Universidade Federal do Pará
Núcleo de Ciências Agrárias e Desenvolvimento Rural
Empresa Brasileira de Pesquisa Agropecuária – Embrapa Amazônia Oriental
Universidade Federal Rural da Amazônia
Programa de Pós-Graduação em Ciência Animal

Nathália Nogueira da Costa de Almeida

Efeito do cortisol na produção *in vitro* de embriões bovinos

Belém-Pará
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Tese apresentada para obtenção do título de Doutor em Ciência Animal. Programa de Pós-Graduação em Ciência Animal. Núcleo de Ciências Agrárias e Desenvolvimento Rural. Universidade Federal do Pará. Empresa Brasileira de Pesquisa Agropecuária – Amazônia Oriental. Universidade Federal Rural da Amazônia
Área de concentração: Produção Animal
Orientador Prof. Dr. Otávio Mitio Ohashi.
Co-Orientador: Dr. Moyses Miranda

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Dedico

Aos meus pais ***Raimundo Nonato*** *e* ***Maria de Fátima****,*
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pela eterna paciência, carinho, suporte e amor dispensados a mim.

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“A mente que se abre a uma nova idéia jamais voltará ao seu tamanho original.”

Albert Einstein

RESUMO

O objetivo dessa tese foi avaliar o efeito do cortisol, na maturação oocitária e cultivo de embriões bovinos. No **ARTIGO 1** foi analisada a distribuição e localização de receptor de glicocorticóide (GR) por imunocitoquímica em oócitos, células do *cumulus*, embrião de 2-4 células, 8-16 células, mórula e blastocisto, e foi verificado também a presença de RNAm (rtPCR qualitativa) para GR nos referidos estádios. Os resultados apontam que o GR está presente em todas as células analisadas. A fim de verificar a funcionalidade de GR no desenvolvimento embrionário pré-implantacional, a tradução do RNAm para GR foi silenciada em zigotos pela técnica de RNAi, e o desenvolvimento embrionário subsequente foi analisado. A diminuição de transcrito e proteínas de GR pela técnica de RNAi prejudicou o desenvolvimento embrionário ($p<0,05$. A presença de GR em oócito e células do *cumulus* nos indica que essas células são sensíveis ao uso de GC. Visto isso, no **ARTIGO 2** foi avaliado o efeito de diferentes concentrações do cortisol durante a maturação *in vitro* (**MIV**) de oócitos bovinos sobre o desenvolvimento embrionário, índice de apoptose e expressão de gênica (*NRF1, COX, TFAM, GLUT1, FASN e HSP70*). As concentrações de cortisol utilizadas foram 0,01; 0,1 e 1 µg/mL.. Não houve diferença estatística em relação ao número de células e taxa de clivagem, porém a concentração de 0,1 µg/ml de cortisol aumentou a taxa de blastocisto quando comparada ao grupo controle (sem cortisol na MIV) (41 ± 10 *versus* 21 ± 1,2; $p<0,05$; respectivamente). A taxa de apoptose e a expressão gênica em oócitos, células *do cumulus*, e blastocistos foi avaliada apenas na concentração de 0,1 µg/ml de cortisol. Não houve diferença estatística com relação ao índice apoptótico, e nem com relação à expressão gênica em oócitos e células do *cumulus* para os genes *COX, NRF1, HSP70 e FASN* ($p>0,05$). Em relação à expressão gênica embrionária, apenas as quantificações relativas de RNAm para *FASN*, *GLUT1* e *HSP70* estavam aumentadas nos blastocistos tratados com 0,1µg/mL durante a **MIV** quando comparados aos embriões do grupo Controle ($p<0,05$), os demais genes não mostraram alteração ($p>0,05$). **No ARTIGO 3** foi analisado o uso do cortisol durante o cultivo *in vitro* **(CIV)** de embriões bovinos. Visto que no Capítulo 1 foi identificado GR em todos os estádios de desenvolvimento embrionário e quando a expressão de GR foi silenciada o desenvolvimento embrionário foi prejudicado. Sendo assim, no experimento 3 foram adicionadas diferentes concentrações (0,01; 0,1 e 1 µg/mL) de

cortisol na CIV e o desenvolvimento embrionário foi avaliado, mesmos parâmetros do Capítulo 2. Não houve diferença estatística nos embriões tratados com Cortisol em diferentes concentrações quando comparados com o Controle para nenhum dos parâmetros analisados ($p>0.05$). A concentração de 0,1 µg/mL foi escolhida para avaliar outros parâmetros de qualidade embrionária. Sendo assim, embriões CIV Com ou Sem 0,1 µg/mL de Cortisol, foram analisados quanto à taxa de apoptose e expressão gênica, não sendo observada diferença estatística em nenhuma das análises ($p>0,05$). Após estes estudos concluímos que oócitos e embriões são responsivos a GC, que a adição de cortisol na MIV melhora a competência oocitária, porém a suplementação com cortisol na CIV não influenciou o desenvolvimento embrionário.

Palavras-Chaves: *Cortisol. Oócito. Embrião. Expressão Gênica.*

ABSTRACT

The aim of this thesis was to evaluate the effect of cortisol in oocyte maturation and bovine embryo culture. In Chapter 1 we analyzed the distribution and location of glucocorticoid receptor (GR) by immunocytochemistry in oocytes, cumulus cells, embryonic cells 2-4, 8-16 cell, morula and blastocyst, and was also verified the presence of mRNA (qualitative RT-PCR) for GR in the stages. The results showed that the GR is present in all cells analyzed. In order to verify the functionality GR in preimplantation embryo development, translation of mRNA for GR zygotes was silenced in the RNAi technique, and subsequent embryo development was analyzed. The embryonic development decreased (P <0.05) after silencing of GR mRNA. The presence of GR oocyte and the cumulus cells indicates that these cells are sensitive to the use of CG. Given this, in Chapter 2, the effects of different cortisol concentrations during in vitro maturation (IVM) of bovine oocytes and embryonic development, apoptosis rate and gene expression (*NRF1, COX, TFAM, GLUT1, FASN and HSP70*) were analiyzed. The concentrations of cortisol used were 0.01, 0.1 and 1 mg / mL . There was no statistical difference in the number of cells and cleavage rate, but the concentration of 0.1 mg/ml of cortisol increased the blastocyst rate when compared to the control group (without cortisol in IVM) (41 ± 10 versus 21 ± 1.2; p <0.05, respectively). The rate of apoptosis and gene expression in oocytes, cumulus cells and blastocysts was only assessed at a concentration of 0.1 mg / ml of cortisol. There was no statistical difference in the apoptotic index, and not with respect to gene expression in oocytes and cumulus cells for COX genes, *NRF1*, *HSP70* and *FASN* (p> 0, 05). Regarding embryonic gene expression, only the measurements relative mRNA *FASN*, *GLUT1* and *HSP70* were increased in blastocysts treated with 0.1 g/ml during IVM when compared to embryos of the Control group (p <0.05), the other genes showed no change (p> 0.05). In Chapter 3, we investigated the use of cortisol during in vitro culture (IVC) of bovine embryos. As in Chapter 1 was identified GR in all embryonic stages and when the GR expression was silenced and embryonic development was impaired. Thus, in the experiment three different concentrations (0.01, 0.1 and 1 mg / mL) of cortisol in IVC and embryonic development was evaluated same parameters of Chapter 2. There was no statistical difference in the embryos treated with cortisol in different concentrations when compared to the control for the parameters analyzed (p> 0.05). The

concentration of 0.1 mg/ml was chosen to evaluate other parameters of embryo quality. Thus, IVC embryos with or without 0.1 mg/mL of cortisol, were analyzed for apoptosis rate and gene expression, not being statistically significant difference in any of the analyzes ($p > 0.05$). After these studies conclude that oocytes and embryos are responsive to GC, and the addition of cortisol in IVM improves oocyte competence, but the supplementation of IVC with cortisol may not have influence on embryo development

Key Words: *Cortisol. Oocyte. Embryo. Gene Expression.*

LISTA DE ABREVIATURAS

%	Porcentagem
℃	Grau Celsius
µg	Micrograma
Na+.	Cátion Sódio
11β-HSD	11β-hidroxi esteróide desidrogenase
AMPc	Adenosina Monofosfato Cíclico
ANOVA	Análise de Variância
ACTH	hormônio adrenocorticotrópico
BSA	Albumina Sérica Bovina
CCO	Complexo-*cumulus*-oócito
CIV	Cultivo *In Vitro*
CO_2	Gás Carbônico
COX	Citocromo c oxidase
CRH	Hormônio liberador de corticotropina
DNAmt	Acido desorribonucleico mitocondrial
DNAn	Acido desorribonucleico nuclear
et al.	*et alli* (e colaboradores)
FASN	Acido graxo sintase
FIV	Fecundação *In Vitro*
FSH	Hormônio Folículo Estimulante
g	Gravidade
GC	Glicocorticoide
GLUT 1	Glicoproteína transportador de glicose 1
GR	Receptor de glicocoirticoide
GRE	Elemento responsivo a glicocorticoide
HDL	Proteína de alta densidade
HEPES	*N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid*
HHA	hipotálamo-hipófise-adrenal
HSP70	*Heat shock protein*
RNAi	Ácido ribonucléico de interferência
IGF-1	Fator de crescimento ligado a insulina

KLF	Fator ligado a Kruppel
LH	Hormônio Luteinizante
MIV	Maturação *In Vitro*
mL	mililitro
MPF	fator promotor da fase M
NaCl	Cloreto de sódio
N_2	Gás Nitrogênio
NRF1	Fator nuclear de respiração 1
O_2	Oxigênio
p	Probabilidade
pH	Potencial Hidrogeniônico
PI3K	Fosfatidilinositol 3,4,5trifosfato
PIVE	Produção *in vitro* de embrião
PFA	Paraformaldeído
PBS	Solução Salina Tamponada
POU5F1	*POU domain, class 5, transcription factor 1*(Domínio POU, classe 5, fator de transcrição 1)
RNAm	Ácido ribonucléico mensageiro
siRNA	*small interfering* Ácido ribonucléico
TFAM	Fator de transcrição mitocondrial
TUNEL	Terminal deoxinucleotidil transferase Uracil
PCR	Reação em cadeia da polimerase
SFB	Soro Fetal Bovino
SOF	*Sintetic Ouviduct Fluid* (Fluido do Oviduto Sintético*)*
TCM	*Tissue Culture Medium* (Meio de Cultura de Tecidos)
TL	TALP (Tyrodes com Albumina, Lactato e Piruvato)
O_2	Oxigênio

SUMÁRIO

1. INTRODUÇÃO

A produção *in vitro* de embriões (PIVE) é uma biotecnologia da reprodução, que envolve etapas de colheita, maturação oocitária, fecundação e cultivo de zigotos *in vitro,* podendo ser aplicada nos estudos para compreensão dos eventos biológicos que ocorrem no início do desenvolvimento embrionário pré-implantacional e também vem sendo incorporada aos programas de melhoramento genético animal como técnica de multiplicação de animais de interesse econômico, em especial os da espécie bovina (CAMARGO et al*.,* 2006; GONÇALVES et al., 2008; LONERGAN, 2008).

Atualmente o Brasil ocupa posição de destaque no cenário mundial com consequente reconhecimento internacional, sendo o líder na produção *in vitro* de embriões bovinos, declarando no ano de 2010 mais de 264.000 embriões (STROUD; CALLESEN, 2013).

Apesar dos avanços em relação à qualidade do embrião produzido *in vitro*, o mesmo ainda é de qualidade inferior em comparação ao embrião produzido *in vivo*. Por este motivo, pesquisas que visem melhorar a qualidade do embrião *in vitro* são ainda necessárias, visto que isso permitirá submeter o referido embrião ao processo da criopreservação, a qual ainda não apresenta resultados satisfatórios e consistentes, especialmente do ponto de vista comercial (DRIVER et al., 2012; KHURANA; NIEMANN, 2000; MACHADO et al., 2013; STROUD; BO, 2011).

Diversos fatores tem sido objeto de pesquisa na tentativa de melhorar a qualidade do embrião produzido *in vitro*, dentre esses, a composição dos meios (íons, tampões, aminoácidos e substratos energéticos) e as condições de cultivo (WRENZYCKI; STINSHOFF, 2013). Nesse sentido grupos de pesquisa buscam o aperfeiçoamento dos meios utilizados na PIVE, na tentativa de mimetizar o microambiente *in vivo*, em diversos aspectos, inclusive na adição de hormônios relacionados ao metabolismo com resultados satisfatórios, como por exemplo, hormônios tireoideanos (ASHKAR et al, 2010; COSTA et al., 2013), leptina (CÓRDOVA et al., 2010; ARIAS-ALVAREZ et al.; 2011; JIA et al., 2012); insulina (CEBRIAN-SERRANO; SALVADOR; SILVESTRE, et al., 2014; MOTA et al., 2014) melatonina (XM et al.; 2014; WANG et al., 2014) e hormônio do crescimento (PERS-KAMCZYC et al., 2010).

Outros compostos, além dos acima citados, podem também contribuir no aperfeiçoamento dos referidos meios, como por exemplo, os hormônios glicocorticóides (cortisol e dexametasona), conhecidos como hormônios do estresse, que também atuam comprovadamente no metabolismo celular, entretanto com relação à reprodução, seus efeitos ainda não estão totalmente elucidados.

Em humano, Keay et al. (2002) verificaram que a administração de dexametasona (análogo sintético do cortisol) nos ciclos de fertilização *in vitro* melhorou a resposta ovariana ao estímulo super-ovulatório, sugerindo assim que os glicocorticoides seriam importantes para a maturação oocitária, bem como, para a implantação embrionária (POLAK DE FRIED et al., 1993; KEAY et al., 2002).

Em bovinos, o cultivo de embriões com dexametasona aumentou a taxa de eclosão e o número de células em blastocistos sem influenciar nos índices de apoptose (SANTANA et al., 2014).

Dada a importância do cortisol na fisiologia reprodutiva em mamíferos, principalmente na ovulação, o qual está presente em altas concentrações no líquido folicular de folículos pré-ovulatórios (KEAY et al., 2002), e durante a gestação, acredita-se que no sistema *in vitro* ele possa ter ações, em nível de maturação oocitária e desenvolvimento embrionário pré-implantacional, dessa forma aperfeiçoando a produção *in vitro* de embriões

2. OBJETIVOS

2.1. OBJETIVO GERAL

- Melhorar a qualidade do embrião bovino produzido *in vitro* através da suplementação dos meios de cultivo com cortisol.

2.2. OBJETIVOS ESPECÍFICOS

- Identificar a presença de transcritos e a distribuição de receptores de glicocorticóides em oócitos bovinos imaturos e maturados *in vitro*, células do *cumulus* de oócitos maturados *in vitro*, e em embriões de diferentes estágios de desenvolvimento embrionário.

- Compreender a importância do receptor de glicocorticoide durante o desenvolvimento embrionário pré-implantacional.

- Avaliar o efeito de diferentes concentrações de cortisol durante a maturação oocitária e o desenvolvimento embrionário *in vitro*.

3. REVISÃO DE LITERATURA

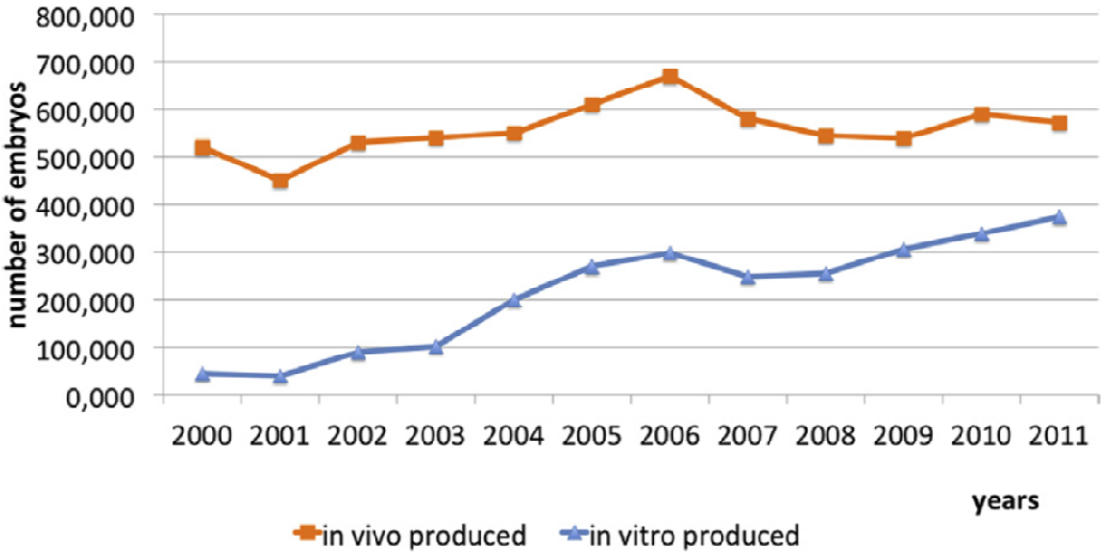
3.1. PRODUÇÃO *IN VITRO* DE EMBRIÕES

Para a produção *in vitro* de embriões (PIVE), os oócitos precisam ser coletados e preparados para a fecundação em laboratório. Após a fecundação os embriões podem ser cultivados até o estádio de blastocisto (MACHATY et al., 2012). É uma biotecnologia da reprodução utilizada para acelerar a produção de animais com genética superior, impedir o descarte de fêmeas geneticamente privilegiadas com infertilidade adquirida, permite o estudo da fisiologia ovariana e embrionária pré-implantacional (GONÇALVEZ et al., 2008).

A PIVE, inicialmente, era vista como uma biotécnica restrita às entidades de pesquisa, sendo algo fora da realidade do setor pecuário (VIANA, 2009).

Atualmente, é uma ferramenta amplamente requisitada pelo setor comercial, sendo indispensável nos programas de melhoramento genético, (CAMARGO et al*.,* 2006). De acordo com os dados da Sociedade Internacional de Transferência de embrião, a IETS, o número de embriões produzidos *in vitro* e transferidos para receptoras aumentou mais de 10 vezes (STROUD, 2012 *apud* GALLI et al., 2014) nos ultimo 12 anos, demonstrando assim seu potencial comercial, como pode ser visto na figura 1.

Figura 1: Resumo dos dados estatísticos da Sociedade Internacional de Transferência de Embrião, que mostra uma comparação dos embriões transferidos in vitro e in vivo por ano. Os embriões produzidos in vitro estão em constante ascensão.



Fonte: STROUD, 2012 apud GALLI et al., 2014.

Em 2011 foram relatados quase 400.000 embriões bovinos PIV em todo o mundo, e o Brasil se destacou por ser o responsável por aproximadamente 85% desse total, reforçando sua liderança absoluta no mercado mundial (STROUD, 2012 *apud* GALLI, et al., 2014).

