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**INFLUÊNCIA DO ESTRESSE TÉRMICO NA PRODUÇÃO DE
INTERFERON TAU E NO ESTRESSE OXIDATIVO DE EMBRIÕES
BOVINOS PRODUZIDOS *IN VITRO***

Santa Maria, RS

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Dissertação apresentada ao Curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária, área de concentração em Sanidade e Reprodução Animal da Universidade Federal de Santa Maria (UFSM, RS), como requisito parcial para a obtenção do título de **Mestre em Medicina Veterinária**.

Orientador: Prof. Alfredo Quites Antoniazzi

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RESUMO

INFLUÊNCIA DO ESTRESSE TÉRMICO NA PRODUÇÃO DE INTERFERON TAU E NO ESTRESSE OXIDATIVO DE EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

AUTORA: Carolina dos Santos Amaral

ORIENTADOR: Alfredo Quitês Antoniazzi

Perdas gestacionais acontecem nos diferentes estágios da gestação por uma variedade de causas, contudo ocorrem com maior frequência nos primeiros 30 dias. Este decréscimo se torna mais grave quando os animais estão sob condições ambientais desfavoráveis. Fatores como a hipertermia podem reduzir as taxas de concepção em torno de 20-30%, quando compararmos aos meses em que o animal se encontra em condições de conforto térmico. Portanto, o estresse térmico é um problema encontrado no manejo de vacas leiteiras nos trópicos e subtropicais, causando reduções na produção e reprodução. O presente trabalho teve como objetivo avaliar a influência do estresse térmico no desenvolvimento embrionário, produção de interferon tau (IFNT) e estresse oxidativo em embriões produzidos *in vitro* (PIV). Para avaliar a influência do estresse térmico sobre a produção *in vitro* de embriões bovinos, foi proposto um modelo *in vitro* de estresse térmico. Esse modelo consiste em alterar a temperatura em fases distintas da PIV de embriões. Para induzir o estresse térmico, a temperatura foi gradualmente aumentada de 38,5°C até chegar aos 40,5°C, onde permaneceu por 6 horas. Após, a temperatura foi diminuída de forma gradativa, até retornar à 38,5°C. Os grupos experimentais foram divididos em cinco: controle; oócitos submetidos ao estresse térmico durante a maturação *in vitro* (IVM HS); oócitos submetidos ao estresse térmico durante a fertilização *in vitro* (IVF HS); zigotos submetidos ao estresse térmico durante o primeiro dia de cultivo *in vitro* (IVC HS); e embriões submetidos ao estresse térmico durante os três primeiros dias de produção *in vitro* (IVM+IVF+IVC HS). Todos os grupos submetidos ao estresse térmico tiveram diminuição nas taxas de clivagem e blastocisto, diminuição da expressão gênica e proteica de IFNT e aumento do estresse oxidativo. O número de células por blastocisto, número de células do trofoblasto e expressão de genes de sobrevivência celular *AKT* e *XIAP* não tiveram diferença entre os grupos. Quanto aos genes de estresse celular, apenas a *HSP70* teve diferença entre os grupos, mostrando-se aumentada em todos os grupos submetidos ao estresse térmico. Apesar do estresse térmico ser amplamente estudado como fator limitante na reprodução em função da diminuição de níveis hormonais, qualidade de gametas e viabilidade embrionária, foi demonstrado pela primeira vez a diminuição da produção de IFNT em embriões submetidos ao estresse térmico nos primeiros dias de desenvolvimento.

Palavras-chave: estresse térmico, hipertermia, reconhecimento materno da gestação, espécies reativas de oxigênio, blastocisto.

