IGF1 cultured embryos probably had similar blastocyst formation and TE stabilization, even embryos cultured with IGF1 had reduced number of TE cells.

Even though *G6PD* is a gene related to metabolism [61], the high expression of this gene, stimulates cellular development and proliferation [62]. *G6PD* is a X-linked gene, its transcripts levels are greater in IVP embryos than embryos produced *in vivo* and *G6PD* transcripts are also higher in female than in male embryos [63]. *G6PD* gene in IGF group was more expressed than in the other two groups, which can be related to proliferative actions of IGF1 with the activation of the MAPK pathway [15,16], improving blastocyst development with an increase in cell proliferation.

IGF1 effects on subsequent embryo survival *in vivo* are more likely the result of differences in gene expression rather than in changes in cell number, allocation or apoptosis [34]. In the present study, transcripts of genes related to embryo development, implantation and glucose metabolism of 6-8 cell stage embryos and expanded blastocysts obtained with the addition of IGF1 to IVM and IVC in chemically semi-defined media showed interesting and revealing results regarding this matter.

Conclusions

In conclusion, present results demonstrated that the addition of IGF1 during oocyte maturation in chemically semi-defined media improves embryo quality, increasing total cell number of blastocysts and embryo culture with the supplementation of IGF1 increases the amount of cells of the inner cell mass. The use of IGF1 during *in vitro* maturation stimulates glucose metabolism of cumulus-oocytes complexes, increasing glucose uptake and lactate synthesis. Gene expression results suggest that FBS removal from embryo culture is favorable to *in vitro* embryo production and that the addition of IGF1 to culture media may be beneficial for embryos as it improves expression of important embryo quality biomarkers, with the possibility to enhance embryo development and survival after transfer.

Supplementary data

Supplementary File 1. Allocation of inner cell mass (ICM) and trophectoderm (TE) cells, ICM:TE ratio and total cell number of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during oocyte maturation, embryo culture or both (Mean ± S. E.)

Supplementary File 2. Array of genes.

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Table 1

Cleavage rate and blastocyst development of *in vitro* produced bovine embryos of 20 replicates (n = 3,819 oocytes) in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during oocyte maturation, embryo culture or both.

Treatment			Embryo Development/Oocytes				
IVM	IVC	Oocytes (n ≈ 20/drop)	Cleavage (%)	BL (%)	BX (%)	BH (%)	Total Blastocyst (%)
FBS	FBS	409	346 (85) [a]	50 (12) [a]	107 (26) [a]	20 (5) [a]	177 (43) [a]
FBS	PVA	421	359 (85) [a]	25 (6) [bc]	40 (10) [d]	2 (0) [c]	67 (16) [d]
FBS	IGF	422	366 (87) [a]	25 (6) [bc]	38 (9) [d]	0 (0) [c]	63 (15) [de]
PVA	FBS	425	302 (72) [b]	35 (8) [b]	88 (21) [b]	6 (1) [bc]	129 (31) [b]
PVA	PVA	425	297 (71) [b]	14 (3) [c]	24 (6) [de]	2 (0) [c]	40 (10) [ef]
PVA	IGF	434	324 (75) [b]	20 (5) [c]	35 (8) [d]	3 (1) [c]	58 (14) [de]
IGF	FBS	416	289 (70) [b]	21 (5) [bc]	66 (16) [c]	15 (4) [ab]	102 (24) [c]
IGF	PVA	424	308 (72) [b]	19 (4) [c]	12 (3) [e]	0 (0) [c]	31 (7) [f]
IGF	IGF	443	312 (71) [b]	15 (3) [c]	25 (6) [de]	2 (0) [c]	42 (10) [ef]

[a, b] Data with different superscripts in the same column differ significantly ($p < 0.05$).

Abbreviations: BL, blastocyst; BX, expanded blastocysts; BH, hatched blastocysts.

Table 2

Allocation of inner cell mass (ICM) and trophectoderm (TE) cells and ICM:TE ratio of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during oocyte maturation (Mean ± S. E.)