A utilização comercial da biotecnologia foi possível porque os sistemas de produção *in vitro* de embrião evoluíram, entretanto as condições ainda são subótimas, como por exemplo, a taxa de blastocisto ainda é baixa (média 40%), e nem todos estes apresentam a capacidade de chegar a termo. Além disso, a qualidade do embrião *in vitro* é inferior quando comparado ao *in vivo*, fato comprovado pela baixa criotolerância, cavitação precoce sem compactação adequada, redução de proteínas de comunicação intercelular, e atrasos nos ciclos celulares atrasados resultando em retardo do desenvolvimento e menor número de celular (THOMPSON, 1997; ENRIGHT et al., 2000; LONERGAN et al., 2003; PLOURDE, et al., 2012).

Por este motivo, pesquisas que visem melhorar a qualidade do embrião *in vitro* são ainda necessárias, visto que isso permitirá submeter o referido embrião ao

processo da criopreservação, a qual ainda não apresenta resultados satisfatórios e consistentes, especialmente do ponto de vista comercial (STROUD; BO, 2011).

Outro ponto relevante está no fato de que a eficiência dos embriões produzidos *in vitro* e congelados provavelmente determinará a aceitação da tecnologia *in vitro* por outros países, pois até agora, a maioria dos embriões transferidos é constituída de embriões PIV frescos (não congelados). Como por exemplo, apenas 7% dos embriões PIV transferidos em 2009 eram congelados (STROUD; BO, 2011). Sendo assim, o Brasil seria o maior beneficiado com o aperfeiçoamento dos meios de cultivo e conseqüente melhora da qualidade embrionária.

3.1.1. Contribuição dos meios de cultivo para o desenvolvimento embrionário

Os embriões exibem uma imensa plasticidade e tolerância, sendo capazes de se adaptar ao ambiente onde são cultivados. São capazes de se desenvolver em meios de cultivo *in vitro* com composição nutricional mínima, e mesmo assim uma proporção razoável de blastocistos são formados e aptos a ponto de levar uma gestação a termo. Entretanto muitos estudos têm mostrado que sim, os embriões são sensíveis as condições de cultivo, ao ambiente, e isso pode afetar o futuro pré e pós nascimento, assim como o potencial de desenvolvimento (LONERGAN; FAIR, 2008; LONERGAN; FAIR, 2014).

A sensibilidade embrionária pode ser verificada pela qualidade inferior dos embriões produzidos *in vitro*, que na maioria das vezes, está relacionada com a composição dos meios e condições de cultivo, demonstrando a necessidade de pesquisas que proponham modificações nos protocolos, visando diminuir as diferenças qualitativas entre o embrião produzidos *in vitro* do *in vivo* (DRIVER, et al., 2012; KHURANA; NIEMANN, 2000).

As evidências apontam que a qualidade do oócito é crucial para a fertilização e produção *in vitro* em blastocistos (ALBUZ et al., 2010; LONERGAN; FAIR, 2008). Entretanto, o microambiente pós fecundação seria o ponto determinante para a aquisição da qualidade embrionária. Por exemplo, o cultivo de zigotos bovino produzido *in vitro* em oviduto de ovelha aumenta consideravelmente a

qualidade embrionária, mensurada em termos de criotolerância, sendo inclusive semelhante ao embrião produzido *in vivo* (RIZOS et al., 2002).

Nesse sentido modificações nas condições de maturação e cultivo *in vitro* podem afetar o desenvolvimento embrionário, em termos quantitativos e qualitativos (LONERGAN et al., 2000; LONERGAN et al., 2003; SIRARD 2011).

Pesquisas na área de reprodução têm buscado elucidar os complexos mecanismos que regem as várias etapas do desenvolvimento embrionário. Os resultados obtidos destacam o relevante papel dos hormônios metabólicos na concretização de muitas dessas etapas, de modo a se constituírem como elementos fundamentais para garantir a sobrevivência do embrião e o crescimento fetal (HARDY; SPANOS, 2002).

A leptina, conhecida como hormônio do metabolismo relacionado ao estado nutricional, exerce influencia na fisiologia reprodutiva em camundongos (ZACHOW; MAGOFFIN, 1997). Em bovinos já foi identificada a transcrição de receptores para o hormônio em oócitos e embriões. A adição de leptina ao meio de maturação promoveu uma melhora na competência oocitária, constatada pela diminuição da taxa de apoptose e aumento no número de zigotos que desenvolvem a blastocisto, entretanto quando adicionado ao meio de cultivo embrionário (10ng/mL e 100ng/mL) não exerceu nenhum efeito positivo no processo (ARIAS-ALVAREZ et al., 2011; BOELHAUVE et al., 2005; CÓRDOVA et al., 2010; JIA et al., 2012).

Um metabólito que tem sido considerado importante regulador do desenvolvimento embrionário é o hormônio do crescimento (GH). Em bovinos, o receptor de GH é expresso a partir do segundo dia do desenvolvimento embrionário, enquanto o GH inicia sua expressão a partir do dia oito (KOLLE et al., 2001). Em bovinos o GH quando adicionado ao meio de cultivo in *vitro* causa aumento no catabolismo de lipídios e de glicogênio verificada em blastocistos (KOLLE et al., 2001) e aumenta o número de células no trofectoderma e na massa celular interna, o que ocorre aparentemente por uma inibição do processo apoptótico (KOLLE et al., 2002).

A utilização da insulina apresenta divergentes efeitos em embriões pré-implantacionais. É um hormônio que promove a entrada de glicose na célula além de exercer efeito mitogênico (GARDNER; KAYE, 1991). Insulina no meio de maturação melhorou a taxa de blastocisto assim como aumentou a expressão de GDF-9, gene relacionado à competência oocitária (MOTA et al., 2014). Já foi observado que a

adição de insulina no meio de cultivo diminuiu a apoptose e aumentou a proliferação celular em embrião de coelho (HERRLER et al., 1998). O mesmo ocorreu em bovinos, a presença de insulina no meio de cultivo não aumentou o número de células embrionárias bovinas demonstrando assim sua atividade mitogênica, além de ter efeito anti-apoptótico em embrião (AUGUSTIN et al., 2003).

O meio de maturação suplementado com triiodotironina (COSTA et al., 2013) por 20 horas aumentou a taxa de eclosão em blastocisto bovinos, o que é considerado como um parâmetro de qualidade embrionária (GORDON, 2003; SUTOVSKY et al., 2001).

3.1.2. Avaliação embrionária

A análise da qualidade embrionária se faz importante, pois auxilia no momento de escolha de qual embrião deve ser transferido para a receptora, por isso a necessidade de aperfeiçoamento dessas avaliações, tornando-as mais confiáveis e menos invasiva, de modo a garantir o sucesso pós transferência embrionária (BO; MAPLETOFT, 2013; EL-SAYED et al., 2007).

A avaliação da qualidade embrionária pode ser feitas de diversas maneiras. Os métodos tradicionais são avaliação do desenvolvimento embrionário, descrita como a proporção de oócitos que clivam após a fecundação (taxa de clivagem) e chegam ao estádio de blastocisto (taxa de blastocisto); outro tipo de análise é taxa de eclosão, ou seja, o número de blastocisto que saem da zona pelúcida. Essas análises podem ser feitas em lupa estereomicroscópica, e é necessário que o avaliador seja experiente (BO; MAPLETOFT, 2013). Além disso, outras técnicas também auxiliam na avaliação de um sistema alternativo como indicativo de qualidade embrionária, como a contagem do numero total de células, através da marcação nuclear por Hoescht ou iodeto de propídio; estando esta avaliação relacionada a habilidade do embrião em sobreviver no útero (HARDY et al., 1989); e a análise da taxa de apoptose, também considerada um indicativo da viabilidade embrionária (GAD et al., 2012; LONERGAN; FAIR, 2014; RUVOLO et al., 2013).

Outra ferramenta valiosa para análise da qualidade embrionária é a expressão de RNA mensageiro (RNAm), na qual genes relacionados a competência

do oócito e do embrião são analisados e quantificados (DRIVER et al., 2012; EL-SAYED et al., 2007; GAD et al., 2012; WRENZYCKI et al., 2007) .

Dentre os vários aspectos indicadores de qualidade embrionária e que podem ser avaliados por meio da analise da expressão gênica podemos verificar a função mitocondrial. A mitocôndria possui papel importante pelo metabolismo energético celular, homeostasia e morte celular, sendo responsável pela geração de ATP através da fosforilação oxidativa (OXPHOS) (HUO; SCARPULLA, 2001; MAY-PANLOUP et al., 2005).

Para desempenhar tantas funções, ela necessita de contribuições tanto do DNA nuclear (DNAn) quanto do DNA mitocondrial (DNAmt). O fator nuclear de respiração 1 (*NRF1*) é importante para manutenção do DNAmt e função da cadeia respiratória, sendo considerado um fator de ativação de vários genes relacionados a mitocôndria, entre eles os fatores de transcrição mitocondrial e o citocromo c oxidase 1 (*COX1*), proteína da cadeia respiratório codificada no DNAmt (HUO; SCARPULLA, 2001). May-Panloup et al. (2005) descreveram que oócito bovinos que não clivaram 28 horas após a fecundação apresentaram número reduzido de transcritos para *NRF1* e *COX1*.

O fator de transcrição mitocondrial A (*TFAM*) regula a replicação e transcrição do DNAmt, importante nas divisões celulares. Ambos NRF1 e TFAM são importantes para embriogênese e evidências apontam que seus transcritos aumentam com a replicação do DNA (HUO; SCARPULLA, 2001; CHIARATTI et al., 2010, MASTROMONACO et al., 2012, MAY-PANLOUP et al., 2005).

Outro ponto do desenvolvimento embrionário que pode ser analisado com o estudo dos transcritos é o metabolismo dos carboidratos. Os transportadores de glicose (GLUT) são uma família de proteínas glicoprotéicas que facilitam a entrada de glicose na célula não necessitando de Na+. A expressão de *GLUT 1* durante o desenvolvimento embrionário pré-implantacional é restrito a membrana basolateral em células do trofoblasto de blastocistos bovinos (AUGUSTIN et al., 2001). Em embriões cultivados *in vitro* a expressão de transcritos é reduzida quando comparada com os embriões desenvolvidos *in vivo* (NIEMANN & WRENZYCKI, 2000). O número expressivo de transportadores GLUT 1, talvez, seja em função da necessidade extra de glicose, estocado na forma de glicogênio , garantindo assim reserva energética para o inicio do desenvolvimento embrionário *in vivo* (GARCIA-HERREROS et al., 2012; THOMPSON et al., 1996).

O metabolismo dos lípidos constitue um aimportante fonte energética em oócitos e embriões (SINCLAIR et al., 2003). O ácido graxo sintase (*FASN*) que catalisa a síntese do palmitato na cadeia saturada de ácidos graxos, contribuindo dessa maneira para os a formação da reserva energética. Colesterol e fosfolipídios são essenciais para a formação de membranas celulares e muito importantes para as divisões celulares após a fecundação (AUCLAIR et al., 2013; GONZÁLEZ-SERRANO et al., 2013).

A análise do estresse celular embrionário é associada à expressão das proteínas do estresse térmico (*HSP*), que são uma família de chaperonas, importantes para o empacotamento de proteínas (ABDOON et al., 2014; EITAM et al., 2010; KAWARSKY et al., 2001). A interpretação sobre a quantificação desse gene deve ser feita com cuidado. Machado et al., 2013 esperavam que o cultivo *in vitro* aumentasse a expressão de HSP70 em embriões bovinos (14º dia de cultivo), entretanto verificaram que embriões *in vivo* no mesmo estádio apresentavam mais RNAm para *HSP70*. Sendo assim, eles sugeriram que o RNAm para *HSP70* em embriões produzidos *in vitro* estaria sendo traduzido de forma mais rápida em proteína HSP70 em resposta as condições de estresse do cultivo. Outras evidências sugerem que a expressão de elevada de HSP70 em embriões bovinos está associada ao aumento nos transcritos de Interferon tau, importante sinalizador embrionário para reconhecimento materno (HICKMAN et al., 2013).

3.2 HORMÔNIOS GLICOCORTICÓIDES

Os glicocorticóides (GC) são hormônios esteróides, sintetizados a partir de uma molécula de colesterol. O nome glicocorticóide (glicose+cortex+esteroide) é derivado da sua função na regulação do metabolismo da glicose, sua síntese no córtex adrenal e sua estrutura esteroidal (DE BOSSCHER, 2010).

Esses hormônios estão envolvidos em uma gama de processos fisiológicos como o metabolismo energético (PECKET, 2011), processos anti-inflamatórios (BARNERS; ADCOCK, 2009) e na reprodução, agindo em processos como luteinização, oogênese e ovulação (TILBROOK, 2000; MICHAEL; PAPAGEORGHIOU, 2008).

O cortisol, principal hormônio GC circulante em humanos e bovinos, também é conhecido como hormônio do estresse, devido o aumento da sua secreção ser um sinal clássico de estresse animal, agindo sobre o metabolismo e visando a mobilização de energia, ou seja, aumentar a disponibilidade de combustível em frente a fatores estressores (BENFIELD, et al 2014; HELLHAMMER; WUST; KUDIELKA, et al., 2009).

3.2.1 Glicocorticóides fisiológicos e sintéticos

O cortisol é o principal hormônio glicocorticóide produzido no córtex da adrenal, exercendo cerca de 95% da atividade glicocorticóide no organismo. A corticosterona é outro importante GC fisiológico, apresentando atividade glicocorticóide menos potente que o cortisol. Contudo, em algumas espécies, como em roedores, é o principal hormônio da classe atuante no organismo.

Vários análogos sintéticos de glicocorticóides têm sido desenvolvidos pela indústria farmacêutica e incluem a betametasona, prednisona, predinisolona e dexametasona (DEX), entre outros. Este último apresenta uma atividade glicocorticóide 30-40 vezes mais potente que o cortisol. Uma das vantagens desses fármacos é que eles não sofrem regulação celular e possuem menos efeitos colaterais com relação aos GC fisiológicos (DE BOSSCHER; HAEGEMAN, 2009).

3.2.2 Regulação da síntese de glicocorticóides

A síntese de GC ocorre na zona *fasciculata* do córtex das glândulas adrenais, a partir do colesterol. As lipoproteínas de baixa densidade (LDL) se ligam a receptores específicos presentes na membrana celular das células da adrenal, os quais são endocitados. O colesterol é liberado após fusão das LDL com lisossomos, e então são esterificados e estocados no interior das células para serem usados na produção de esteróides pelas células adrenocorticais (GRIFFIN; OJEDA, 1996; GUYTON; HALL, 2011).

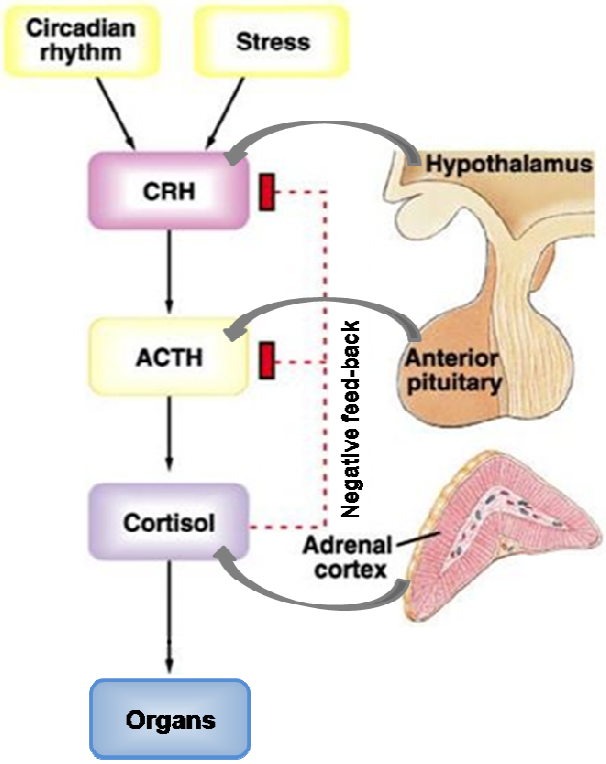
A regulação na produção e secreção de GC é conduzida pelo eixo hipotálamo-hipófise-adrenal (HHA), o sistema nervoso autônomo e glândulas endócrinas atuam em conjunto com efeitos parácrinos e autócrinos. O hormônio liberador de corticotropina (CRH) estimula a secreção de hormônio adrenocorticotrófico (ACTH), o qual induz a secreção de glicocorticóides no córtex adrenal (GRIFFI; OJEDA, 1996).

Tal hormônio irá atuar sobre as células da zona *fasciculata* do córtex adrenal promovendo uma maior produção dos receptores de LDL e das enzimas envolvidas na cascata de síntese dos GC, induzindo, assim, a síntese destes hormônios por estas células (GUYTON; HALL, 2011).

Os glicocorticóides são liberados imediatamente na circulação por difusão (GETTING, 2006). Os efeitos da ACTH sobre a supra-renal ocorrem de forma imediata e crônica. Os efeitos agudos acontecem em poucos minutos após estimulo da esteroidogênese através de um aumento da proteína Star, mediadora na entrega do colesterol para a primeira enzima da cascata. O efeito crônico, dentro de 24-26h, leva ao aumento na síntese de todas as enzimas esteroidogênicas, assim como também estimula a síntese do colesterol LDL (MILLER; AUCHUS, 2011).

Há, desta forma, um *feedback* positivo por parte dos hormônios hipotalâmico e hipofisário para produção de GC. Quando a concentração plasmática de cortisol atinge o limite máximo fisiológico, o cortisol passa a promover um *feedback* negativo sobre o eixo HHA, inibindo assim sua própria síntese (Figura 2) (BENFIELD, et al., 2014).

Figura 2: Esquema representando a regulação da síntese de cortisol. CRH: Hormonio liberador de corticrotopina. ACTH: Hormonio adrenocorticotrópico. Fonte:



Fonte: Adaptado de GUYTON; HALL, 2011.

3.2.3 Fatores que regulam a biodisponibilidade dos glicocorticóides

Em nível celular, a atividade de glicocorticóides circulantes é determinada por fatores que regulam o acesso livre do hormônio ao seu receptor. Tais fatores incluem globulinas ligante de corticosteróide (CBG) e 11β-HSD (11β-hidroxi esteróide desidrogenase). Esses fatores modulam as ações dos glicocorticóides em um nível pré-receptor (GUYTON; HALL, 2011).