ABSTRACT

INFLUENCE OF HEAT STRESS IN INTERFERON TAU PRODUCTION AND OXIDATIVE STRESS OF BOVINE EMBRYOS PRODUCED *IN VITRO*

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Pregnancy losses occur at different stages caused by several conditions, however it occurs more frequently in the first 30 days. This decrease becomes more severe when animals are under unfavorable environmental conditions. Factors such as hyperthermia can reduce conception rates around 20-30% when compared to months where animals are under thermal comfort conditions. Therefore, heat stress (HS) is a problem found in the management of dairy cows in the tropics and subtropics, causing production and reproduction losses. The aim of the present study was to evaluate the influence of HS on development, interferon tau production (IFNT) and oxidative stress of bovine embryos produced *in vitro*. To evaluate the influence of HS on the *in vitro* production of bovine embryos, an *in vitro* model of HS was proposed. This model consists of altering the temperature in some specific stages of the embryo production. To induce HS, the temperature was gradually increased from 38.5°C to 40.5°C, where remained for 6 hours. Then, the temperature was gradually decreased until it returned to 38.5°C. The experimental groups were: control; oocytes submitted to HS during *in vitro* maturation (IVM HS); oocytes submitted to HS during *in vitro* fertilization (IVF HS); zygotes submitted to HS during the first day of *in vitro* culture (IVC HS); and embryos submitted to HS during the first three days of *in vitro* production (IVM + IVF + IVC HS). All groups submitted to HS had cleavage and blastocyst rates decreased, gene and protein expression of IFNT diminished and oxidative stress increased. The number of embryonic cells per blastocyst, number of trophoblastic cells and cell survival genes expression did not differ among groups. Regarding to cellular stress genes, only HSP70 had a difference among groups, showing an increase in all groups submitted to HS. Although HS is widely studied as a limiting factor in reproduction due to the decrease of hormone levels, gametes quality and embryonic viability, it was demonstrated for the first time the decrease in the production of IFNT in embryos submitted to HS in the early days of development.

Keywords: heat stress, hyperthermia, maternal recognition of pregnancy, reactive oxygen species, blastocyst.

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1 INTRODUÇÃO

Perdas gestacionais acontecem nos diferentes estágios da gestação por uma variedade de causas, contudo ocorrem com maior frequência nos primeiros 30 dias. Em média, apenas 30% das vacas em lactação concebem após uma única inseminação artificial, e essa taxa se reduz ainda mais quando estes animais estão sob condições ambientais desfavoráveis (Wiltbank et al., 2016). O decréscimo nas taxas de concepção durante os meses de calor pode estar entre 20-30% comparado aos meses de conforto térmico (De Rensis and Scaramuzzi, 2003), e ainda pode ser observado durante o outono, devido a ação das altas temperaturas sobre a foliculogênese (Roth et al., 2001). Portanto, o estresse térmico é um problema encontrado no manejo de vacas leiteiras nos trópicos e subtropicais, causando reduções na produção e reprodução.

O ambiente tem grande influência sobre a fisiologia animal. O ambiente calórico é determinado pelo clima de uma região. Neste ambiente encontram-se os fatores climáticos como: temperatura, umidade relativa do ar, radiação solar e velocidade do ar, que são reunidos em uma única variável para determinar a temperatura efetiva (Dikmen and Hansen, 2009). Os prejuízos causados pelo estresse térmico possuem maior influência das temperaturas elevadas e da umidade relativa do ar (Kadokawa et al., 2012). Em vacas de leite, a hipertermia pode ocorrer mesmo em temperaturas de 25 a 28°C, quando a umidade relativa do ar está elevada (Sartori et al., 2002). Segundo o estudo desenvolvido por Schuller et al. (2014), a temperatura retal de 39°C é um parâmetro classificado dentro dos limites fisiológicos para a espécie, porém já causam perdas na produção de leite e na fertilidade.

A redução da fertilidade consequente do estresse térmico é um problema de ordem multifatorial, pois acomete o bom funcionamento de vários tecidos e tipos celulares. Sabe-se que o estresse térmico compromete a produção de hormônios reprodutivos (Roth et al., 2000; De Rensis and Scaramuzzi, 2003), desenvolvimento oocitário (Al-Katanani et al., 2002) e embrionário na fase de pré-implantação (Sakatani, 2017). Com relação ao embrião no período de pré-implantação, foi demonstrado que quando o estresse térmico ocorre anterior ao período de ativação do genoma os efeitos são mais intensos; e posterior a transição materno-zigótica, os embriões adquirem uma certa tolerância ao estresse térmico (Sakatani, 2017).