Treatment	Cell analysis			
IVM	ICM (%)	TE (%)	ICM:TE ratio	Total cell N
FBS	35.4 ± 2.0 [b]	64.6 ± 2.0 [a]	1: 3.68 [a]	54.0 ± 2.8 [b]
PVA	48.0 ± 3.0 [a]	52.0 ± 3.0 [b]	1: 1.72 [b]	59.6 ± 4.1 [ab]
IGF	43.3 ± 3.0 [a]	55.6 ± 3.1 [b]	1: 1.63 [b]	67.4 ± 4.2 [a]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

Table 3

Allocation of inner cell mass (ICM) and trophectoderm (TE) cells and ICM:TE ratio of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA), or PVA + IGF1 (IGF) during embryo culture (Mean ± S. E.).

Treatment	Cell analysis			
IVC	ICM (%)	TE (%)	ICM:TE ratio	Total cell N
FBS	37.8 ± 1.7 [b]	62.1 ± 1.7 [a]	1: 2.50 [a]	64.8 ± 2.3 [a]
PVA	44.1 ± 3.4 [ab]	55.9 ± 3.3 [ab]	1: 2.17 [a]	55.3 ± 4.6 [a]
IGF	44.8 ± 2.8 [a]	54.1 ± 2.9 [b]	1: 2.37 [a]	60.9 ± 3.9 [a]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

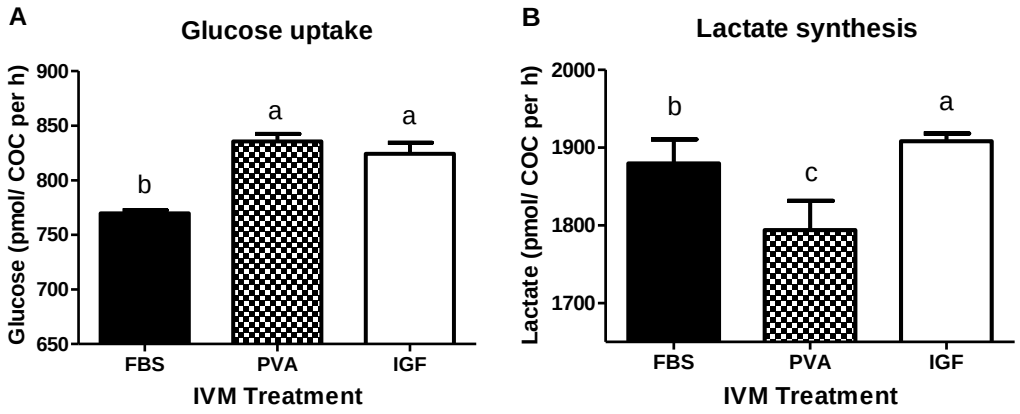

Figure 1. Mean ± SEM of glucose uptake (A) and lactate synthesis (B). Experimental groups of IVM with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

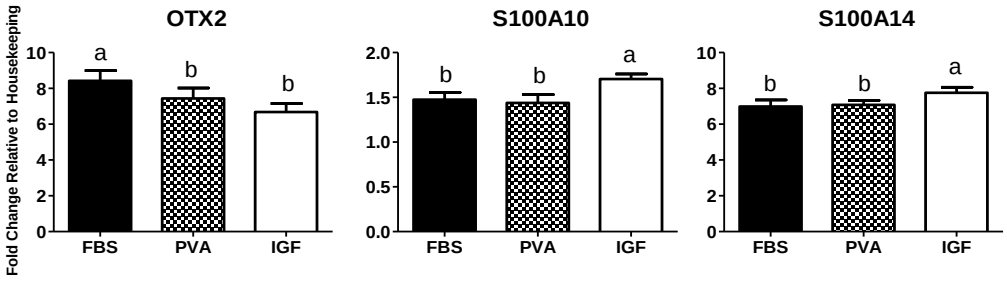

Figure 2. Mean ± SEM of transcripts of genes for embryo development of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVM with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