A maioria dos GC's encontra-se ligado a proteínas transportadoras, predominantemente CBG e, em certa medida, à albumina. Assim, apenas 2-5% do total de cortisol circula na forma livre. Em humanos os níveis de CBG permanecem relativamente constante após a adolescência e não têm variações diurnas (COOLENS; BAELEN; HEYNS, et al., 1987, HEYNS; COOLENS, 1988). Esta globulina parece ter diferentes afinidades à corticosterona em diferentes temperaturas (CAMERON, 2010). Mutações no gene CBG causando diminuição da

ligação a CBG tem sido associadas com baixa pressão arterial (BREUNER; ORCHINIK, 2002; TORPY et al., 2001)

Existem duas isoformas da enzima 11β-HSD, o tipo 1 e o tipo 2. A 11β-HSD-2 converte cortisol em cortisona, esta por sua vez possui baixa afinidade pelos receptores de glicocorticóide, enquanto que a 11β-HSD-1 catalisa o oposto da reação; transformando cortisona em cortisol (GRIFFIN; OJEDA, 1996).

A 11β-HSD 1 é encontrada em muitos tecidos, incluindo o fígado, o tecido adiposo, o músculo, o cérebro e o sistema vascular, enquanto 11β-HSD 2 converte o cortisol em cortisona e é encontrado no tecido sensível à aldosterona (WAKE; WALKER, 2004). A 11β-HSD 2 protege o receptor mineralocorticóide da ação competitiva do cortisol mais abundante, que tem a mesma afinidade de ligação para o receptor de aldosterona, assegurando, assim, um efeito de mineralocorticóides apenas pela aldosterona (MASUZAKI et al., 2001).

3.2.4 Receptores de glicocorticóides

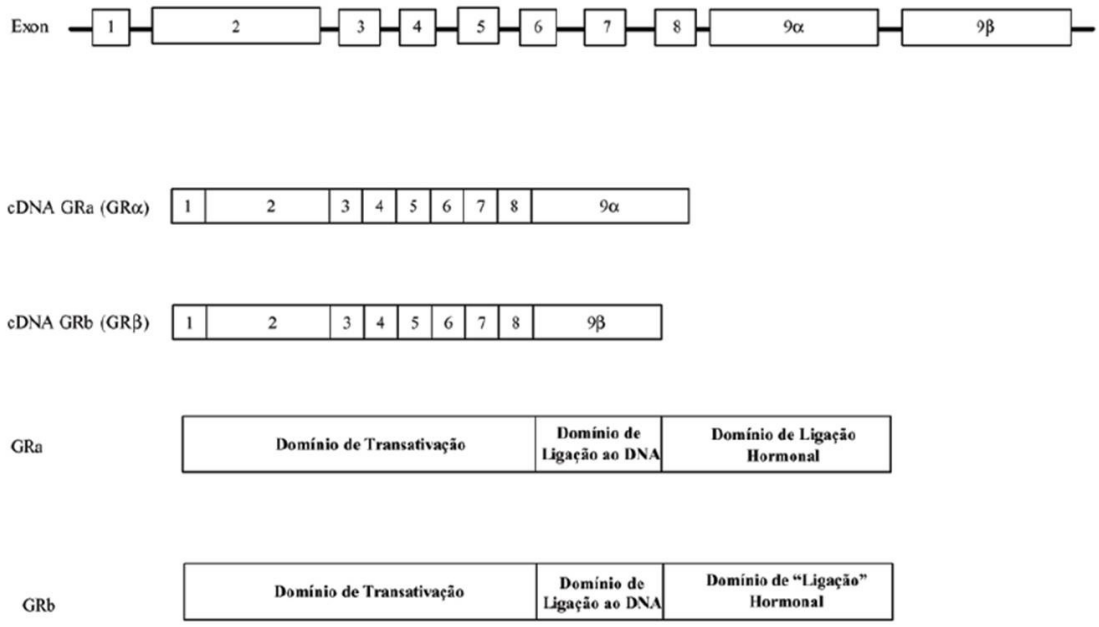
A maioria das ações dos glicocorticóides são controladas por receptores de glicocorticóides (GR), também conhecidos como receptores nucleares da superfamília 3, grupo C, membro 1 (NR3C1), membro da superfamília de receptores hormonais nucleares que se ligam a fatores de crescimento.

O receptor de glicocorticóide é uma proteína codificada por um único gene, e seu transcrito é formado pela transcrição de nove éxons deste gene. Sua estrutura é constituída por três domínios, a) o domínio N-terminal ou domínio de transativação, o qual interage com a maquinaria de transcrição; b) o domínio de ligação ao ligante (LBD), que irá se ligar a molécula de hormônio; e c) o domínio de ligação ao DNA (DBD), o qual irá se ligar ao elemento de resposta a glicocorticóides (GRE) presentes no genoma (MERKULOV; MERKULOVA, 2012; VANDEVYVER et al, 2012).

Ainda que exista apenas um gene codificante para o GR, há numerosas isoformas para este receptor, o que se apresenta como resultado de *splicing* e iniciação de transcrição alternativos (OAKLEY; CICLOWSKI, 2011; MERKULOV; MERKULOVA, 2012).

Em humanos as principais isoformas são GRα e o GRβ (Figura 3), sendo que este primeiro apresenta-se como a forma clássica e é biologicamente ativo, pois em contato com o hormônio é capaz de se ligar à GRE de genes responsivos a glicocorticóides, atrair co-ativadores, e influenciar na transcrição gênica (FARIA; LUNGUI, 2006; MERKULOV; MERKULOVA, 2012). Em bovino foi descrito o GRα (JAMES et al., 2003; NAYEBOSADRI et al., 2012; TETSUKA et al., 2010).

Figura 3: Representação da estrutura do gene de receptor glicocorticoide e das isoformas proteicas GR alfa e GR beta. O gene do GR apresenta nove *exons*, e um *splincing* alternativo no nono *exon* gera as isoformas alfa (777 aminoácidos) e beta do GR (742 aminoácidos). Os dominios de transativação, de ligação ao DNA e de ligação hormonal estão representados.



Fonte: FARIA; LUNGUI, 2006.

Embora as isoformas GRα e GRβ sejam 94% idênticas, a isoforma β é incapaz de ligar-se aos glicocorticóides e de ativar a transcrição gênica. Esta isoforma possui 742 aminoácidos e, apesar de também formar um complexo protéico com as HSP (*"heat shock proteina"*, proteína do choque térmico), localiza-se primariamente no núcleo celular, mesmo na ausência do ligante, e tem como função regular negativamente a ação do GRα (OAKLEY; CIDLOWSKI, 2011; WANG et al., 2014).

Ambas as isoformas estão presentes em praticamente todos os tipos celulares, sendo necessários para o desenvolvimento pós-natal. Contudo a distribuição celular dos diferentes tipos de GR difere entre os tipos celulares dependendo do estágio de desenvolvimento, diferenciação ou fase do ciclo celular, sendo essas varias isoformas do receptor responsáveis pelas diferentes respostas fisiológicas e farmacológica dos glicocorticoides (OAKLEY; CIDLOWSKI, 2011).

Existem ainda outras isoformas não tão comuns e com pouca atividade transcricional. Além disso, pouco se sabe a respeito da sua real função, são resultado do *splicing* alternativo entre os éxons 2 e 7 (GR-P) e exons 3 e 4 (GR-γ), estes receptores possuem pouca atividade transcricional (FARIA; LUNGUI, 2006).

Além do *splicing* alternativo para geração de diferentes isoformas, novas proteínas podem ser geradas, através da tradução alternativa. O RNAm do *GRα* pode gerar novas formas de proteinas, as quais diferem em tamanho na região N-terminal. Todas as proteinas têm a mesma afinidade pelo glicocorticoide, são capazes de se ligar aos GREs e estimular a expressão gênica, mas variam na eficiência de indução pelo hormônio (MERKULOV; MERKULOVA, 2012; OAKLEY; CIDLOWSKI, 2011).

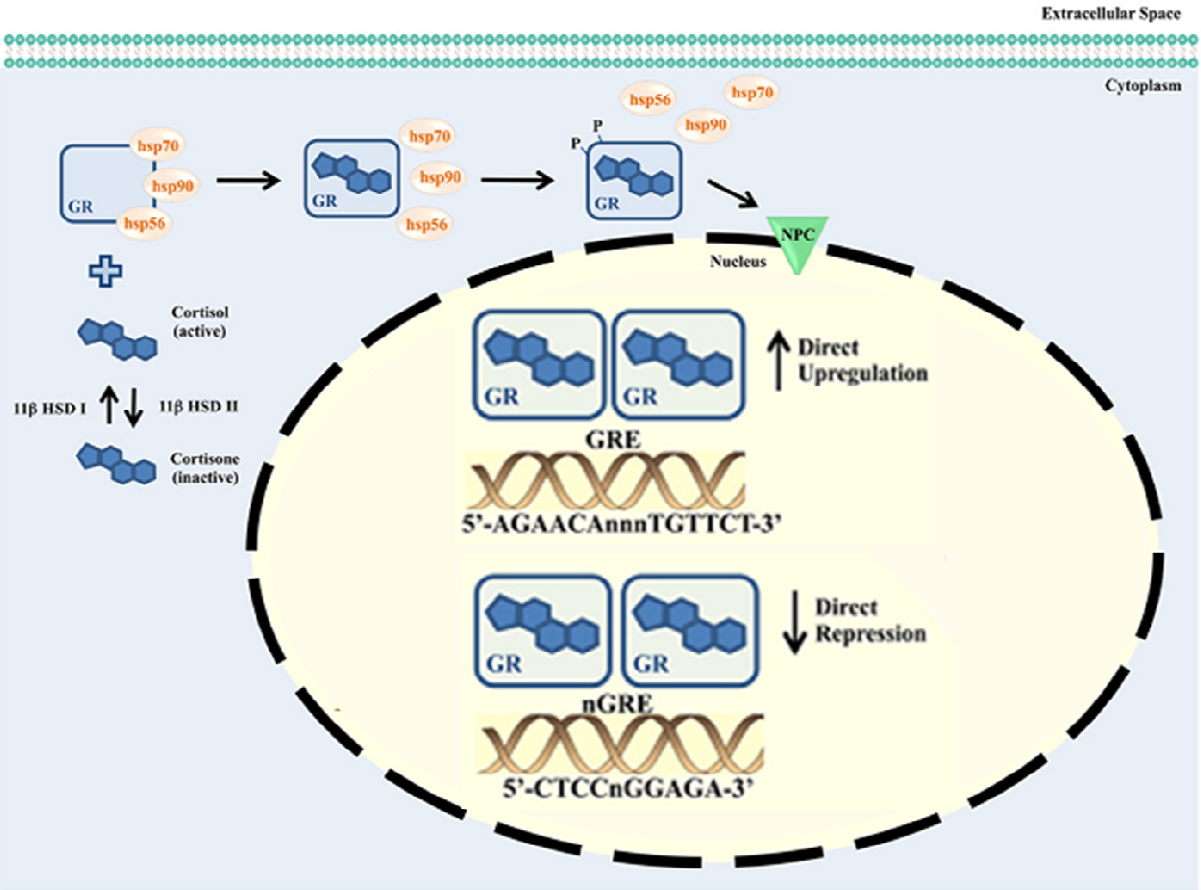
3.2.5 Mecanismo de ação

O principal mecanismo de ação dos GC ocorre através da sua ação genômica. Os glicocorticóides são capazes de se difundir pela membrana plasmática e se ligar a receptores específicos e de alta-afinidade, os receptores de glicocorticoides (GR*)*. Tais receptores na forma inativa então localizados no citosol ligados a proteínas HSP e imunofilinas, formando um complexo. Quando o cortisol se liga ao receptor, induz uma mudança conformacional na proteína, a qual provoca sua liberação do complexo e revelando um sitio de ligação nuclear, que facilita sua translocação para o núcleo (BARNES, 1998) ou ainda para a mitocôndria (LEE et al., 2013; PSARRA; SEKERIS, 2011).

Os GRs ligados ao hormônio formam dímeros, que se ligam a domínios de ligação específicos no genoma (elementos de reposta a glicocorticóides), e agem promovendo ou inibindo a transcrição de RNAm que originarão as proteínas

mediadoras das ações fisiológicas do GC (Figura 4). Além de atuar diretamente sobre o genoma, os GRs podem agir se ligando a outros fatores de transcrição, modulando sua ação, tanto de forma positiva quanto negativa (BAXTER, 1976; REDDY et al., 2009).

Figura 4: Esquema representando o mecanismo de ação do hormônio glicocorticóide (GC). O receptor de glicocrtiocide (GR) está no citoplasma acoplado a proteinas do choque térmico (hsp) e com a sua ligação ao GC, este se desloca para o núcleo, onde vai ativar ou inibir a expressão gênica. GRE: elemento responsivo a glicocorticóide localizado no DNA.



Fonte: Whirledge; Cidlowski, 2013.

O número de genes regulados diretamente (transativação) pelos glicocorticoides, por célula, é estimado entre 10 e 100. Sabe-se do papel desses genes no metabolismo da glicose e de lipídios, seja aumentando a expressão de transportador de glicose do tipo 1 (*GLUT 1*) em enterócitos e ou de ácido graxo sintetase (*FASN*, que participa da lipogênese) em adipócitos (GATHERCOLE et al., 2011; REICHARDT et al., 2012; SHAO et al., 2013).

Os glicocorticóides aumentam também a expressão de genes responsáveis pela resposta celular ao estresse, a *HSP* (proteínas do choque térmico). As HSPs estão envolvidas no processo de síntese, translocação e empacotamento de proteínas (EITAM et al., 2010; GEETHING; SAMBROOK, 1992; RAVIKUMAR; MUTHURAMAN, 2014). Outra função das HSP, em especial da HSP70, é inibir a apoptose celular induzida por diferentes fatores estressantes (HSU et al., 2014; KENNEDY et al., 2014), dessa forma os glicocorticóides desempenham sua função anti-apoptótica de forma indireta.

Entretanto muitos genes também são inativados de forma direta ou indireta (transrrepressão), regulados através de uma interação com outros fatores de transcrição e coativadores, representando o principal mecanismo pelo qual os glicocorticóides inibem a atividade de fatores de transcrição que regulam a resposta imune, bloqueando a expressão de NF- Kb (nuclear factor Kappa-B) e AP-1 (proteína ativação 1 do complexo dos fatores de transcrição) (HAYASHI et al., 2004).

O complexo glicocorticóide mais receptor pode ser translocado também para a mitocôndria, e se ligar a seqüências semelhantes à GRE em seu DNA, sugerindo assim a ação direta de GC na transcrição mitocondrial (DEMONACOS et al., 1995; LEE et al., 2013). Psarra e Sekeris (2011) verificaram que o cultivo de hepatócitos com dexametasona por 24 horas aumentou a expressão da proteína citocromo c oxidase subunidade 1 (COX 1), afetando assim a biosíntese das enzimas da fosforilação oxidativa . Além disso, os GCs superexpressam genes mitocondriais por meio da indução nuclear de fatores transcricionais da mitocôndria, como o fator de transcrição mitocondrial A (*TFAM*) e fator de respiração nuclear (*NRF 1*) (DATSON et al., 2008; LEE et al., 2013; PSARRA; SEKERIS, 2011).

A teoria genômica tradicional de ação dos glicocorticoides não explica completamente alguns efeitos rápidos desses hormônios. E assim começaram as especulações a respeito das ações não-genômicas, no qual foi identificado um tipo de receptor de membrana plasmática, que tem a mesma capacidade de ligação ao hormônio, e que provavelmente está relacionado a vias de sinalização celular, que desencadeiam efeitos mais rápidos como fosforilação de enzimas e estabilização de organelas (CHEN; QUI, 1999; EVANS; MURRAY; MOORE, et al., 2000).

3.2.6 Ação fisiológica

Hormônios glicocorticoides regulam inúmeros processos fisiológicos, como a regulação do metabolismo dos carboidratos, lipídios e proteínas, e exercem ação anti-inflamatória e imunossupressora.

O principal alvo dos GC é o metabolismo energético, tendo como principais objetivos a manutenção do nível de glicose circulante no sangue e a indução da gliconeogênese. O cortisol não age de forma direta sobre tais processos, mas sim ao estimular a síntese de enzimas participantes nessas vias metabólicas (GRIFFIN; OJEDA, 1996; GUYTON; HALL, 2011).

Além de atuar sobre o metabolismo, o cortisol age sobre outras funções fisiológicas como nas respostas anti-inflamatória e imunossupressora (BARNERS; ADCOCK, 1993; BOSSCHER; VANDEN; HAEGEMAN, 2000; BARNERS; ADCOCK, 2009). Dependendo do tipo celular, os GC promovem tanto a apoptose, como em timócitos e linfócitos, quanto exerce um efeito antiapoptótico em diferentes tipos celulares, como em célula epitelial pulmonar (WEN et al., 1997), neutrófilos humanos (KATO et al., 1995), células da granulosa (SASSON; AMSTERDAM, 2003) e células endoteliais glomerulares (MESSMER et al., 2000).

3.2.7 Glicocorticoides e Reprodução

A secreção de glicocorticóide em resposta ao estresse inibe o eixo hipotálamo-hipófise-gonadal (HHG) diminuindo a síntese de hormônios sexuais femininos e masculinos. Entretanto na ausência do estresse, acredita-se que os níveis fisiológicos de GC afetam de forma positiva a função reprodutiva. Além disso, estudos *in vivo* e *in vitro* indicam que outros componentes do sistema reprodutor também são regulados por glicocorticóides (WHIRLEDGE; CIDLOWSKI, 2013).

No sistema reprodutor masculino os GC além de inibirem a produção e liberação de testosterona, atuam aumentando a expressão de *BAX* e *Fas* (proteínas pró-apoptóticas) na linhagem germinativa testicular. Nesse sentido a enzima 11β-HSD 2, que regula ação do cortisol, é expressa em altos níveis nas células de Leydig, protegendo assim essas células do efeito negativo dos GC's (GE; HARDY, 2000; SINHA; SWERDLOFF, 1999).

No sistema reprodutor feminino o entendimento da atuação do cortisol na regulação da esteroidogênese através do eixo HHA é muito bem compreendido nos dias atuais, entretanto ainda há controvérsia com relação a sua ação nas células ovarianas (WHIRLEDGE; CIDLOWSKI, 2013).

Exposição de células ovarianas humanas durante a vida fetal à glicocorticóides resultou na diminuição da densidade de células germinativas como conseqüência do aumento da apoptose na oogônia (POULAN et al. 2012). No entanto, os glicocorticóides também atuam como potente citoprotetor no ovário, pois diminui a apoptose nas células luteínicas em bovinos, preservando assim a função do corpo lúteo (DUONG et al., 2012; KOMIYAMA et al., 2008).

Além disso, os GC's desempenham papel chave no inicio da gestação, pois regulam o sistema imune materno, a invasão e fixação do embrião no útero, e ainda o crescimento e desenvolvimento fetal (MICHAEL; PAPAGEORGHIOU, 2008). Tanto que atualmente a medicina reprodutiva humana utiliza desse conhecimento para administrar GC sintéticos a pacientes submetidas à fertilização *in vitro* e assim obter melhores taxas de gestação (BOOMSMA; KEAY; MACKLON et al., 2007; POLAK DE FRIED et al., 1993).