O ambiente uterino também sofre efeitos do estresse térmico. Em vacas submetidas a estresse térmico há uma redução no fluxo sanguíneo uterino com diminuição da troca de calor e consequentemente aumento da temperatura interna do útero (Gwazdauskas et al., 1981). Essas mudanças inibem o desenvolvimento embrionário e impedem o sucesso de inseminações, além

de aumentarem a taxa de perda embrionária. Em um estudo realizado na Espanha observou-se que vacas holandesas que conceberam durante o verão apresentaram 12,3% de perdas embrionárias em contraste com as que emprenharam no inverno e tiveram apenas 2,1% de perdas (Garcia-Ispierto et al., 2006).

Embriões em fase inicial de desenvolvimento são mais susceptíveis aos efeitos do estresse térmico (De Rensis et al., 2015; Sakatani, 2017) e vacas submetidas ao estresse térmico têm corpo lúteo menor e produzem menos progesterona (Macias-Cruz et al., 2016). Dessa forma, o estresse térmico impacta diretamente no período de desenvolvimento embrionário inicial, na formação e desenvolvimento de um corpo lúteo competente e no reconhecimento materno da gestação.

O reconhecimento materno da gestação em ruminantes é o período em que o conceito sinaliza sua presença para a mãe, através da secreção de interferon tau (IFNT) (Bazer and Thatcher, 2017). Para ocorrer o reconhecimento materno da gestação em ruminantes é essencial a formação de um corpo lúteo especializado para a síntese de progesterona. O tamanho do corpo lúteo é diretamente proporcional à concentração sérica de progesterona, alongação e produção de IFNT pelo embrião (Ribeiro et al., 2016). É o momento que ocorre a maior mortalidade embrionária em bovinos (Thatcher et al., 2001). Conseqüentemente, o entendimento dos efeitos diretos e indiretos do estresse térmico no desenvolvimento embrionário inicial e sua sinalização para o reconhecimento materno da gestação adequado é fundamental para minimizar perdas econômicas e melhorar índices de produção.

Desta maneira, nossa hipótese é que existe diferença na expressão de IFNT nos embriões bovinos submetidos ao aumento de temperatura durante a PIVE, e essa variação na temperatura induz a ocorrência de estresse oxidativo no embrião. Os objetivos deste estudo foram: 1) avaliar as taxas de desenvolvimento embrionário em embriões bovinos produzidos sob efeito do estresse térmico; 2) demonstrar a influência do estresse térmico na expressão de IFNT e produção de ROS; 3) correlacionar a ocorrência do estresse térmico com o estresse oxidativo e a expressão de genes de sobrevivência e estresse celular.

2 REVISÃO DE LITERATURA

2.1 IMPACTO DO ESTRESSE TÉRMICO NOS ÍNDICES REPRODUTIVOS

O estresse térmico é definido como o resultado da inabilidade do animal dissipar ou produzir calor para manter sua temperatura fisiológica. Isso acontece quando o animal se encontra fora da sua zona de conforto térmico, consequência tanto de temperaturas elevadas (também conhecido como estresse calórico), como de temperaturas baixas (Slimen et al., 2016). O estresse térmico representa uma das maiores causas de perdas produtivas e reprodutivas em um sistema de produção de leite. Vacas leiteiras possuem maiores exigências metabólicas, o que influencia na eficiência reprodutiva, ocasionando em baixos índices nos rebanhos (De Rensis and Scaramuzzi, 2003).

Os prejuízos ocorridos pelo estresse térmico possuem influência das temperaturas elevadas e da umidade relativa do ar (Kadokawa et al., 2012). Em vacas de leite, a hipertermia pode ocorrer mesmo em temperaturas de 25 a 28°C, quando a umidade relativa do ar está elevada (Sartori et al., 2002). Segundo o estudo desenvolvido por Schuller et al. (2014), a temperatura retal de 39°C é um parâmetro classificado dentro dos limites fisiológicos para a espécie, porém já causam perdas na produção de leite e na fertilidade.