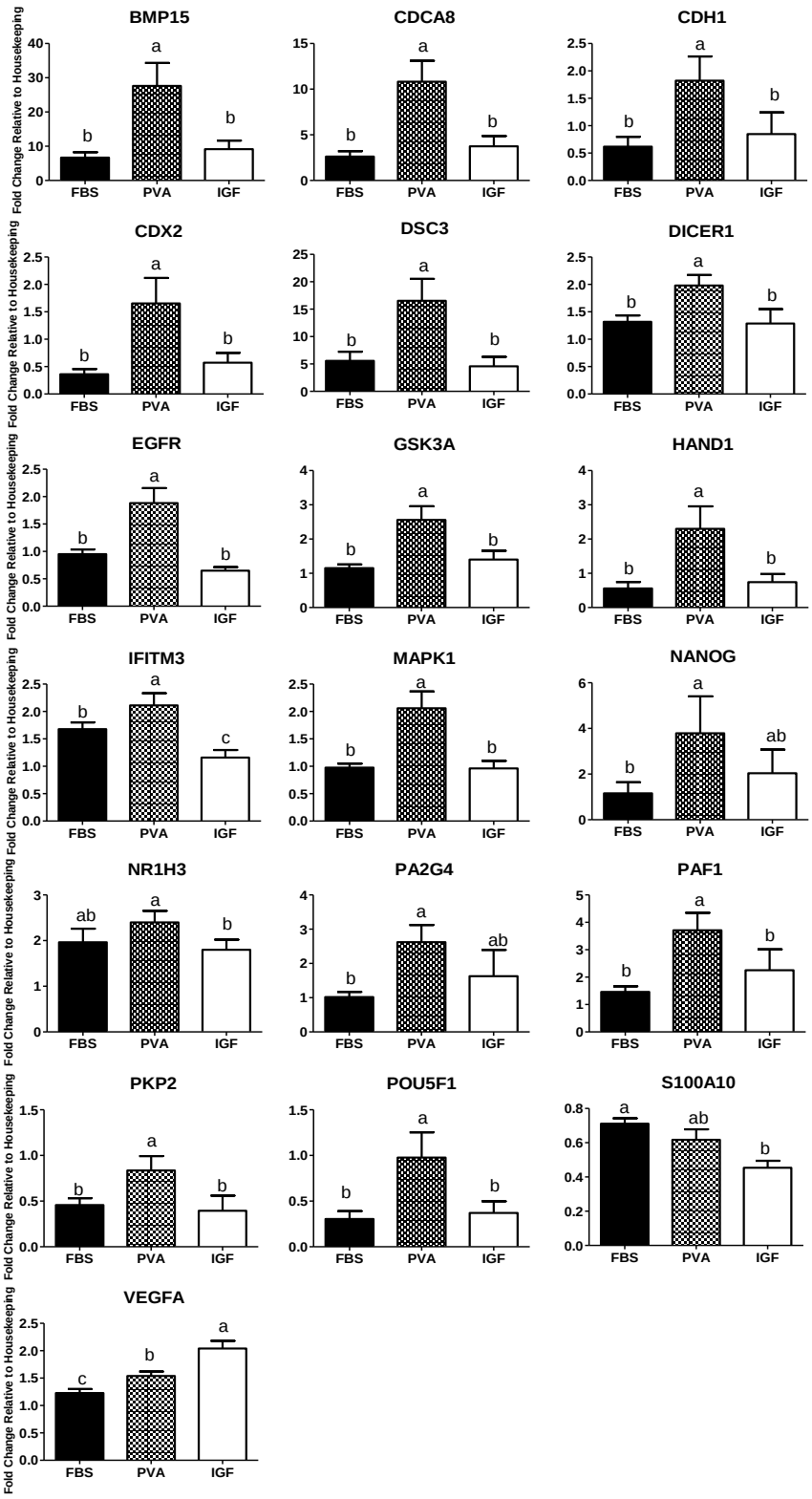

Figure 3. Mean ± SEM of transcripts of genes for embryo development and implantation of 6-8 cell stage embryos. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

