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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
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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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Comissão Examinadora

Profa. Dra. Fernanda da Cruz Landim
Presidente e Orientadora
Departamento de Reprodução Animal e Radiologia Veterinária
FMVZ – UNESP – Botucatu

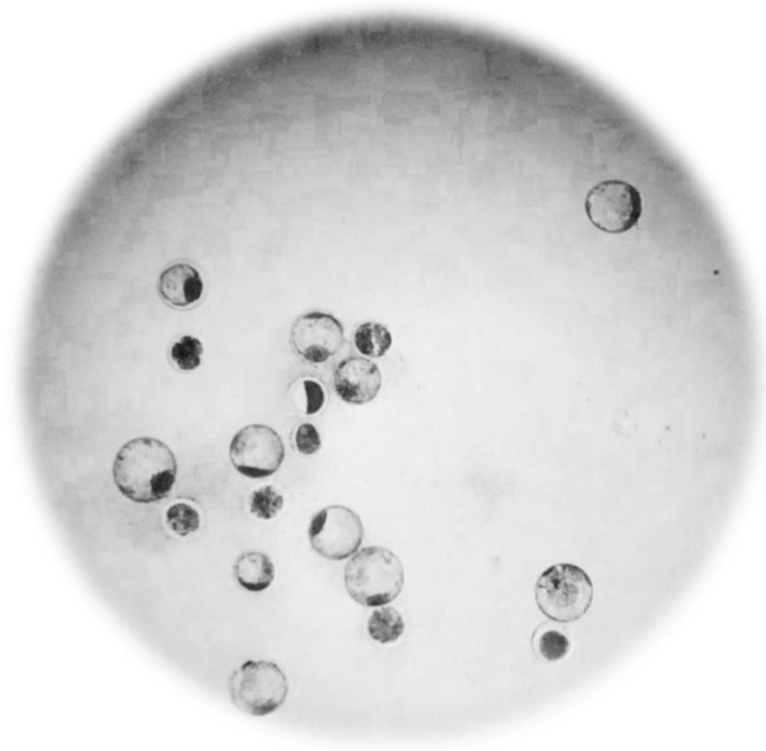
Prof. Dr. João Carlos Pinheiro Ferreira
Departamento de Reprodução Animal e Radiologia Veterinária
FMVZ – UNESP – Botucatu

Profa. Dra. Fabiana Ferreira de Souza
Departamento de Reprodução Animal e Radiologia Veterinária
FMVZ – UNESP – Botucatu

Prof. Dr. Anthony César de Souza Castilho
Programa de Pós-graduação em Ciência Animal
Universidade do Oeste Paulista (UNOESTE) – Presidente Prudente

---

Prof. Dr. Mateus José Sudano
Curso de Medicina Veterinária
Universidade Federal do Pampa - Uruguaiana



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.

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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

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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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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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.