3.3 GLICOCORTICOIDES E SUA RELAÇÃO COM A PRODUÇÃO *IN VITRO* DE EMBRIÕES

3.3.1 Glicocorticoides e maturação oocitária *in vitro*

Em bovinos e humanos os glicocorticoides presentes na circulação se difundem para o fluido folicular, levando a níveis intrafoliculares de cortisol ativo geralmente mais elevados que os do sangue (HARLOW et al., 1997; ANDERSEN, 2002; THURSTON et al., 2002) e segundo Keay et al. (2002) estão associados com uma melhora na taxa de implantação embrionária em mulheres submetidas a técnicas de reprodução assistida sem ciclos de superestimulação, sugerindo assim que os GC influenciam no desenvolvimento folicular e maturação oocitária. Em estudos com células da granulosa de pacientes submetidos a fertilização *in vitro,* a

detecção da atividade da enzima moduladora de cortisol, 11β-HSD tipo 1, contribuiu para predizer o sucesso da transferência embrionária (MICHAEL et al., 1993).

Esses achados levaram a estudos que indicam que o cortisol está envolvido no final da maturação oocitária em humanos (HARLOW et al., 1997; KEAY et al., 2002; MICHAEL et al., 1997, 1999).

Apesar do efeito positivo dos GC na maturação oocitária em humanos, em outros mamíferos os efeitos ainda são contraditórios. Em um estudo mais detalhado sobre o mecanismo de ação dos glicocorticóides na maturação oocitária *in vitro* em suínos, foi demonstrado que o cortisol e seu análogo sintético, a dexametasona, tem efeito inibitório na progressão da meiose dependendo da concentração (YANG et al., 1999). A menor dose de cortisol com efeito inibitório foi 100ng/mL, semelhante a concentração encontrada em suínos após administração de ACTH, assim podemos concluir que esse efeito é mais farmacológico, ou seja , tóxico que fisiológico (YANG et al., 1999).

Além disso, este efeito negativo pôde ser impedido pelo antagonista do receptor de glicocorticóide, a droga mifepristona (RU-486), sugerindo que a retomada da meiose e maturação oocitária podem ser reguladas via receptor de glicocorticoide (YANG et al., 1999).

É interessante considerar por quais mecanismos os GC provocaram o bloqueio da maturação meiótica. Sabe-se que a regulação do ciclo celular, em especial a meiose, é controlada pelo fator promotor da fase M (MPF), formado pelo complexo $p34^{cdc2}$ e ciclina B1 (CHOI et al., 1991), e que o MPF fosforila várias proteínas envolvidas na formação da membrana nuclear, condensação da cromatina e reorganização de microtubulos (VERDE et al., 1992; DEKEL, 1996). Indicando assim uma possível rota pela qual os glicocorticóides podem atuar na maturação oocitaria, já que no estudo de YANG et al. (1999) os GC bloquearam a quebra da vesícula germinativa.

Em outro estudo realizado pelo mesmo grupo de Yang, os pesquisadores identificaram em oócitos suínos o mecanismo responsável para as ações inibitórias da dexametasona, que na concentração de 1ug/mL diminuiu os níveis da proteína ciclina B1, mas manteve os de $p34^{cdc2}$ em oócitos suinos maturados por 48 horas. A exposição dos gametas a DEX também diminuiu o complexo $p34^{cdc2}$-ciclina B1, impedindo assim a dissolução do envoltório nuclear (CHEN et al., 2000). O metabolismo de glicocorticóides no microambiente folicular pode ser controlado pela

enzima 11β-HSD, que converte cortisol em cortisona, sendo esta forma a biológica do hormônio que possui pouca afinidade pelo receptor de GC (LI et al., 2012). Webb et al. (2008) demonstraram que oócitos suínos que alcançam o estágio de metáfase II apresentam taxas elevadas desta, protegendo, assim, o microambiente ovariano dos efeitos inibitório dos GC durante a maturação.

Em ratos, Andersen (2003) testou concentrações fisiológicas e suprafisiológicas de cortisol e dexametasona durante a maturação oocitária com ou sem suplementação do hormônio folículo estimulante (FSH) por 24 horas, observando que os GC exerceram um efeito mínimo ou não influenciaram na regulação da maturação oocitária. Vale ressaltar que a presença de receptores para GC já foi comprovada em ovários de ratas, sendo este um possível mecanismo para uma reposta ao hormônio (SCHREIBER et al., 1982; TETSUKA et al., 1999).

Merris et al. (2007) avaliaram a função da dexametasona na foliculogênese e maturação oocitária de camundongos, observando que concentrações menores que 40ug/mL não afetam a foliculogênese e nem a oogênese, entretanto doses maiores que esta prejudicam a diferenciação folicular e a progressão dos oócitos para a metáfase II. Em altas doses de GC, a produção de andrógenos, estrógenos e progestina diminuíram drasticamente, o que pode ter comprometido a maturação nuclear e o metabolismo das células da granulosa.

Apesar de trabalhos sobre o efeito de GC em roedores já terem sido relatados (ANDERSEN, 2003; MERRIS et al., 2007), os mesmos não analisavam o efeito na maturação oocitária com subseqüente desenvolvimento embrionário. Além disso, em camundongos, o principal GC é a corticosterona e não o cortisol. Gonzales et al. (2010) analisaram o efeito de corticosteróides na maturação *in vitro* e mudanças nos padrões da via da quinase regulada por sinal extracelula (ERK), que são proteínas importantes para a progressão do ciclo celular, assim como também foram avaliados fertilização e progressão até ao estádio de blastocisto.

Estes pesquisadores verificaram um efeito diferencial de glicocorticóides naturais e sintéticos em oócitos de roedores. Concentrações suprafisiológicas (250 uM) de corticosterona diminuíram a ativação de ERK-1/2 e consequentemente a taxa de maturação nuclear oocitária, levando a um decréscimo no desenvolvimento embrionário. Fato este não observado no tratamento com dexametasona, que, mesmo em altas concentrações, não exerceu nenhum efeito na produção *in vitro* de embriões (GONZALES et al., 2010).

São várias as funções desenvolvidas por ERK-1/2. Além de atuarem diretamente no ciclo celular, essas quinases em altos níveis mantêm fosforiladas a proteína Bad (proteína pró-apoptótica), suprimindo assim a apoptose celular (GEBAUER et al., 1999). Esse efeito anti-apoptótico das ERK-1/2 pode ser regulados por glicocorticóides. SASSON et al (2003) verificaram que a dexametasona em doses baixas (10 nM) foi capaz de induzir a fosforilação de ERK-1/2 em células da granulosa bovinas e assim mediar o efeito anti-apoptótico nessas células.

Em peixe, os efeitos benéficos dos GC durante a maturação oocitária são melhores compreendidos. Diferentes espécies de peixes teleósteos mostraram ser capazes de induzir o oócito à maturação no seu estágio final, utilizando cortisol isolado ou combinado com metabólitos hidroxilados da progesterona, e esses efeitos ainda persistirem para o desenvolvimento embrionário (SUNDARARAJ et al., 1971, MILLA et al., 2006; KLEPPE et al., 2013).

LI et al. (2012) mostraram que as taxas de fertilização, clivagem e eclosão de embriões derivados de oócitos de peixe maturados em meio suplementado com cortisol foram maiores em relação ao grupo controle, apresentando embriões com maior número de células e melhorando assim a qualidade embrionária, através da ativação de vias de sinalização do sistema IGF (fatores de crescimento relacionados a insulina, do inglês “insulin-like growth factors”). Desta forma, a inclusão de cortisol na maturação *in vitro* aparentemente promoveu um efeito duradouro no desenvolvimento embrionário.

Embora o conjunto de regulação responsável pela maturação de oócitos em mamíferos seja semelhante podem existir variações mais sutis entre as espécies (ANDERSEN, 2003; BILODEAU-GOESEELS, 2012), além disso, diferentes respostas a GC podem ser atribuídas principalmente a níveis de receptores para glicocorticóides e sua afinidade, assim como a cinética da proteína durante maturação oocitária.

3.3.2 Glicocorticóides e o cultivo *in vitro* de embriões

São poucos os trabalhos que descrevem o efeito de glicocorticoides no desenvolvimento embrionário pré-implantacional. Siemieniuch et al. (2010) identificaram transcritos para receptores de glicocorticoides e as duas isoformas das

enzimas 11β- hidroxiesteroide desidrogenase (11β–HSD) no oviduto e no endométrio gravidico assim como em embriões pré-implantacionais produzidos *in vitro.* Nesse estudo a expressão de RNAm para 11β-HSD1 era maior em embriões bovinos produzidos *in vitro* no 4º dia (5-16 células) de cultivo quando comparados com trofoblastos e endometrio no 16º dia de gestação. Dessa forma pode-se supor que, durante as fases iniciais da gravidez, os GC atuam como um fator autócrino ou parácrino e assim mediam a comunicação entre o ambiente materno e o embrião.

Já que o embrião pré-implantacional é capaz de responder a presença de glicocorticóides, Merris et al. (2007) testaram o efeito embriotóxico da dexametasona (5 a 80 µg/mL) no início do cultivo *in vitro* de embriões de camundongos e verificaram que este não afetava o estádio de desenvolvimento de 2 células nem as clivagens iniciais, porém, a expansão e a capacidade de eclosão dos blastocistos foram prejudicadas a partir de 10 µg/mL de dexametasona, sendo que os embriões expostos a 80 µg/mL apresentaram, a partir do 5°dia de cultivo, características degenerativas.

Em outro estudo foi verificado o efeito anti-apoptótico da dexametasona no cultivo *in vitro* de embriões bovinos. Sabe-se que embriões produzidos *in vitro* têm elevadas taxas de apoptose, resultando assim na qualidade inferior dos mesmos (SANTANA et al.,2014).

Santana et al. (2014) avaliaram o possível envolvimento da dexametasona na diminuição da apoptose em embriões bovinos e observaram que o tratamento de embriões com dexametasona não diminuiu a taxa de apoptose observada em embriões de D4, mas melhorou a qualidade embrionária, acelerando a eclosão e aumentando o número de células, sugerindo os efeitos positivos do GC na proliferação celular (SANTANA et al., 2014). Esse estudo corrobora com o estudo de LI et al., 2012 em embrião de peixe no qual a adição de cortisol no fluido folicular aumentou o número de células embrionárias devido a hiperexpressão de genes relacionados a fatores de crescimento.

Mais estudos são necessários para se identificar a melhor dose benéfica de GC, assim como o estádio de desenvolvimento embrionário no qual estes hormônios atuam induzindo o melhoramento da qualidade do embrião. Tais informações podem servir de base para se compreender a função dos GC na fase inicial de gestação e como ele pode ser utilizado dentro das biotecnologias reprodutivas.

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4. ARTIGO 1: Expression and functional significance of the glucocorticoid receptor in bovine oocytes and embryos.

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Abbreviations: GC, glucocorticoid; GR, glucocorticoid receptor; siRNA; small interference RNA; IVC, in vitro culture; IVM, in vitro maturation; IVF, in vitro fertilization; COCs, cumulus-oocytes complexes

ABSTRACT

Glucocorticoids (GC) are the hormones involved in various physiological processes, because their main mechanism of action occurs through interaction with specific receptors, the identification of the response may indicate that the CG play a major role in the production of embryos in vitro. Thus the aim of this work was to study the glucocorticoid receptor (GR) in vitro production of bovine embryos. First we identify the presence of mRNA and protein for GR (using RT-PCR analysis and immunocytochemistry, respectively), cumulus oocyte complexes (COC), 2-4 oocytes and embryo cells 8-16 cell, morula and blastocyst. All stages presented transcripts for GR and positive staining of the corresponding protein, however there was a difference in the labeling pattern as the location of the GR (cytoplasm and nucleus) between the analyzed stages. In the second experiment GR was analyzed for early embryonic development silencing the translation of mRNA for GR, using the technique of RNAi. Zygotes were injected with siRNA to GR 16 hours after in vitro fertilization, and then cultured for 8 days for analysis of embryonic development and analysis of the amount of mRNA and protein for GR in blastocyst. Cleavage rate, blastocyst, relative quantification of mRNA and fluorescence for GR were submitted to ANOVA . Gene expression for GR decreased in blastocysts, and quantification of immunofluorescence in blastocysts ($p < 0.05$), revealing that there was block the translation. Regarding the rate of cleavage and blastocyst decreased in the injected group (58 ± 4.4%; 9% ± 1.4, respectively) compared to control (81% ± 8.3; 25% ± 7, respectively) ($p < 0.05$). We conclude therefore that the GR is present in the cytoplasm and nucleus of COC and pre-implantation bovine embryos, these GR are important for proper embryonic development.

INTRODUCTION

Glucocorticoids modulate many physiological processes including energy metabolism (Pecket et al., 2011), immune and cardiovascular function (Barners and Adcock, 2009) and the stress response (Aschbacher et al., 2013; Whirledge and Cidlowski, 2013). Beyond their effects upon the hypothalamic-pituitary-adrenal/gonadal axis, glucocorticoids play important roles in reproduction including onset of puberty timing, spermatogenesis, luteal function as well as in pregnancy establishment and maintenance (Dupuis et al., 2007; Hikim and Swerdloff, 1999; Waddell et al., 1996).

Few studies have evaluated the effect of GCs on gametes and pre-implantation embryos despite some evidence for a direct role upon ovarian function and early embryo development. For instance, in human follicular fluid the cortisol:cortisone ratio is higher in preovulatory than immature follicles (Fateh et al., 1989; Harlow et al., 1997; Keay et al, 2002), suggesting a positive effect of cortisol (the active metabolite) on oocyte maturation.

Moreover, higher cortisol:cortisone concentration ratios in follicular fluid have been associated with IVF success in women undergoing unstimulated assisted reproduction cycles (Keay et al., 2002). In a recent study, supplementation of the in vitro culture medium with the synthetic GC dexamethasone improved the developmental kinetics and cell number of in vitro produced bovine blastocysts (Santana et al., 2014). Altogether, these studies support a role of GCs upon oocyte/embryo developmental competency. Most actions of GCs are mediated by intracellular signaling through the glucocorticoid receptor (GR), a member of the family of transcription factors linked to nuclear receptors. Several isoforms are generated by alternative splicing of the GR gene. In humans, alternative splicing in exon 9 results in two transcripts that yield the hGRα and hGRβ isoforms, respectively (Merkulov and Merkulova, 2012; Oakley and Ciclowski, 2011).

Furthermore, post-transcriptional changes resulting from multiple translational initiation sites also lead to eight hGRα isoforms (Lu and Cidlowski, 2005; Oakley and Ciclowski, 2011) . Most unbound hGRα is found in the cell cytoplasm as part of a large protein complex including several chaperone proteins (Grad and Picard, 2007). After binding to its ligand hGRα changes its conformation and translocates to the

nucleus, where it binds to gene responsive elements (GREs) or other transcription factors, hence modulating the transcription of target genes (Beato, 1989).

In accordance with a role of glucocorticoids in oocyte maturation and early embryo development, GR expression has been identified in ovarian granulosa cells (Hirst et al., 1990; Schreiber et al., 1982), in oocytes (Belikov et al., 2007; Milla et al., 2009) and in embryos (Driver et al., 2001; Lu et al., 2012; Manceau et al., 2012) in laboratory species and humans.

In the bovine species, expression of mRNA for the GRα isoform has been shown in embryos and the endometrium (Siemieniuch et al., 2010), suggesting a role in signaling during early embryo development. However, given the complex transcriptional and translational regulation of the GR, the presence of mRNA alone does not necessarily translate to the presence of functional GR protein (Adams et al., 2000). Given that the main actions of GCs occur via binding to its GR, in this study we sought to identify the GR both at the transcript and protein level in all cumulus cells, oocytes and embryos. The goal of this study is to understand the potential role of GCs via the GR during oocyte maturation and early embryo development.

RESULTS

Experiment 1: GR mRNA and protein expression in oocytes, cumulus cells and embryos.

All samples analyzed displayed specific amplification for GR mRNA as evidenced by a single peak in the dissociation curve prior to 35 cycles. Similarly, all samples displayed positive immunostaining for GR protein, which was absent in negative controls (Figure 1).

Cumulus cells from GV oocytes displayed predominantly difuse cytoplasmic immunostaining (Figure 1D). Similarly, cumulus cells from MII oocytes also presented cytoplasmic staining although with a more uniform distribution, but with nuclear stained too (Figure 1G). Immature oocytes showed a uniform GR distribution in the cytoplasmic and nucleus (Figure 2C). However in matured oocytes GR displayed a patchy appearance in MII oocytes, prioritizing one pole of the oocyte, and in the limit between metaphase and polar body showed a GR stained too (Fig. 2F).

For embryos, we analyzed GR immunolocalization at different developmental stages (Figure 3). At the 2-4 cell stage, the GR was evenly distributed in both the

cytoplasm and nuclei of blastomeres (Figure 3A and 3C). Conversely, blastomeres in embryos at the 8-16 cell stage displayed more intense immunostaining in the nucleus than cytoplasm (Figure 3D). At the morula and hatched blastocyst (HB) stages, the GR was again evenly distributed throughout the cells (Figure 3I and 3J).