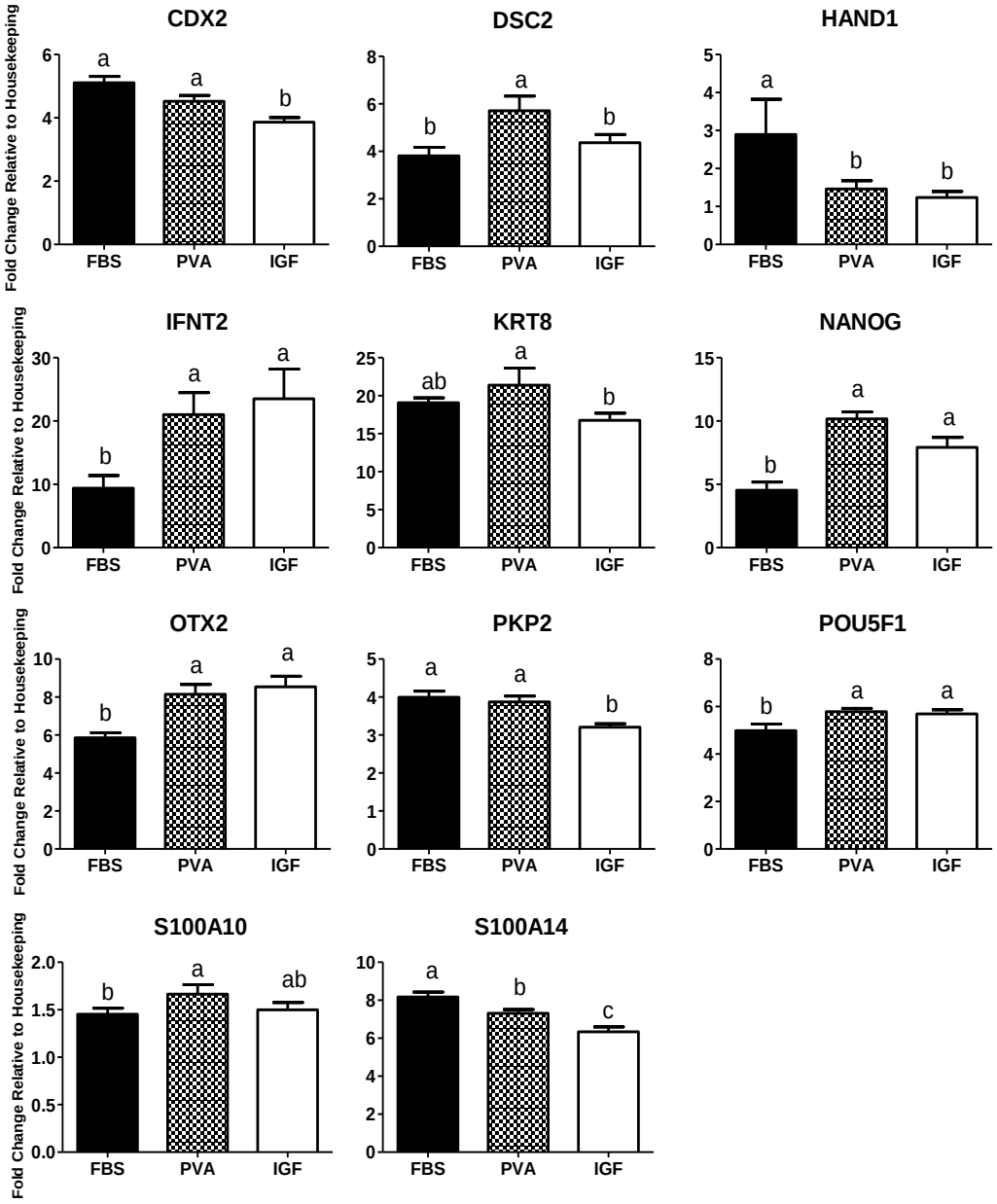

Figure 4. Mean ± SEM of transcripts of genes for embryo development and implantation of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