Dentre todas as categorias de animais em uma fazenda leiteira, vacas em lactação possuem maior ingestão de matéria seca. Esses nutrientes são absorvidos, metabolizados e, conseqüentemente, geram calor que precisa ser dissipado. Quando o animal se encontra em uma situação de estresse térmico, torna-se desfavorável dissipar o calor produzido (De Rensis and Scaramuzzi, 2003; Kadokawa et al., 2012). Esse fato se comprova no estudo feito por Sartori et al. (2002), que demonstrou que vacas têm maior aumento de temperatura corporal em resposta à temperatura ambiental do que a categoria de novilhas.

Vacas sob o efeito do estresse térmico diminuem o consumo de matéria seca (Ammer et al., 2017), o que altera a atividade ruminal e pode causar balanço energético negativo. Como conseqüências, ocorrem distúrbios metabólicos associados, como a acidose ruminal subaguda e a cetose (Polsky and von Keyserlingk, 2017). Sendo assim, a categoria de vacas em lactação é a mais susceptível aos prejuízos em decorrência ao estresse térmico por calor (Sartori et al., 2002; Hansen, 2009).

Estudos comprovam que vacas expostas ao estresse térmico no ato da concepção e aos 60 dias iniciais de gestação foram mais susceptíveis a perdas gestacionais quando comparadas com vacas que não sofreram estresse térmico nesse mesmo período (De Rensis et al., 2015). O

decréscimo nas taxas de concepção durante os meses de calor pode estar entre 20-30% comparado aos meses de conforto térmico (De Rensis and Scaramuzzi, 2003). Essa queda na taxa na concepção ainda é observada durante o outono, onde as vacas já não se encontram mais sob estresse térmico, devido a ação das altas temperaturas sobre a foliculogênese da estação anterior (Roth et al., 2001).

O estresse em decorrência das altas temperaturas diminui os níveis circulantes de progesterona, estradiol, gonadotrofinas (De Rensis and Scaramuzzi, 2003; Das et al., 2016), e da citocromo P450 SCC mitocondrial, enzima responsável pela clivagem da cadeia lateral do colesterol em pregnenolona (McCracken et al., 2015). Assim, com essa diminuição dos níveis de progesterona, a possibilidade de perdas durante o início da gestação aumenta. O estresse térmico ainda pode afetar o peso e prejudicar o desenvolvimento vascular fisiológico da placenta (Dunlap et al., 2015).

2.1.1 Efeito do estresse térmico sobre a maturação oocitária

Vacas expostas ao estresse térmico por calor têm redução da qualidade dos oócitos, o que afeta o desenvolvimento subsequente do embrião (Sugiyama et al., 2007). Complexos Cumulus-oócitos (CCOs) em fase de vesícula germinativa expostos a temperaturas elevadas (40°C-41°C) mostram maturações citoplasmática e nuclear deficientes, além do aumento da formação anormal do fuso e diminuição da competência de desenvolvimento embrionário após fertilização (Roth, 2015).

O estresse térmico de CCOs em fase de vesícula germinativa compromete as funções de oócitos pela indução da atividade da caspase, ocasionando apoptose (Roth and Hansen, 2004), e altera a transcrição materna e funções mitocondriais (Roth, 2015). O estresse térmico muda não somente a função de oócitos, mas também das células do *cumulus*, com a diminuição da produção de metaloproteinase de matriz 9 (Rispoli et al., 2013) e de hialuronidase (Lenz et al., 1983).

O estresse por calor excessivo também induz o estresse oxidativo levando ao aumento de níveis de espécies reativas de oxigênio (do inglês *Reactive Oxygen Species* – ROS) em oócitos (Ozawa et al., 2002). As ROS danificam o DNA e induzem apoptose ou disfunção de organelas celulares, como as mitocôndrias (Roth, 2015). No entanto, pesquisadores mostraram que a suplementação com antioxidantes durante a maturação *in vitro* (MIV) com choque térmico aumenta a taxa de maturação de oócitos, bem como a competência dos embriões

futuros. A glutathiona (GSH) é um importante componente antioxidante no oócito e atua diminuindo os níveis de ROS (Edwards et al., 2001).