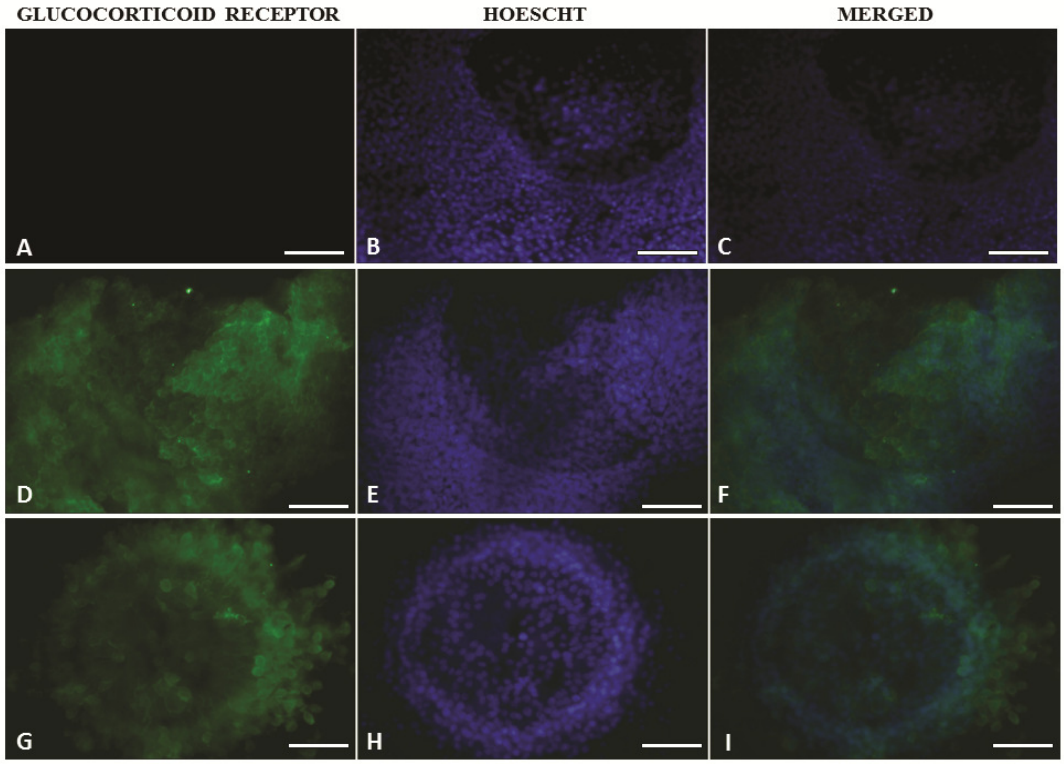

Figure 1: Immunocytochemistry for the glucocorticoid receptor (GR) in cumulus-oocyte complexes. (A,B,C) Negative controls without primary antibody. (D,E,F) Cumulus cells from inmature (GV) oocytes. (G,H,I) Cumulus cells from mature (MII) oocytes. GV: germinal vesicle. MII: metaphase II. Bar scale 50 µM

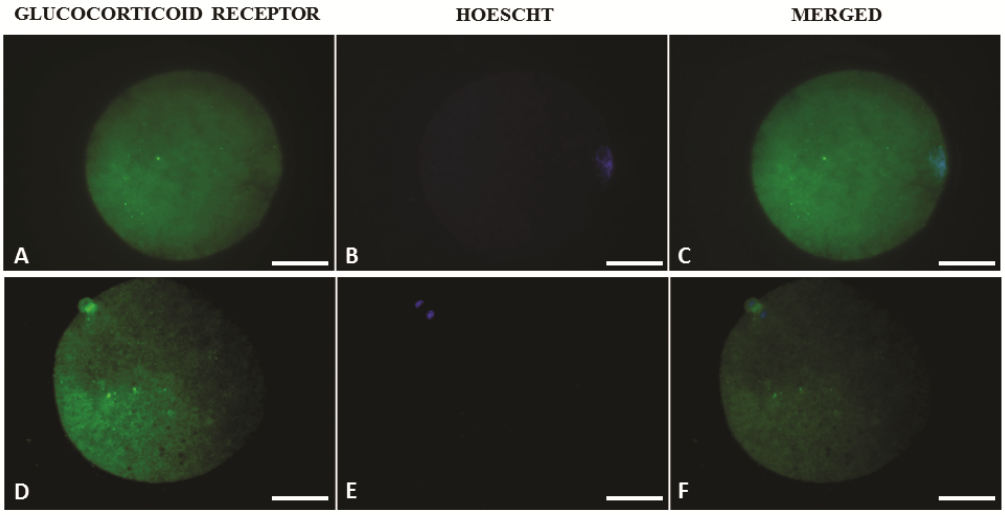

Figure 2. Immunocytochemistry for the glucocorticoid receptor (GR) in oocytes. (A,B,C) Inmature (GV) oocytes. (D,E,F) Mature (MII) oocytes. Bar scale 50 µm.

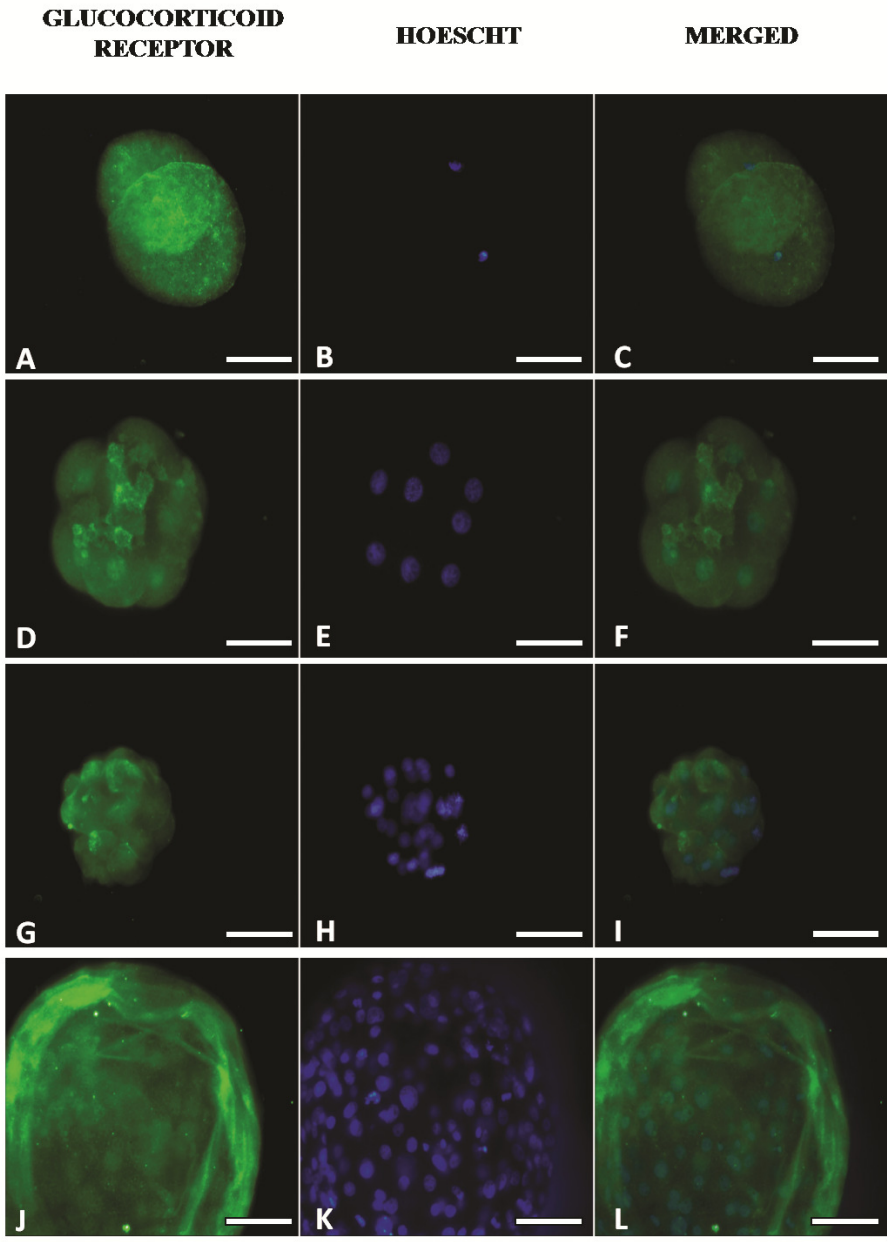

Figure 3: Immunocytochemistry for the (GR) in embryos at different developmental stages. (A,B,C) 2 cell embryo (representing 2-4 cell embryos). (D,E,F) 8 cell embryo (representing 8-16 cell embryos). (G,H,I) Morula. (J,K,L) Hatched blastocyst. Bar scale 50 µm.

Experiment 2: Effect of GR siRNA microinjection in zygotes on the subsequent embryo development.

To analyze the functional significance of the GR during bovine embryonic development, a GR-specific siRNA was microinjected into zygotes at 16 hours after IVF, and cleavage and blastocyst rates were then assessed (Table 1). Interestingly, embryos injected with GR-specific siRNA displayed lower ($P<0.05$) cleavage and blastocyst developmental rates. This was not due to microinjection itself since zygotes injected with a scrambled siRNA showed development rates similar ($P>0.05$) to those in the uninjected control group.

Table 1: Cleavage and blastocyst rates following zygote microinjection with a siRNA specific for the glucocorticoid receptor.

Groups	**n**	**Cleaved %** **Mean ± SD**	**Blastocyst %** **Mean ± SD**
Control	176	81%±8.3[a]	25%±7.0[a]
Injection Control	144	80%±3.6[a]	17%±4.6[ab]
siRNA Injection	147	58%±4.4[b]	9%±1.4[b]

[a, b] Different lowercase superscripts within column denote significant differences ($p<0.05$)

n = sample size; SD = standard deviation

Cleavage was evaluated at 24 hr of in vitro culture.

Control: uninjected zygotes. Injection control: zygotes injected with a scrambled control siRNA.

The efficiency of downregulating GR expression was evaluated in blastocysts () at 8 days post siRNA microinjection. A siRNA concentration of 20 µM was sufficient to reduce GR expression at both the transcript (Figure 4) and protein (Figure 5) levels, when compared to both uninjected controls or controls injected with a scrambled siRNA ($P<0.05$).

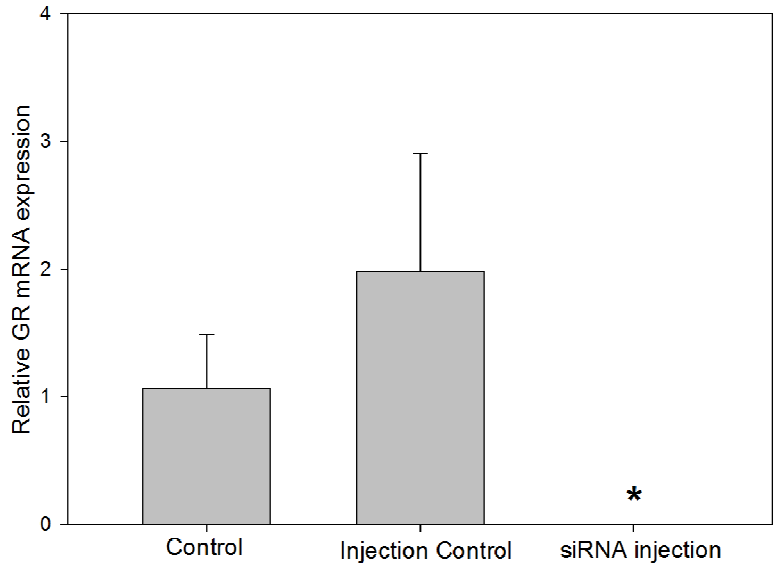

Figure 4: Relative concentration of mRNA for the glucocorticoid receptor (GR) blastocysts following microinjection of a GR-specific siRNA. Control: uninjected zygotes. Injection control: zygotes injected with a scrambled siRNA. *P<0.001.

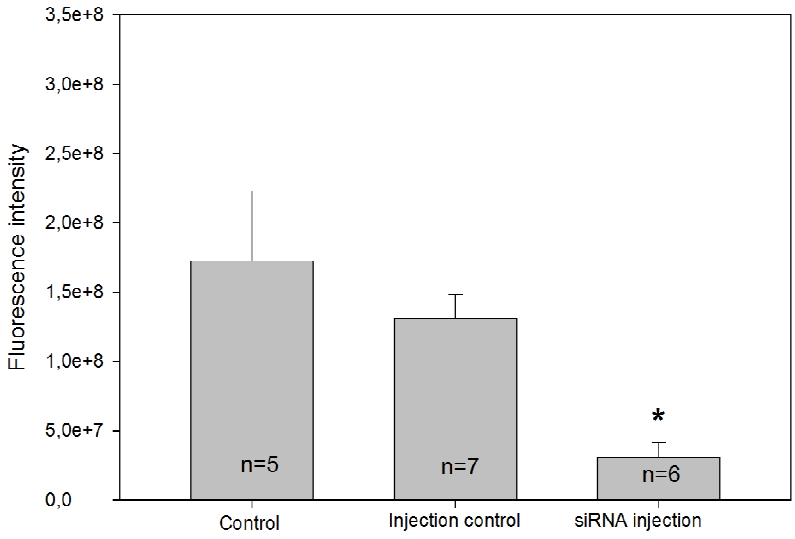

Figure 5: Relative fluorescence intensity for glucocorticoid receptor (GR) immunoblotting in bovine blastocysts. Zygotes were injected with a GR-specific siRNA. Control: uninjected zygotes. Injection control: zygotes injected with a scrambled siRNA. *P<0.001

DISCUSSION

Herein we report expression of GR at the protein level, as well as changes in the pattern of GR expression during oocyte maturation and embryo development. This is particularly relevant given that previous studies had only shown expression at the mRNA level and that microRNAs can post-transcriptionally regulate the expression of various genes with impact on protein levels (Papadopoulos et al., 2015; Siemeniuch et al., 2010). Moreover, since most effects of glucocorticoids are driven via its receptor (Burgess et al., 2010), we hypothesized that the expression of GR should have functional significance.

To further explore this we knocked down GR expression in zygotes using siRNA technology and recorded embryo development rates. Interestingly, lower GR levels correlated to a decrease in cleavage and blastocyst rates as compared to uninjected and injected controls. Altogether, these experiments provide evidence for a role of glucocorticoids in preimplantation embryo development. Much has been said about the importance of GRs at the end of oocyte maturation and ovulation (Andersen, 2002; Keay et al 2002), In regards oocyte maturation in pig glucocorticoid may exert negatively effects (Chen et al., 2000) and in mice decrease ERK -1/2 leading to a poor blastocysts rates (Gonzales et al., 2010), but little is understood about the mechanisms by which these hormones exert their function.

The nuclear GR translocation and its localization are important factors to understand the glucocorticoid actions (Robertson et al., 2013). Therefore, in this study, we assessed the distribution of the GR in both GV and MII oocytes, as well cumulus cells. Cumulus cells (CC) are cells of follicular lineage that directly surround the oocyte (Chian et al., 2004; Gilchrist et al, 2008) connected among them and with the oocyte by cytoplasmic projections with gap junctions (Albertini et al, 2001). Cumulus cells play a crucial role in oocyte maturation, and the acquisition of oocyte competence (Macaulay et al, 2014; Regassa et al, 2011).

Interestingly, cumulus cells from GV oocytes showed both nuclear and cytoplasmic GR distribution . Previous studies have also shown that translocation or presence of the GR in the nucleus may be independent from glucocorticoids in the medium, James et al. (2003) showed in bovine lens epithelial cells that GR was marginally in the nucleus without a presence of glucocorticoid and in the cauda epididymis of rat, that exhibited a predominant GR-positive nuclear/perinuclear staining and a diffuse cytoplasmic immunolocalization (Silva et al., 2010). Although in many reports the predominant location of unbound GR appears to be cytosolic,

based on the classic mechanism that following binding, the glucocorticoid-GR complex translocates to the nucleus to affect gene expression (De Bosscher et al. 2000; Oakley et al., 1999; Raddatz, et al, 1996). So we speculate that GR in the nucleus may have actually bound to glucocorticoids present in follicular fluid prior to oocyte retrieval for our experiments (Spicy et al., 1987).

During maturation, the oocyte acquires competence or the ability to become fertilized and support embryonic development (Sirard et al., 2006). This competence acquisition requires all nuclear, cytoplasmic and molecular maturation, with the corresponding storage of transcripts, proteins and nutrients to be used at the time of activation of the embryonic genome (Sirard , 2011). In this study we report the presence of GR in immature oocytes, at a time when the DNA chromatin is decondensed and accessible to gene transcription, presumably generating mRNAs to be stored after germinal vesicle breakdown (Rodgers et al, 2003; Assou et al, 2010). Conversely, GR was not observed in the the metaphase plate in MII oocytes, although a stained close to metaphase and polar body was observed; which is compatible with the condensed chromatin and lower transcription activity observed at this stage (Lequarre et al., 2004; Sirard et al., 1998). From these findings, we infer that supplementation of maturation medium with glucocorticoids may contribute to the acquisition of higher oocyte competence.

In this study we confirm that GR transcripts (Siemeniuch et al., 2010) are translated into the corresponding protein in bovine preimplantation embryos, further supporting a potential role for glucocorticoids in embryo development. Indeed, 2-4 cell embryos displayed both cytoplasmic and nuclear staining for GR. In cattle, it is generally considered that activation of the embryonic genome takes place at the 4 to 8 cell stage (Jiang et al., 2014). However, some studies reported activation of some genes already at the 4-cell stage, with eight embryonic proteins already detected at this stage against the 23 present in 8-cell embryos (Barnes and First, 1991). Thus, the nuclear localization of GR in 4-cell embryos may be involved in early embryonic genome activation in cattle, the developmental stage at which the maternal transcripts and proteins begin to be degraded, and the embryo becomes dependent upon transcription of its own genes (Memili and First., 2000; Graf et al, 2014).

Nuclear GR immunostaining became further evident in blastomeres from 8-16 cell embryos, and was also observed at later stages. This may also correlate to the increased gene transcription occurring during these initial stages of embryo

development. ,which may be required to sustain the corresponding high cleavage activity (Graf et al., 2014).

To further analyze the functional significance of the GR during embryonic development, we knocked down GR mRNA translation using siRNA technology. This should allow for the analysis of the effects of decreased amounts of the corresponding GR protein during early embryonic development (Driver et al, 2013;. Favetta et al, 2007;Furuya et al,. 2007; Yamanaka et al, 2011; Xiong et al, 2013).

Interestingly a decrease in GR expression resulted in a 16% decrease in the blastocyst rate observed on the 8th day of culture. Our study did not address the reason by which GR downregulation may have hampered blastocyst development. However, human adenocarcinoma cells transfected with siRNA for GR displayed a significant suppression of 44 glucocorticoid-regulated genes, including genes important for early embryonic development, such as transcription factors, Klf4 and POU5F1. Therefore, further research should analyze the effect of GR downregulation upon expression of developmental genes.

In summary, the GR is expressed both at the mRNA and protein levels in bovine GV and MII oocytes and their corresponding cumulus cells, as well as in embryos at various stages of preimplantation development, albeit its distribution pattern varies at these different stages. The fact that GR downregulation reduced embryo development rates supports a crucial role for this receptor in embryo preimplantation events . We speculate that reduced GR expression may impact embryos by affecting the expression of genes important for development, although this requires further investigation.

MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

Experimental design

Two experiments were conducted to study the presence of functional GRs in bovine oocytes and embryos. Experiment 1 aimed to identify the presence of GR transcripts (qualitative analysis by real-time PCR) and protein (immunocitochemistry) in oocytes and cells of the immature and matured cumulus embryo cells 2-4, 8-16

cell, morula and blastocyst. In Experiment 2, GR expression was knocked down via microinjection of the corresponding siRNA into zygotes, and embryo development was subsequently monitored. The efficiency of GR downregulation was evaluated by relative quantification via immunofluorescence using a primary antibody against the GR.