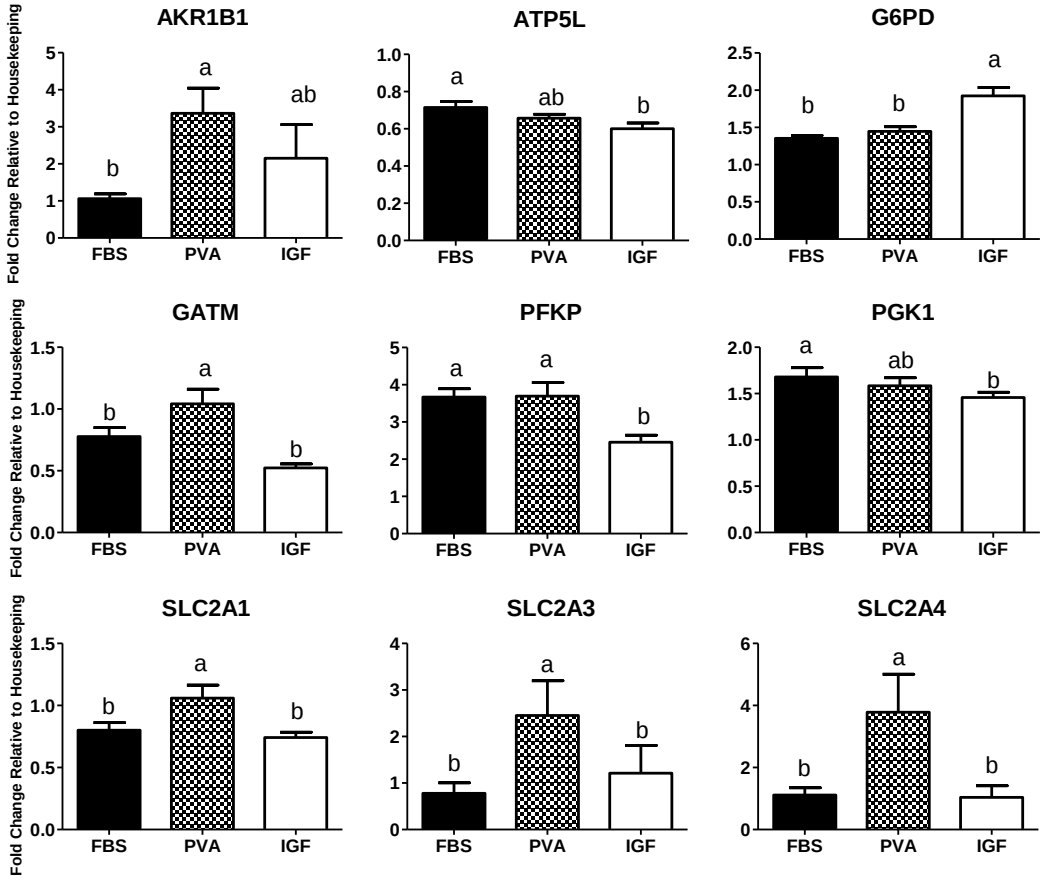

Figure 5. Mean ± SEM of transcripts of genes for glucose metabolism and oxidative phosphorylation of 6-8 cell stage embryos. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

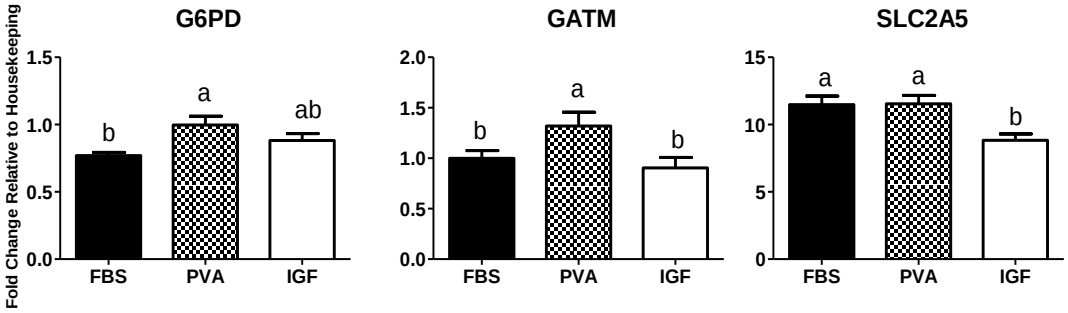

Figure 6. Mean ± SEM of transcripts of genes for glucose metabolism and oxidative phosphorylation of expanded blastocysts. Data represent the fold-change of level of expression relative to housekeeping genes. Experimental groups of IVC with the respective addition of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF). Data with different superscripts differ significantly ($p \leq 0.05$).