2.1.2 Efeito do estresse térmico sobre a fertilização e a mortalidade embrionária

O estresse térmico está entre os fatores que comprometem a eficiência dos gametas femininos e masculinos, assim como a viabilidade embrionária. Estudos *in vivo* sugerem uma relação entre a temperatura ambiente no dia da inseminação artificial e a taxa de morte de embriões (De Rensis et al., 2015). Estudos *in vitro* também relataram que a alta temperatura durante a fertilização reduziu a competência embrionária (Sakatani, 2017).

A qualidade do oócito (Sugiyama et al., 2007), a motilidade, integridade e função dos espermatozoides são diminuídas pela elevação de temperatura (Sakatani, 2017). A incubação de espermatozoides de 40°C a 42°C por 4 horas diminui a sua motilidade e integridade (Sakatani et al., 2015). Resultados indicam que o mecanismo anti-polispermia dos oócitos é interrompido pelo estresse térmico. Além dos efeitos no mecanismo anti-polispermia, o dano aos zigotos também pode suprimir o sucesso da fertilização e competência de desenvolvimento (Sakatani et al., 2015).

2.2 RESPOSTA CELULAR AO ESTRESSE TÉRMICO

A mitocôndria é o principal local de metabolismo do oxigênio, responsável por aproximadamente 85-90% do oxigênio celular consumido. As ROS são subprodutos do metabolismo do oxigênio, portanto sua formação acontece de forma fisiológica no metabolismo orgânico (Shigenaga et al., 1994). As ROS participam de diversas funções biológicas, como na produção de energia, na fagocitose, na regulação do crescimento celular e sinalização intracelular (Droge, 2002; Slimen et al., 2014; Soysal et al., 2017).

Embora pequenas concentrações de ROS sejam benéficas, o aumento descontrolado de ROS podem causar dano celular, pela ativação das vias de apoptose e da inflamação (Khan et al., 2017). Vários tipos de estresse celular induzem a ocorrência do estresse oxidativo, incluindo o estresse pelo calor e frio. O estresse térmico induz o estresse oxidativo (Slimen et al., 2014; Zachut et al., 2017). Quando o embrião é submetido a altas temperaturas, ocorre a formação excessiva de ROS e o sistema antioxidante se torna incapaz de estabelecer o equilíbrio (Correa et al., 2008; Simoes et al., 2013; Sakatani, 2017).

Para manter as concentrações de ROS balanceadas e obter um metabolismo oxidativo estável, a mitocôndria dispõe do seu próprio sistema de defesa antioxidante cuja função é controlar a produção de ROS e estabelecer o equilíbrio oxidativo. Neste sistema estão incluídos 2 principais grupos: antioxidantes enzimáticos e não enzimáticos. Antioxidantes enzimáticos são compostos principalmente pelas enzimas superóxido dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) e glutathione redutase (GR). Dentre os antioxidantes conhecidos como não enzimáticos estão compostos como as vitaminas A, C e E, selênio e carotenoides, entre outros (Slimen et al., 2014). Alguns grupos de pesquisa têm desenvolvido trabalhos relacionados com a suplementação de antioxidantes não enzimáticos no meio de maturação, fertilização e cultivo de embriões, a fim de aumentar as taxas de clivagem e desenvolvimento embrionário em bovinos (Takahashi et al., 2002; Rocha-Frigoni et al., 2016), suínos (Li et al., 2016), ovinos (Mishra et al., 2016) e ratos (Lian et al., 2013).

Os marcadores de estresse oxidativo mais utilizados para células reprodutivas em bovinos são a GSH, SOD e a mensuração da quantidade total de ROS (Ascari et al., 2017). Também é possível o uso de técnicas de biologia molecular para quantificação de ROS e as principais enzimas antioxidantes (Mishra et al., 2016). Outro marcador de estresse térmico *in vitro* e *in vivo* é a presença de proteínas induzidas por choque térmico (do inglês *Heat Shock Proteins* – HSPs), (Sakatani, 2017). Embora a síntese da maioria das proteínas seja acometida pelo estresse térmico, esse fato não se aplica às HSPs. As HSPs fazem parte de um grupo heterogêneo de proteínas denominado de chaperonas, que possuem peso molecular e função biológica específicas. De modo geral, as chaperonas auxiliam no remodelamento de uma proteína lesionada, evitando um dano celular letal (Hightower, 1990).