In Vitro Embryo Production

Oocyte collection and in vitro maturation (IVM)

Ovaries were obtained from a slaughterhouse and transported in 0.9% sodium chloride solution at room temperature for a maximum of 2 hours. Once in the laboratory, 2-8 mm antral follicles were punctured using a using a syringe and 18Ga needle and follicular fluid was aspirated. Only cumulus-oocyte complexes (COCs) with compact cumulus cells and good morphological appearance were selected (Leibfried and First, 1979). Groups of 10-15 COCs were then placed into 100-µL droplets of TCM 199 supplemented with 25 mM sodium bicarbonate, 10% FBS (Gibco BRL, Grand Island, NY, USA), 11 mg/mL pyruvate, 50 µg/ml gentamicin, 0.5 µg/mL FSH (Folltropin, Bioniche Animal Health, Belleville, Ont., Canada), and 5 µg/mL LH (Lutropin, Bioniche Animal Health). Drops were overlaid with sterile mineral oil, and cultured in an incubator at 38.5℃ in a 5% CO_2, 20% O_2 and 75% N_2 atmosphere in humidified air for 22 hours.

In vitro fertilization (IVF) and in vitro culture (IVC)

Frozen semen from one bull (*Bos indicus*) of proven fertility was used for IVF.

After thawing, semen was centrifuged at 180 *g* for 7 minutes through a discontinuous density gradient Percoll column (GE Healthcare Bio-Sciences, Uppsala, Sweden).

In vitro fertilization was performed in 80-µL droplets of TALP-Fert modified medium (Parrish et al., 1988) supplemented with 30 µg/ml heparin, 1.8 µM epinephrine, 18 µM penicillamine, 10 µM hypotaurine, and 4 mg/ ml bovine serum albumin (BSA) under the same conditions described for IVM. In each droplet, groups of 20 COCs were co-incubated with 2 x 10^6 sperm/ml frozen-thawed washed sperm. At 26 hours following insemination presumptive zygotes were incubated in a cumulus

cell monolayer in 100-µL droplets of synthetic oviductal fluid (SOF) culture medium (Holm et al. 1999). Incubation proceeded in the same conditions as described for IVM. Length of time in culture depended upon the following experimental step.

Microinjection of GR-specific siRNA into zygotes

The GR gene sequence was obtained from GenBank (access number: NM_001206634). Both the sense and antisense sequence for the corresponding siRNA were designed and synthesized by Life Techonologies (Burlington, ON, Canada), siRNA primer sense: GGAGAUGAUAACUUGACUUTT, siRNA primer antisense: AAGUCAAGUUAUCAUCUCCTT and for control, a scrambled siRNA primer sense: GGAGUUAAAUCGUAGACUUTT, and anti sense: AAGUCUACGAUUUAACUCCTT. The siRNA was dissolved in RNase free water according to the manufacturer's instructions.

At 16 hours following IVF, presumptive zygotes were randomly allocated to one of three experimental groups (20-30 per group): 1) Control or not microinjected; 2) Injection Control or embryos microinjected with scrambled siRNA not targeting GR mRNA; and, 3) Injection group or zygotes injected with the siRNA targeted to GR. The control group was incubated in SOF medium and stored under the same conditions of IVM.

For micromanipulation, zygotes were transferred to 20-µL droplets of TALP HEPES medium supplemented with 5% FBS overlayed with light mineral oil. Microinjection was performed with sterile injection capillary (Femtotip II; Eppendorf, Hamburg, Germany) using a FemtoJet Microinjector (FemtoJet express, Eppendorf, Hamburg, Germany). Approximately 15 pL of GR-specific siRNA duplex (20 mM) or its scrambled control were delivered into the cytoplasm of a zygote. Lysed embryos were discarded, and viable zygotes were washed 3 times in SOF medium, and cultured for 8 days.The efficience of GR downregulation following siRNA microinjection was estimated by the relative fluorescence intensity reduction following immunoblotting as well as by the relative transcript expression using qRT_PCR (see below).

Immunocytochemistry

Immature (GV) and mature (MII) oocytes, as well as matured and immature CCO/Oocyte and embryos (of 15 pools each) at different stages of development (2-4

cells, 8-16 cells, morula and hatched blastocyst) were washed 3 times in wash buffer (0.1% PBS / PVP) and fixed in 4% paraformaldehyde (PFA) for 15 minutes. After fixation, samples were washed 3X and stored in wash buffer at 4 °C. Oocytes / COO / embryos were then permeabilized in 400-µL drops of 0.25% Triton X-100 in wash buffer for 10 minutes and rinsed 3X for 3 minutes each in wash buffer. Samples were then incubated in 400 µL of blocking buffer (1% BSA, 0.3M glycine, 0.1% PVP in PBS) for 30 minutes and rinsed 3X for 3 minutes each in wash buffer. After blocking, samples were incubated in 25-µL drops of blocking buffer containing a mouse raised monoclonal primary antibody against the GR (1:50; ab2768, ABCAM, Cambridge, USA) at 4℃, overnight in a humid chamber. Negative controls were incubated in blocking buffer devoid of primary antibody. After rinsing 3X in wash buffer for 3 minutes each, samples were incubated in 40-µL drops of blocking buffer with a FITC-conjugated goat anti-mouse secondary antibody (1:200; sc2010; Santa Cruz, Dallas, USA) for 1 hour in a dark chamber. For nuclear staining, , samples were incubated in 100 µl of 1:100 Hoechst in blocking buffer for 10 minutes and then rinsed 3X in wash buffer. Drops with about 10 oocytes/embryos were placed onto a glass slide and overlaid with 5µL of anti-fade (VECTASHIELD, Ingold Road USA). Visualization was performed using a H550S fluorescence microscope (NIKON, Tokyo, Japan) at 400X using the appropriated excitation filters. The relative quantification of the specific fluorescent was calculated using the Corrected Total Cell Fluorescence (CTCF), which is a measurement of intensity of the specific fluorescent against the background (Burgess et al., 2010). *CTCF = Integrated Density – (Area of selected cell X Mean Fluorescence of background readings). The measurement of the images for the calculation was obtained using Image J software (ImageJ 1.32j analysis software, NIH, USA).

Gene expression: Storage of samples

Cumulus cells of both GV and MII oocytes were removed by repeated pipetting in a 0.5% hyaluronidase solution. Only oocytes with a first polar body were considered mature. Embryos at the 2-4 cell (day 2), 8-16 cell (day 3), morula (day 5) and hatched blastocyst (day 7) stage were also collected for analysis. For zona pellucida removal, oocytes and embryos were incubated in a 1.5 mg/ml protease solution in PBS for 5 minutes. Immature oocytes matured in pools of 20 and their respective cumulus cells Groups of 20 immature or mature oocytes, their

corresponding cumulus cells, as well as groups of 30-60 embryos were each placed separately in 0.2-mL microtubes containing 5 µl of RNAlater (provide source information) and stored at -20°C until the time of RNA extraction.

Gene expression: RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Micro kit kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was treated with DNAse I (provided with the kit) to avoid contamination by genomic DNA. Reverse transcription was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Gene expression: Real time PCR

Real time PCR was performed using a StepOne plus Real Time PCR system (Applied Biosystems). Primers were designed using Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) based on cDNA sequences available in GenBank (Table 2). Primer specificity was checked by BLAST. The PCR total reaction volume was 10 µL containing 5 µL of SYBR Green Master Mix (Applied Biosystems), 0.5 mM of each primer, and 4 µL of DNA diluted 1: 8. The thermocycling conditions consisted of 95 °C for 10 minutes, followed by 45 cycles at 60 °C for 1 minute. The specificity of PCR products was verified by evaluating the corresponding dissociation curves.

The amplification efficiency for each gene was calculated using a relative standard curve of serial dilutions of cDNA (1: 4, 1: 8; 1:16; 1:32; 1:64). The relative quantity of each target gene was corrected relative to the expression of the housekeeping genes histone 2A1 (*H2A1*) for Experiment 1, or the tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide (*YWHAZ*) and succinate dehydrogenase flavoprotein subunit (*SDHA*) (Goossens et al., 2005) in Experiment 2. After normalizing to endogenous gene expression ($\Delta Ct = \text{target gene } Ct - \text{endogenous gene}$) and then to the control group ($\Delta\Delta Ct = \text{treatment } \Delta Ct - C\ \Delta Ct$), relative quantification using $2^{-\Delta\Delta Ct}$ was calculated as a fold change of target mRNA expression vs. control. Each experiment was performed in triplicate.

Table 2: Primer sequences, expected fragment size and GenBank access code for genes evaluated by RT-PCR.

Gene	Sequence	Size (pb)	Genbank
YWHAZ	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	120	BM446307
SDHA	GCAGAACCTGATGCTTTGTG CGTAGGAGAGCGTGTGCTT	185	NM_174178
H2a1	CCAGCACTTCAGACAAAATCCTA AGCGGCTGACTCTTCGTTG	182	U62674
GR	TGACTTCCTTGGGGACTTTG TGAGGAACTGGATGGAGGAG	107	XM_612999.4

YWHAZ: Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide. *SDHA*: Succinate dehydrogenase flavoprotein subunit. *H2A1*: Histone 2A1. *GR*: glucocorticoid receptor. bp: base pairs

Statistical Analysis

Data for average embryo development rates, as well as relative gene expression and immunofluorescence values were analyzed by ANOVA followed by Bonferroni post-test ($p < 0.05$) using SigmaPlot 11.0 software (Systat Software, San Jose, SC, USA).

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5. ARTIGO 2: EFFECT OF CORTISOL ON BOVINE OOCYTE MATURATION AND EMBRYO DEVELOPMENT IN VITRO

O artigo foi submetido a revista Theriogenology e segue as normas da revista

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ABSTRACT

Glucocorticoids (GCs) are important mediators of key cellular events. Herein we investigated the effect of adding cortisol to the in vitro maturation (IVM) medium on the acquisition of developmental competency in bovine oocytes. Cortisol (0.01, 0.1 or 1 µg/mL) had no effect on cleavage rates or cell numbers of resulting blastocysts, although supplementation with 0.1 µg/mL during IVM increased blastocyst rates of in vitro fertilized (IVF) bovine oocytes as compared to untreated controls (41 ± 10 vs. 21 ± 1.2%, $p < 0.05$, respectively). This concentration was chosen to assess changes in the relative expression of potential GC target genes. Oocytes matured in the presence of cortisol and their corresponding cumulus cells did not show changes in expression for genes analyzed as compared to untreated controls. Notably, blastocysts from oocytes matured in cortisol-supplemented medium expressed higher relative levels of glucose transporter 1 (*GLUT1*), fatty acid synthase (*FASN*), and heat shock protein 70 (*HSP70*).

This study supports a role for cortisol in the acquisition of bovine oocyte competence. This is evidenced by increased blastocyst development rates and presumably related to increased embryonic transcripts with roles in glucose and lipid metabolism, as well as the cellular response to stress.

Keywords: cortisol, oocyte competence, gene expression, embryo, bovine

1. Introduction

Oocyte maturation is a long and complex process during which the female gamete acquires the competence to be fertilized as well as to sustain in vitro embryo development to the blastocyst stage and, likely, in vivo development to term [1-3]. In vivo, oocyte maturation occurs within the follicle driven by multiple local and systemic signalling events that require interaction between the oocyte and surrounding somatic cells [4]. Given the inherent challenge involved in trying to replicate these events, in vivo matured oocytes display higher developmental competency than their in vitro counterparts [5,6].

Certain mRNA and protein expression patterns determine oocyte quality [7,8]. In this regard, it is known that in vitro culture conditions used for oocyte maturation can influence gene expression and thus developmental competency [8, 9]. Moreover, while much progress has been made in optimizing maturation media, in vitro matured bovine oocytes rarely yield more than 30-40% blastocyst rates as compared to almost two times higher rates obtained with in vivo matured oocytes under the same fertilization and embryo culture conditions [6,10]. Therefore, optimization of the oocyte maturation conditions via the addition of hormones and/or growth factors to the culture medium is still a research priority [9, 11-14].

Glucocorticoid hormones (GC), in particular cortisol, are important mediators in many cellular events such as apoptosis modulation [15, 16], response to stress [17,18], lipid and carbohydrate metabolism [19,20], and mitochondrial activity [21-23]. However, their role in ovarian physiology remains unclear. Studies in pigs and mice showed that supplementation of maturation medium with cortisol inhibited or had no effect on oocyte developmental competency, respectively [24,25]. Conversely, a

higher cortisol:cortisone ratio in follicular fluid of women undergoing IVF cycles was associated with higher embryo implantation rates [26,27]. Furthermore, in humans, GCs diffused from general circulation into follicular fluid where they were metabolized so that intrafollicular levels of the active metabolite cortisol were higher than those in blood, especially close to the LH peak [28]. Based upon these findings, low doses of dexamethasone (a synthetic GC) are often administered to women as a co-treatment to increase the ovarian response in assisted reproduction cycles [29]. Altogether, these studies suggest that GCs may have a positive effect on oocyte maturation.

Transcripts for the GC receptor (GR) and 11β-Hydroxysteroid dehydrogenase (11β HSD) type I, the enzyme that catalyzes the conversion of cortisone to its active metabolite cortisol, have been identified in bovine granulosa and theca interna cells from mature ovarian follicles [30]. Moreover, the concentration of cortisol in follicular fluid is highest around the LH peak in cow preovulatory follicles [31]. This suggests that GCs may also play an important role during folliculogenesis and oocyte maturation in the bovine species. Given the importance of improving in vitro embryo production methods and the evidence in regards to a potential role of GCs in the acquisition of oocyte developmental competency, the goal of this study was to evaluate the effect of different concentrations of cortisol during in vitro oocyte maturation and subsequent bovine embryo development.

2. Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA) unless otherwise stated.

2.1 Experimental design

Two experiments were conducted in order to understand the potential role of cortisol on bovine oocyte maturation. In experiment 1, embryo development rates were evaluated following IVF of oocytes incubated in in vitro maturation medium containing different cortisol concentrations. Endpoint evaluations included cleavage and blastocyst rates, as well as blastocyst cell numbers. The cortisol concentration providing the best outcomes in experiment 1 was chosen for in vitro oocyte maturation in Experiment 2. Mature oocytes, their corresponding cumulus cells, and resulting day 8 blastocysts were used to assess the transcript levels for potential GC target genes, which included: two genes related to mitochondrial function, cytochrome c oxidase subunit (*COX1*) and nuclear respiratory factor 1 (*NRF1*) [21,32-34]; one gene related to lipid metabolism or fatty acid synthase (*FASN*) [19,35]; and, one gene related to cellular stress or heat shock protein 70 (*HSP70*) [17,36,37]. In addition, only for embryos, we evaluated the expression of two additional genes with roles in: carbohydrate metabolism or glucose transporter 1 (*GLUT1*) [20,38]; and, cellular respiration or mitochondrial transcription factor (*TFAM*) [22,32].

2.2 In Vitro Embryo Production

2.2.1. Oocyte collection and in vitro maturation (IVM)

Ovaries were obtained from a slaughterhouse and transported in 0.9% sodium chloride solution at room temperature for a maximum of 2 hours. Once in the laboratory, 2-8 mm antral follicles were punctured using a syringe and 18Ga needle and follicular fluid was aspirated. Only cumulus-oocyte complexes (COCs) with compact cumulus cells and good morphological appearance were selected [39].

Groups of 35-41 COCs were matured in 400-µL droplets of TCM 199 supplemented with 25 mM sodium bicarbonate, 10% fetal calf serum (FCS) (Gibco BRL, Grand Island, NY USA), 11 mg/mL pyruvate, 50 µg/mL gentamicin, 0.5 µg/mL FSH (Folltropin, Bioniche Animal Health, Belleville, Ont., Canada), and 5 µg/mL LH (Lutropin, Bioniche Animal Health). A cortisol (Sigma H0135) stock solution was prepared at a concentration of 50 µg/mL in ethanol, and 50-µL aliquots were stored at -20 °C until use. The final ethanol concentration did not exceed 0.1%, which should not affect embryonic development [24]. Thus, COCs were matured for 20 hours in the absence of cortisol (control group) or in the presence of different cortisol concentrations (0.01, 0.1 or 1 µg/mL). Incubations were performed at 38.5°C in a 5% CO_2, 20% O_2 and 75% N_2 in humidified air atmosphere. Given that GC hormones may bind to plastic, COC maturation was performed in custom made (1 cm in diameter) glass Petri dishes [40].

2.2.2. In vitro fertilization (IVF) and in vitro culture (IVC)

Frozen semen from one bull (Bos indicus) of proven fertility was used for IVF. After thawing, semen was centrifuged at 180 *g* for 7 minutes through a discontinuous density gradient Percoll column (GE Healthcare Bio-Sciences, Uppsala, Sweden).

In vitro fertilization was performed in 80-µL droplets of TALP-Fert modified medium [41] supplemented with 30 µg/mL heparin, 1.8 µM epinephrine, 18 µM penicillamine, 10 µM hypotaurine, and 4 mg/mL bovine serum albumin (BSA) under the same conditions described for IVM. Groups of 20 oocytes per droplet were inseminated with washed sperm at a final concentration of 2 x 10^6 sperm/mL.

At 26 h following insemination, presumptive zygotes were denuded from their cumulus cells by repeat pipetting. Groups of 20 were then incubated over a cumulus

cell monolayer in 100-µL droplets of synthetic oviductual fluid (SOF) culture medium supplemented with 3 mg/mL BSA, 50 µg/mL gentamicin, and 5% FBS [42]. Incubations proceeded under the same conditions described for IVM. Cleavage and blastocyst rates were evaluated on days 2 and 7 of culture, respectively (Experiment 1). Thirteen to 16 embryos per group were labeled with the fluorochrome Hoechst 33342 [43] and examined under a fluorescence microscope (Eclipse TE 300, Nikon Corporation, Tokyo, Japan; X 400) to assess cell numbers. For Experiment 2, day 8 blastocysts were stored in RNAlater (Applied Biosystems, Foster City, CA) at -20°C following the manufacturer's directions.

2.3 Gene expression

2.3.1 Acquisition and storage of samples for RNA isolation

For RNA isolation, oocytes were matured as above and cumulus cells removed by repeat pipetting in a 0.5% bovine testis hyaluronidase solution in PBS. Only MII oocytes, as evidenced by first polar body extrusion, were selected. Zona pellucidae were removed from oocytes by incubation in a 1.5 mg/mL protease solution in HEPES-buffered TCM199, for 5 minutes. Cumulus cells were also saved for analysis. Embryos were collected on day 8 of in vitro culture at the hatched blastocyst stage. Triplicate samples consisting of 20 mature oocytes, their corresponding cumulus cells or 10 hatched blastocysts were placed into microcentrifuge tubes containing 0.2 mL of RNAlater (Applied Biosystems, Foster City, CA) and stored at -20°C for later use.