Supplementary File 1.

Allocation of inner cell mass (ICM) and trophectoderm (TE) cells, ICM:TE ratio and total cell number of *in vitro* produced bovine embryos in the presence of fetal bovine serum (FBS), polyvinyl-alcohol (PVA) or PVA + IGF1 (IGF) during oocyte maturation, embryo culture or both (Mean ± S. E.)

Treatment		Cell analysis			
IVM	IVC	ICM (%)	TE (%)	ICM:TE ratio	Total cell N
FBS	FBS	36.6 ± 2.5 [b]	63.4 ± 2.5 [ab]	1: 2.92 [a]	62.7 ± 3.5 [ab]
FBS	PVA	32.8 ± 4.2 [b]	67.2 ± 4.2 [a]	1: 4.36 [a]	48.4 ± 5.8 [c]
FBS	IGF	36.8± 3.5 [b]	63.2 ± 3.5 [ab]	1: 3.76 [a]	51.0 ± 4.9 [c]
PVA	FBS	37.4 ± 3.2 [b]	62.6 ± 3.1 [ab]	1: 2.68 [a]	58.7 ± 4.4 [bc]
PVA	PVA	53.6 ± 6.5 [a]	46.4 ± 6.4 [c]	1: 0.89 [a]	49.7 ± 8.9 [bc]
PVA	IGF	53.0± 5.3 [a]	47.0 ± 5.3 [c]	1: 1.60 [a]	70.4 ± 7.3 [ab]
IGF	FBS	39.5 ± 3.1 [b]	60.4 ± 3.1 [ab]	1: 1.88 [a]	73.1 ± 4.3 [a]
IGF	PVA	45.8 ± 6.5 [ab]	54.2 ± 6.4 [abc]	1: 1.28 [a]	67.8 ± 8.9 [abc]
IGF	IGF	44.6 ± 5.6 [ab]	55.4 ± 6.0 [abc]	1: 1.75 [a]	61.4 ±7.7 [abc]

[a, b] Data with different superscripts in the same column differ significantly ($p \leq 0.05$).

Considerações finais

Foram analisados os efeitos de IGF-I durante a maturação oocitária e cultivo embrionário *in vitro* sem a presença de soro fetal bovino e os resultados foram comparados com produção comumente realizada com a utilização do mesmo. Apesar de que os resultados de produção embrionária com IGF1 e SFB tenham sido divergentes, foi possível observar que existem de fato efeitos benéficos da adição de IGF-I à produção de embriões *in vitro*.

Poucos efeitos positivos foram observados quando IGF-I foi adicionado ao meio de maturação, mas aparentemente, estes efeitos dependem da concentração utilizada. Provavelmente concentrações menores à utilizada no presente estudo podem levar a melhores resultados, já que mesmo com melhores características celulares nos blastocistos, a expressão gênica apresentou resultados que se relacionam com baixa qualidade embrionária.

Por outro lado, a presença de IGF-I no meio de cultivo embrionário apresentou maior quantidade de efeitos benéficos. Foi observada uma alteração nas características celulares, o que demonstrou uma melhor qualidade embrionária a mesma que foi compatível com os resultados obtidos na expressão gênica. Os genes analisados indicaram um melhor desenvolvimento embrionário associados a um possível aprimoramento no reconhecimento materno e que o IGF-I é capaz de regular os genes relacionados à morte celular. Alterações nos genes relacionados ao metabolismo de glicose e de lipídios foram observadas. No entanto, outras avaliações são necessárias para uma melhor compreensão dos resultados.