2.3.2 RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Micro kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was treated with DNAse I (provided with the kit) to avoid contamination by genomic DNA. Reverse transcription was performed using the High Capacity Reverse Transcription kit (Applied Biosystems, Foster City, CA) also according to manufacturer's instructions.

2.3.3 Real time PCR

Real-time PCR was performed using a StepOne plus Real Time PCR system (Applied Biosystems). Primers were designed using Primer Premier software (Premier Biosoft International, Palo Alto, CA, USA) based on cDNA sequences available in GenBank (Table 1). Primer specificity was checked by BLAST. The PCR total reaction volume was 10 µL, containing 5 µL of SYBR Green Master Mix (Applied Biosystems), 0.5 mM of each primer, and 4 µL of DNA diluted 1:8. The thermocycling conditions consisted of 95℃ for 10 minutes followed by 45 cycles at 60℃ for 1 minute. The specificity of PCR products was verified by evaluating the corresponding dissociation curves.

Table 1: Primer sequences, expected fragment size and Genbank access code for genes evaluated by RT-PCR.

Gene	Sequence	Size (pb)	Genbank
YWHAZ	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	120	BM446307
HSP 70	GAACGCGCTGGAGTCGTAC ATGGGGTTACACACCTGCTC	202	NM_174550.1
GLUT 1	TGCTCATTAACCGCAACGA TGACCTTCTTCTCCCGCATC	133	M60448
TFAM	GACCTCGCTCAGCTTATAACATT CAGCTTTACCTGTGATGTGCC	146	NM_001034016.2
NRF1	TGACCATCCAGACAACGCAA CTCCACCTGTTGAGTGCCAT	127	NM_001098002.2
COX 1	TTTGATGCTTGGGCCGGTAT ACAAATGCGTGTGCGGTTAC	128	YP_209207.1
FASN	CACTCCATCCTCGCTCTCC GCCTGTCATCATCTGTCACC	181	AY343889

YWHAZ: Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide. *HSP70:* heat shock protein 70. *GLUT1*: glucose transporter 1. *TFAM:* mitochondrial transcription factor A. *NRF1*: nuclear respiratory factor 1. *COX1*: cytochrome c oxidase subunit 1 *FASN*: fatty acid synthase. bp: base pairs.

The amplification efficiency for each gene was calculated using a relative standard curve of serial dilutions of cDNA (1: 4, 1: 8, 1:16, 1:32, 1:64). The relative quantity of each target gene was corrected relative to the quantity of the endogenous gene Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ) [44], using the comparative delta CT method ($2^{\Delta\Delta Ct}$). Each experiment was performed in triplicate.

2.4 Statistical Analysis

Data for cleavage and blastocyst rates, as well as for relative gene expression levels were analyzed by ANOVA and Bonferroni post-hoc test, with significance set at $P < 0.05$, using the SigmaPlot 11.0 software (Systat Software, San Jose, CA, USA).

3. Results

3.1 Experiment 1: Effect of cortisol concentration during IVM on subsequent embryonic development

Four replicates were performed to assess the effect of cortisol addition to the oocyte maturation medium on later embryonic development. There were no differences in cleavage rates among groups ($p > 0.05$; Table 2) Conversely, addition of 0.1 µg/mL cortisol to the IVM medium did improve blastocyst rates over those in the control group ($P<0.01$). Cortisol treatment during IVM had no effect on the cell numbers of the resulting blastocysts (Table 3).

Table 2: Effect of supplementing in vitro maturation medium with cortisol on embryo development rates of in vitro fertilized bovine oocytes

Groups	**N**	**Cleaved %Mean ± SD**	**Blastocyst % Mean ± SD**
Control	162	69 ± 9.8	21 ± 1,2 [a]
0.01 µg/mL[*]	158	85 ±10.2	31 ± 6,1 [a,b,c]
0.1µg/mL[*]	164	80 ± 5.5	41 ± 10 [b]
1 µg/mL[*]	165	84 ± 8.7	26,7 ± 2,8 [a, c]

N = sample size; SD = standard deviation.

% blastocyst is calculate as percentage of total oocytes.

[*]Concentration of cortisol added to the medium used for in vitro oocyte maturation.

[a, b, c] Different superscripts within column denote significant differences ($p < 0.05$).

Table 3**:** Effect of supplementing in vitro bovine oocyte maturation medium with cortisol on cell numbers of the resulting in vitro produced blastocysts.

Groups	**N**	**Number of cells Mean ± SD**
Control	13	87.2 ± 8.9
0.01 µg/mL*	17	78.5 ± 20.8
0.1 µg/mL *	14	93.3 ± 20.4
1 µg/mL*	16	83,3 ± 5,4

N = sample size; SD = standard deviation.

* Concentration of cortisol added to the medium used for in vitro oocyte maturation.

3.2 Experiment 2: *Gene expression analysis in COCs and embryos*

Given that supplementing the IVM medium with 0.1 µg/mL of cortisol improved embryo development rates, we used this concentration in subsequent experiments to ascertain expression levels of genes that are reportedly regulated by cortisol [18,19,23, 45,46] and may also be important for embryonic development. No differences in relative gene expression levels were detected for oocytes or cumulus cells when comparing those matured in the presence or absence of cortisol (Figs. 1, 2).

Oocytes matured with or without the addition of 0.1 µg/mL of cortisol were fertilized and cultured in vitro for 8 days to the blastocyst stage. Three replicates consisting of 10 hatched blastocysts each were used for analysis of gene expression levels. Blastocysts that did not hatch were not use for analysis. Interestingly, embryos from oocytes matured in cortisol-containing medium displayed increased

expression levels for *HSP70* (p <0.001), *GLUT1* (p = 0.027) and *FASN* (p = 0.008) (Figure 3).

Figure 1: Comparison of gene expression levels between oocytes matured in vitro in the absence or presence of 0.1 µg/mL cortisol. Data is reported as mean ± SD. The relative quantity of each target gene was corrected relative to the quantity of the endogenous gene tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ). No differences were detected in gene expression levels between treated and control oocytes: *HSP70* = heat shock protein (p = 0.211); 70 *NRF1*: nuclear respiratory factor 1 (p = 0.818); *COX*: cytochrome c oxidase subunit 1 (p = 0.18); *FASN*: fatty acid synthase (p = 0.212).

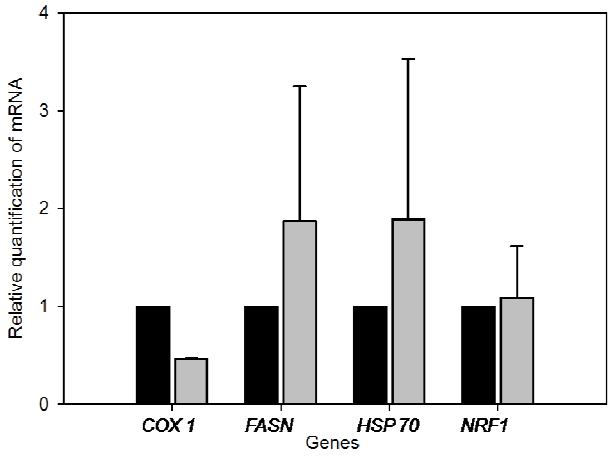


Figure 2: Comparison of gene expression levels between cumulus cells obtained from oocytes matured in vitro in the absence or presence of 0.1 µg/mL cortisol. Mean ± SD. The relative quantity of each target gene was corrected relative to the quantity of the endogenous gene tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ). No differences were detected in gene expression levels between treated and control cumulus cells: *HSP70* = heat shock protein (p = 0.699); *NRF1*: nuclear respiratory factor 1 (p = 0.937); *COX*: cytochrome c oxidase subunit 1 (p = 0.18); *FASN*: fatty acid synthase *FASN* (p=0.485).

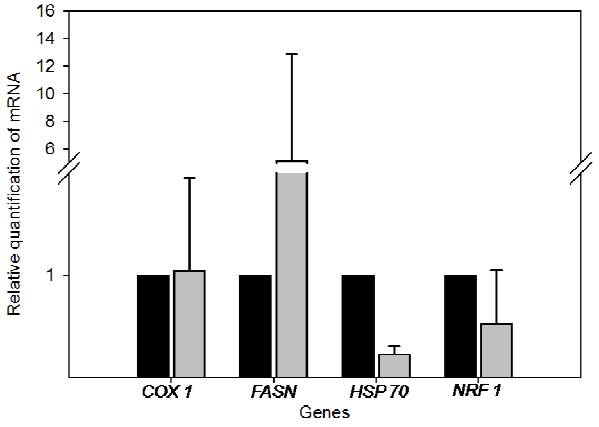
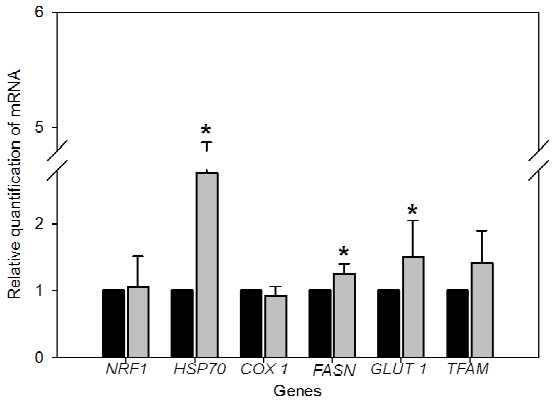


Figure 3: Comparison of gene expression levels between hatched blastocysts obtained from the fertilization of oocytes matured in vitro in the absence or presence of 0.1 µg/mL cortisol. Mean ± SD. The relative quantity of each target gene was corrected relative to the quantity of the endogenous gene tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide (YWHAZ). No differences were detected in gene expression levels for: *NRF1*: nuclear respiratory factor 1 (p = 0.746); *COX*: cytochrome c oxidase subunit 1 (p = 0.289); or, *TFAM*: mitochondrial transcription factor (p<0.06). Gene expression levels between treated and control samples differed for: *HSP70* = heat shock protein (p <0.001); *FASN*: fatty acid synthase (p=0.008); and, *GLUT1*: glucose transporter 1 (p<0.027). * Asterisk denotes p<0.05.



4. Discussion

This is the first study reporting a beneficial effect of GC addition to the in vitro oocyte maturation medium in mammals. Indeed, addition of cortisol to the IVM medium yielded higher blastocyst rates that were paired with increased embryonic expression of *FASN*, *GLUT1* and *HSP 70*. These findings are consistent with cortisol exerting a beneficial effect via the modulation of lipid and carbohydrate metabolism (*FASN* and *GLUT1)*, and the response to stress in treated oocytes (*HSP70*), both pathways susceptible to in vitro conditions and related to embryo quality.

Previous studies have yielded contrasting results in regards to the effect of GC on mammalian oocyte competence. For instance, addition of cortisol or dexamethasone to porcine oocyte maturation medium decreased the percent of oocytes undergoing germinal vesicle breakdown, with no effect on subsequent embryo development [25]. In the mouse, cortisol or dexamethasone had no effect on oocyte maturation rates, although embryo development was not assessed [24]. Conversely, in a different study, corticosterone exerted a concentration-dependent negative effect on mouse oocyte maturation, presumably via inhibition of the extracellular signal-regulated kinases (ERKs), and on blastocyst development. In the same study, dexamethasone had no effect on oocyte maturation or embryo development [47]. While GC concentrations tested in these studies were similar to those used herein, it is difficult to establish a direct comparison given differences in activity of different GCs among species. In addition, oocyte culture conditions also differ between studies. Moreover, albeit subtle, there are reported differences in the regulation of oocyte maturation among species [24, 48] that could account for differences in the response to GC treatment.

Beneficial effects of GCs for in vitro maturation of oocytes have also been reported in non mammalian species. Cortisol is involved in different aspects of oocyte and embryo growth and development in fish species [49-52]. For instance, increasing cortisol levels inside rainbow trout oocytes by its addition in maturation medium resulted in higher embryonic cell numbers at 48 hours following fertilization [49-50]. This effect appeared to be mediated via the activation of insulin growth factor (IGF) related pathways [50]. The fact that in our study we did not detect changes in blastocyst cell number despite a beneficial effect of cortisol in embryo development rates further suggests species-specific differences in the actions of GCs in gametes and pre-implantation embryos.

Most of the actions carried out by GCs are mediated by interaction with the corresponding glucocorticoid receptor (GR). In fact the GR is expressed throughout all stages of bovine follicular development by both granulosa and theca interna cells [30], as well as by the oocyte and cumulus cells before and after IVM (unpublished observations), providing further evidence of the importance of GC in follicular and oocyte physiology. Given that binding of GCs to their GR involves translocation to the nucleus and activation or repression of gene expression [53], we investigated whether cortisol affected oocyte competence via a genomic pathway. However, we found no effect of cortisol on gene expression in either oocytes or cumulus cells. This may be partially due to the relatively large standard deviations observed, possibly linked to the inherent heterogeneity resulting from the use of oocytes from abattoir ovaries, paired with a relatively low sample size [54, 55]. Conversely, cortisol treatment did affect transcript levels for key genes in embryos, suggesting a long-term delayed effect upon gene transcription [53]. For instance, GLUT1, one of the genes upregulated by cortisol treatment, modulates glucose entry into cells. Glucose

can then be used as an energy source via the pentose or glycolytic pathways, both of which are important in embryogenesis [56,57]. This finding also highlights the role of GCs in carbohydrate metabolism.

Moreover, embryos from treated oocytes also expressed higher levels of FASN, an enzyme important for lipogenesis that acts by catalyzing the synthesis of palmitate into long-chain fatty acids to form lipid droplets that represent an energy reserve [35]. Embryos from obese mice expressed less FASN and this was associated with alterations in the regulation of cholesterol biosynthesis [58].

Embryos from treated oocytes also displayed increased *HSP70* expression. Heat shock proteins (HSPs) are a family of chaperones, that participate in important cellular functions, related to stabilization of protein and DNA repair, cell cycle control, as well as apoptosis and elimination of damaged proteins, important to cell viability in response to stresses [18,59,60]. In cattle embryos, HSP expression is also related to the activation interferon tau, which is crucial for maternal recognition and maintenance of pregnancy [36]. Notably, HSPs regulate the cellular response to stress conditions and, in this context, to cortisol [61]. Cortisol also regulates HSP expression in somatic cells such as adipocytes [18] and recent studies suggest that subjecting a cell to a sublethal stress (increased hydrostatic pressure, osmotic stress, oxidative stress), may confer an adaptation and increased tolerance to additional stressful conditions [62-64]. In fact, Du et al. [64] observed that the sublethal stress caused by exposure of immature swine oocytes to high hydrostatic pressure resulted in increased blastocyst rates with higher cell numbers following parthenogenetic activation. Hence we speculate that the mechanism of action of cortisol via increased expression of HSP70 in this study may be related to the preparation of the oocyte to sustain the upcoming stress related to the in vitro production process, thus ultimately

benefiting embryo development, similar to what was observed when submitting oocytes to sublethal stressors [65]. Worth noting is the fact that heat stress in zebu cows promoted deleterious effects on ovarian follicular dynamics, with concomitant decrease in oocyte competence; however this was not accompanied by cortisol elevation in follicular fluid, arguing that the physiological stress detrimental for oocyte competence is not mediated by cortisol [66].

In summary, this study provides evidence for cortisol playing a role in the acquisition of bovine oocyte competence when added to the maturation medium. This resulted in increased blastocyst development rates paired with increased transcripts for genes related to energy metabolism and stress. Future research should elucidate the cortisol-driven oocyte cytoplasmic modifications presumably contributing to improved embryonic development.

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6. ARTIGO 3: EFFECT OF CORTISOL ON BOVINE EMBRYO DEVELOPMENT IN VITRO

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ABSTRACT

The cortisol hormone, major glucocorticoid (GC) found in human and bovine, operates in several physiological processes, including playing a key role at the beginning of pregnancy, regulates mechanisms involved in the embryo in the endometrium deployment process. The objective of this study was to evaluate the effect of different concentrations of cortisol (0.01 mg / mL, 0.1 mg / mL and 1 mg / ml) in the in vitro bovine embryos culture, embryonic development was evaluated as well as the number of cell and relative quantitation of mRNA (*NRF1, COX TFAM, HSP70, FASN, GLUT1*). Oocytes were matured, fertilized in vitro, and subsequently cultured in vitro with 0; 0.01µg/ml; 0.1 µg/ml and 1 ug/ml cortisol. There was no statistical difference in the embryos treated with cortisol compared to the control for the parameters analyzed ($p > 0.05$). The only difference was in the group where embryos were treated with 1 mg/ml cortisol with respect to counting the number of cells when compared to the no treatment group (64.7±11.4 vs. 96.2±20.4, respectively, $p < 0.05$). Although was not observed difference in relation to the improvement of the system, we chose the concentration of 0.1 mg/mL to be evaluated by other paramather. Thus, in vitro embryos were incubated since the 1st day of cultivation with or without 0.1 µg/mL of Cortisol, and the embryos on day 8 were analyzed for gene expression. However there was no difference with respect to gene expression for any of the transcripts ($p > 0.05$). Therefore, we concluded that cortisol have a little or no influence on the regulation of embryo development in the bovine.

Keywords: cortisol, gene expression, embryo, bovine.

INTRODUCTION

The beginning of embryonic development period starts with post-fertilization and blastocyst formation is one of the most critical stages in this process, involves several morphological changes related to genomics activity. During this period the embryo needs to control various events, and many of them are regulated by gene expression, which suffers strong influence of culture conditions (Jiang et al, 2014; Lonergan et al., 2003; Vigneault, et al., 2009).

Many of the products of these genes are involved various cellular processes, and can be regulated by glucocorticoid (GC) as energy metabolism, stress adaptation, transcription, translation, apoptosis, compactation and blastocyst differentiation (Gad et al., 2012; Reddy et al., 2009). The cortisol hormone, main GC found in human and bovine, operates in various physiological processes (Whirledge and Cidlowski, 2013). In addition, the GC's play key role at the beginning of pregnancy, regulating mechanisms involved in embryo implantation process in humans (Brann and Mahesh, 1991; Michael and Papageorghiou, 2008) and in the maintenance of early pregnancy in cattle (Lee et al, 2007; Mariko et al, 2013.).

It well know that the in vitro culture conditions strongly influences on embryonic development, especially with he quality of these embryos (Jiang et al., 2014; Lonergan et al., 2003; Vigneault, et al., 2009). This influence is noted with the wide divergence in standards of embryonic gene expression between the embryo produced in vivo and in vitro (Gad et al., 2012; Jiang et al., 2014). Thus changes in the composition of the culture media can change this in vitro embryo profile (Gad et al., 2012).

However, there are few studies describing the glucocorticoid effect on embryo development in vitro. Siemieniuch et al. (2010) identified mRNA to glucocorticoid receptors and two isoforms of enzymes 11β- hydroxysteroid dehydrogenase (11β-HSD) which control the availability of cortisol in the oviduct and endometrium during early pregnancy. In this study the mRNA expression for 11β-HSD1 was higher in bovine embryos produced in vitro when compared to endometrio at 16th days of gestation. Thus it can be assumed that during the early stages of pregnancy, CG act as an autocrine or paracrine growth factor and thus mediate communication between the embryo and maternal environment.

Other studies employ a synthetic GC, dexamethasone, during embryonic development in vitro (Merris et al, 2007; Santana et al., 2014). In cattle, Santana et al. (2014) observed that dexamethasone when added to in vitro culture improved the embryo quality. However, to date there are no reports on the effect of a physiological GC, such as cortisol, in the cultivation of pre-implantation bovine embryos. Thus the use of a physiological GC can contribute to the understanding of the metabolic behavior of bovine embryo. In this sense the objective of this study is to investigate the effect of different concentrations of cortisol in vitro culture of bovine embryos

MATERIALS AND METHODS

All reagents were from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA), if not they are cited.

Experimental Design

Two experiments were conducted in order to understand the role of cortisol in the culture of bovine embryos produced in vitro. In first experiment the rate of cleavage and blastocyst, as well as the total cell number were evaluated in embryos cultured with or without various concentrations of cortisol. The best concentration found in Experiment 1 was used in Experiment 2, in which was analyzed gene expression.

In vitro production of embryos

In vitro oocyte maturation (IVM)

Ovaries were obtained from a slaughterhouse and transported in refrigerated solution of 0.9% sodium chloride at room temperature for up to 2 hours. Antral follicles 2-8 mm were punctured. Only COC presenting good characteristics were selected (Leibfried and First, 1979) and matured in drops with 100 uL of IVM medium [TCM 199 supplemented with 25 mM of sodium bicarbonate, 10% of fetal bovine serum, 11 mg/ml of pyruvate, 50 ug/ml of gentamicin, 0.5 ug/ml FSH (Folltropin, Bioniche Animal Health, Belleville, Ont., Canada), and 5 ug/ml LH (Lutropin, Bioniche Animal Health)]. COCs were matured for 20 hours incubator with 5% CO2, 20% O2 and 75% N2 under humid atmosphere and temperature at 38.5 ℃.

In vitro fertilization (IVF) and in vitro culture (IVC)

Frozen semen from one bull (Bos indicus) of proven fertility was used for IVF. After thawing, semen was centrifuged at 180 *g* for 7 minutes through a discontinuous density gradient Percoll column (GE Healthcare Bio-Sciences, Uppsala, Sweden).

In vitro fertilization was performed in 80 uL drops of TALP-Fert modified medium (Parrish et al., 1988) supplemented with 30 ug/ml heparin, 1.8 uM epinephrine, 18 uM penicillamine, 10 uM hypotaurine and 4 mg/ml of BSA (bovine serum albumin) under the same conditions described for in vitro maturation.

After 26 h of in vitro fertilization, cumulus cells were removed by repeated pipetting, and 30-40 per group presumptive zygotes were then incubated with 400 uL of SOF culture medium (synthetic oviductal fluid) as described by Holm et al. (1999), with 3mg /ml BSA, 50 ug/ml gentamicin, and 5% SFB, in the absence of cortisol (control group) or presence of different concentrations of cortisol (0.01mg /ml; 0.1 mg /ml or 1mg /ml), previously described by Santana et al (2014a). A stock solution of cortisol (Sigma H0135) was diluted with ethanol, stored in 50 uL aliquots at -20 ° C until the moment of dilution in SOF. The final ethanol concentration did not exceed 0.1%, which does not affect embryonic development (Andersen, 2003). As the plastic has been shown to interact and decrease the amount of steroid hormones in culture media, was used a glass dish (Kennedy and Besses, 1967; Macaulay et al., 2013; McDonald et al., 2008). The atmosphere conditions were the same as described in IVM and FIV.

Cleavage rate was assessed at day 2 of culture and blastocyst rate on the 7th day of culture (Experiment 1). To assess the total number of cells, 9 to 21 embryos per group were labeled with fluorochrome Hoechst 33342 as described previously (Manser et al., 2004) and were examined under a fluorescence microscope (Eclipse TE 300, Nikon Corporation, Tokyo, Japan). On day 8 of culture the embryos were stored in RNAlater (Applied Biosystems, Foster City, CA) at -20 ° C for later RNA extraction (Experiment 2).

Storage of samples

Hatched blastocyst were collected on the 8th day of, and were stored in 0.2 mL microtubes with 5 µl of RNAlater (Applied Biosystems, Foster city, CA) containing 10 embryos (3 pools each). Samples were kept in a freezer -20 ° C until the time of extraction.

RNA extraction and cDNA synthesis

Total RNA was extracted using RNeasy Micro kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The RNA was treated with DNAse I (provided with the kit) to avoid contamination by genomic DNA. Reverse transcription was performed using the High Capacity Reverse Transcription Kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions.

Analysis of gene expression

Real-time PCR was performed in StepOne plus Real Time PCR system (Applied Biosystems). Primers were designed using Primer Premier software (Premier Biosoft International, USA) based on cDNA sequences available in GenBank (Table 1). The specificity of the primer was checked by BLAST. For the PCR reaction consisted was used 5 µL of SYBR Green Master Mix (Applied Biosystems), 0.5 mM of each primer, and 4 uL of DNA diluted 1:8. The thermocycling conditions were: 95 ° C for 10 minutes, and 45 cycles at 60 ° C for 1 minute. The specificity of PCR products was verified by evaluating dissociation curve.

Table 1: Primer sequences, expected fragment size and Genbank access code for genes evaluated by RT-PCR.

Gene	Sequence	Size (pb)	Genbank
YWHAZ	GCATCCCACAGACTATTTCC GCAAAGACAATGACAGACCA	120	BM446307
HSP 70	GAACGCGCTGGAGTCGTAC ATGGGGTTACACACCTGCTC	202	NM_174550.1
GLUT 1	TGCTCATTAACCGCAACGA TGACCTTCTTCTCCCGCATC	133	M60448
TFAM	GACCTCGCTCAGCTTATAACATT CAGCTTTACCTGTGATGTGCC	146	NM_001034016.2
NRF1	TGACCATCCAGACAACGCAA CTCCACCTGTTGAGTGCCAT	127	NM_001098002.2
COX	TTTGATGCTTGGGCCGGTAT ACAAATGCGTGTGCGGTTAC	128	YP_209207.1
FASN	CACTCCATCCTCGCTCTCC GCCTGTCATCATCTGTCACC	181	AY343889

YWHAZ: Tyrosine 3-monooxygenase / tryptophan 5-monooxygenase activation protein zeta polypeptide. HSP70: heat shock protein 70. GLUT1: glucose transporter 1. TFAM: mitochondrial transcription factor A. NRF1: nuclear respiratory factor 1. COX: cytochrome c oxidase subunit 1 FASN: fatty acid synthase. bp: base pairs.

For the analysis of embryonic gene expression was observed the relative quantification of possible target genes of glucocorticoids, which are important for embryonic development. Three genes related to mitochondrial function, mitochondrial transcription factor (*TFAM*), cytochrome C ocidase 1 (*COX*) and nuclear respiratory factor (*NRF1*) (Chiaratti et al , 2010; Datson et al, 2008; Demonacos et al . 1995; Lee et al., 2013; Mastromonaco et al , 2012); one related to lipid metabolism, fatty acid synthase (*FASN*) (Gathercole et al , 2011; Auclair et al , 2013); another related to cellular stress, heat shock protein (*HSP70*) (Eitam et al., 2010; Hickman et al., 2013; Machado et al.,2013); and another one related to carbohydrate metabolism , *GLUT1* (Lopes et al , 2007; Reichardt et al , 2012) .

The efficiency of each gene was calculated using a standard curve relating cDNA with serial dilutions (1: 4 , 1: 8; 1:16 ; 1:32 ; 1:64) . All samples were performed in triplicate. The relative quantification of the target gene was corrected by the quantification of endogenous gene *YWHAZ* (Goossens et al . , 2005), using the comparative CT method (ΔΔCt).

Statistical analysis

Cleavage and blastocyst The data and relative gene expression were analyzed by ANOVA , Bonferroni post- test using the software SigmaPlot 11.0 (Systat Software, San Jose, SC , USA) . Level of significance was $p < 0.05$.

RESULTS

Experiment 1: Effect os cortisol concentrations during IVC

Four replications were conducted to assess the effect of cortisol addition to the embryo culture medium. There were no difference in cleavage and blastocyst rates among groups ($p > 0.05$) (Table 2).

Table 2- embryonic development rate with different concentrations of cortisol in vitro culture.

Groups	N	Cleaved % Mean ± SD	Blastocyst % Mean ± SD
Controle	145	67.11 ± 11	34.8 ± 9.8
0,01 ug/mL	140	66.6 ± 6	30.5 ± 8.9
0,1ug/mL	160	70.0 ± 5	35.6 ± 10.1
1ug/mL	130	70.0 ± 11	27.5 ± 4.5

N = sample size; SD = standard deviation.
% blastocyst is calculate as percentage of total oocytes.

Cortisol treatment during IVC had no effect on the cell numbers of the resulting blastocysts, excepted to groups with 1µg/mL of cortisol that decreased the total number of cells in when compared to control group (Table 3)

Table 3: Effet of supplementing in vitro bovine embryo culture medium with cortisol on cell numbers of the resulting in vitro produced blastocysts

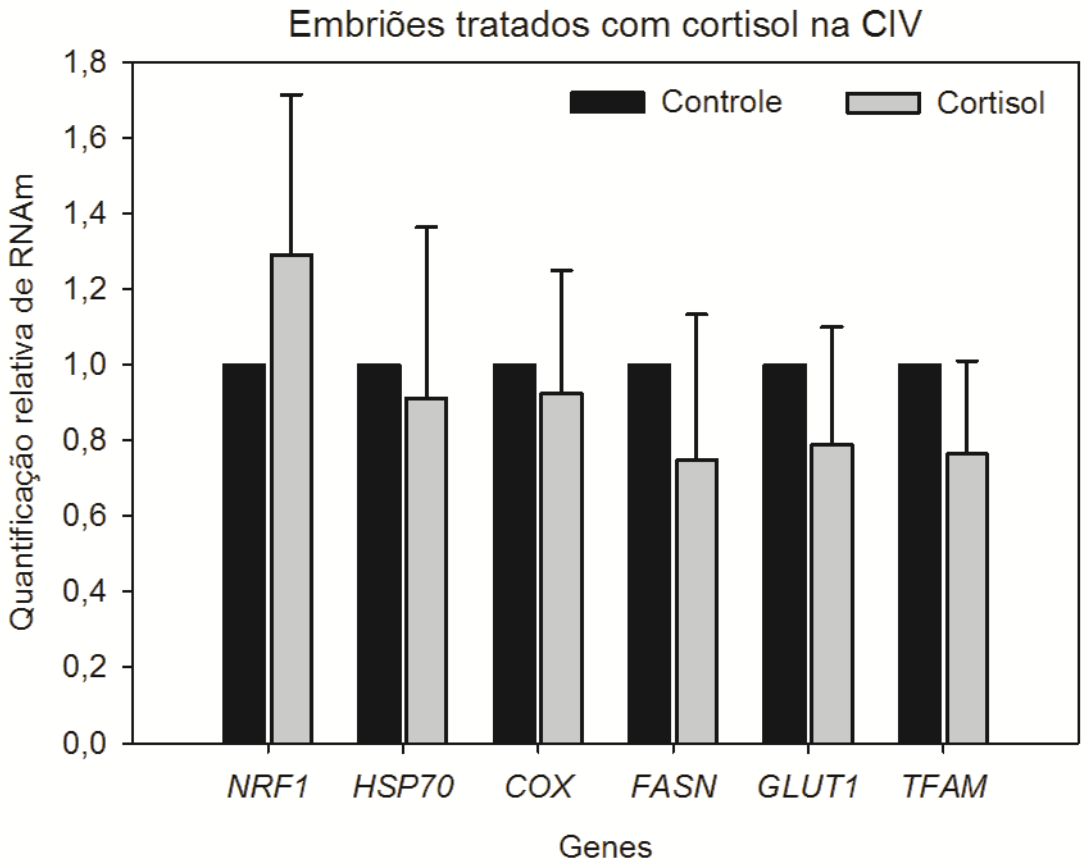
Groups	N	Number of cells Mean ± SD
Control	21	96.2 ± 20.4 [A]
0.01 ug/mL	16	86.4± 11.6 [A]
0.1ug/mL	17	84.7 ± 11.4 [A]
1ug/mL	9	64.7 ± 11.4 [B]

N = sample size; SD = standard deviation.
[A,B] Different superscripts within column denote significant differences ($p < 0.05$).

Experiment 2: Analysis gene expression

We used the cortisol concentration 0.1µg/mL in subsequent experiments to ascertain expression levels of genes that are reportedly regulated by cortisol and may also be important for embryonic development. No differences in relative gene expression levels were detected for embryos when comparing those blastocysts in the presence or absence of cortisol (Figure 1).

Figure 1: Analysis of gene expression in embryos cultured or not with 0.1 µg /mL of cortisol. The control group (without Cortisol) was the calibrator. *GLUT1*: glucose transporter 1. *TFAM:* mitochondrial transcription factor. *HSP70* : heat shock protein 70. NRF1: nuclear respiratory factor 1. *COX*: cytochrome c oxidase subunit 1. *FASN*: fatty acid synthase. p> 0.05 .



DISCUSSION

The GC hormones can act regulating many functions in cells as energy metabolism, stress adaptation, transcription, translation and apoptosis (Gad et al , 2012; Reddy et al., 2009). Thus, the present study was the first to test the effect of cortisol on bovine embryos development. The main GC mechanism of action is through genomic pathway, that it is activated by binding of the hormone to glicorticoide receptor (GR), and this has been described (at least the presence of mRNA) in bovine embryos (Siemieniuch et al.,, 2010). Experiments performed in our laboratory (preliminary results) showed that there is also the protein to GR with large-scale distribution in the cytoplasm and nucleus of bovine embryos in all preimplantation stages. These results showed that the physiological GC , cortisol, is able to perform their effects during early embryonic development.

However, in this study, different concentrations of cortisol in IVC medium did not change the rates of embryonic development and cleavage and blastocyst nor gene expression on embryos. In a previous study (Santana et al., 2014) the presence of dexamethasone, a synthetic GC, improved blastocyst rates in cattle embryos (0.1ug / mL) differing from our results. However, these differences may be due to the affinity of the hormone once dexamethasone is 30 times more potent than cortisol, and dexamethasone did not suffer any type of cellular regulation (Almeida et al., 2000; Menshanov et al, 2013). The cortisol can be regulated by the presence of the enzyme 11βHSD 1, which transforms cortisone to cortisol, its active form, enabling the interaction of GC with its GR and whose presence of the transcripts has been reported in bovine embryos and pregnant endometrium (Siemieniuch et al., 2010).

Merris et al. (2007) also tested the synthetic GC in embryonic development. In this case they analyzed teratogenic effect of dexamethasone (5 to 80 mg / ml) at the first day of culture in vitro of mouse embryos, and that not affect the development of 2-cell stage or the early cleavage, however, the rates of expansion in blastocysts treated with 10 mg/mL of dexamethasone decreased, and the embryos exposed to 80 mg/mL had degenerative characteristics. In our study, the concentration of cortisol 1ug /ml had no effect in the proportion of in vitro produced embryos, but impared the embryo quality. These data corroborate the fact that the growing conditions affect the embryo profile and the probability of a zygote develop into blastocyst (Rizos et al., 2002a, 2002b) .

Often embryo morphology hides molecular differences that are not perceptible to the common light stereomicroscope (Jiang et al, 2014; Van Soom et al., 2003). So we chose the MIC cortisol group with 0.1 mg / mL, which showed similar results to the control group, to investigate other parameters related to embryo quality.

It is known that pre-implantation embryos have specific metabolic requirements for its development (Hashimoto et al., 2000). In a recent study Santana et al (2014b) reported that the use of arginine used throughout the in vitro culture of bovine embryos did not alter development rates or total number of cells, but when it was used in the specific stage, at morula, increased hatching rate and embryo quality. Thus, we speculate that cortisol can have a similar effect specific stage, since there CG receptors at all stages of embryonic development (Siemieniuch et al., 2010).

Regarding the analysis of gene expression was expected that the addition of GC regulate the genes *NRF1*, *TFAM* , *COX*, since the GC acts directly in mitochondrial activity (Lee et al , 2013; Psarra and Sekeris, 2011), but was not observed statistical difference between embryos in the control group and the group treated with 0.1 mg/mL of cortisol in IVC. Although cortisol action in mitochondria may occur via control transcription of nDNA (*NRF1* and *TFAM*) or via regulation of mitochondrial protein transcription (*COX*) , as analyzed in this study , other mechanisms can be activated by GC affecting mitochondrial function in a indirect way, such as through the cytoplasmic signaling proteins (HSP 90), or by blocking pro-apoptotic protein or second messenger which activates phosphorylase enzymes (Alzamora and Harvey, 2008; Song and Buttgereit , 2006).

The *FASN* and *GLUT1* gene expression did not suffer regulation by GCs, then there is the possibility that the regulation of carbohydrate and lipid metabolism by GC is not regulated by those genes, as in the adipose tissue that GC stimulate lipolysis by activating the hormone-sensitive lipase, or enhance the utilization of carbohydrates by an increase in glucose transporter expression of insulin-dependent (GLUT4) (Long et al., 2012; Reddy et al, 2009; Wang et al , 2014).

The relative quantification of *HSP70* gene is not regulate in hatched blastocysts treated with 0.1 µg/mL of Cortisol. This gene is related to cellular stress (Eitam et al., 2010), however its transcription also occurs under physiological conditions (Ravikumar and Muthuraman, 2014), and also plays antiapoptotic function (Kennedy et al., 2014). In this case we can assume that the responsiveness to stress is similar for both control and cortisol group.

In conclusion, the present study demonstrates that glucocorticoids are unable to affect positive the early embryo development.

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7. CONCLUSÕES GERAIS

Concluímos que:

Há receptor de glicocorticóide em complexo *cumulu*s oócito imaturo e maturado, embrião de 2-4 e 8-16 células, mórula e blastocisto, e que esse receptor é importante para o desenvolvimento embrionário.

A suplementação do meio de maturação oocitária *in vitro* com 0,1 ug/mL de cortisol aumenta a proporção de blastocistos, além de promover a regulação gênica embrionária de genes relacionados à capacidade de resposta ao estresse celular (*HSP70*) e metabolismo de lipídios (*FASN*) e carboidratos (*GLUT1*).

A adição de cortisol no cultivo de embriões *in vitro* não teve efeito positivo sobre o desenvolvimento embrionário.