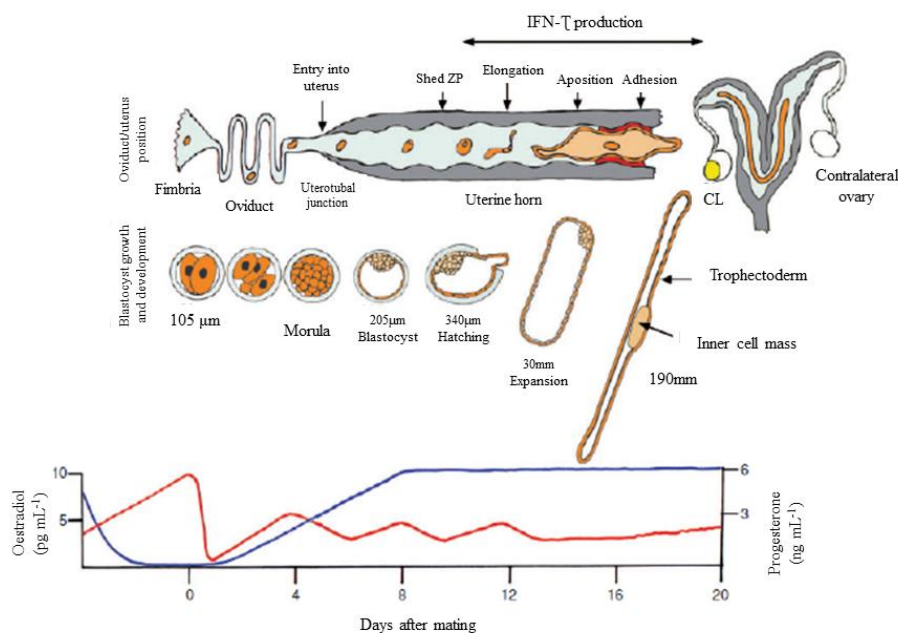
O mecanismo de ação das HSPs inicia com a ativação do seu fator de transcrição (do inglês *Heat Shock Transcription Factors* – HSFs). HSFs estão presentes no citoplasma de forma inativa. Os monômeros de HSFs sofrem o processo de fosforilação por proteínas quinases e são ativados, sendo translocados até o núcleo da célula, onde se ligarão a sítios específicos para transcrever o RNAm da HSP. Embora a ação das HSPs não seja restrita ao estresse térmico, acredita-se que essas proteínas utilizem energia da hidrólise de ATP para desnovelar proteínas lesionadas pela hipertermia, possibilitando novo enovelamento, dessa vez na forma correta ou no lugar correto (Sreedhar et al., 2000; Slimen et al., 2014).

2.3 RECONHECIMENTO MATERNO DA GESTAÇÃO EM RUMINANTES

O reconhecimento materno da gestação é o período em que o concepto sinaliza sua presença para a mãe com a finalidade de aumentar a vida útil do corpo lúteo e consequentemente evitar o retorno à ciclicidade (Farin et al., 1990; Niswender et al., 2000). Em ruminantes, o período de sinalização coincide com o alongamento do embrião e a máxima produção IFNT (Spencer et al., 2007; Antoniazzi et al., 2010).

Após a fecundação, inicia-se o processo de desenvolvimento embrionário. O embrião passa pelas fases de zigoto, quando ocorre o estabelecimento da singamia. Logo após sofre sucessivas mitoses, até chegar à fase de mórula. Nessa fase, que acontece de 4 a 6 dias após a fecundação, o embrião entra no útero. Em seguida, evolui para o estágio de blastocisto inicial, com a formação da blastocele e o estabelecimento de 2 tipos celulares: trofoblasto e massa celular interna (embrioblasto). O blastocisto inicial se desenvolve e expande, até o dia 8-9 quando ocorre a eclosão da zona pelúcida. Então o blastocisto passa a se alongar de forma tubular, formando um filamento capaz de preencher todo o espaço intrauterino (Figura 1). A expressão de IFNT aumenta à medida que ocorre o processo de alongação (Hirayama et al., 2014).

Figura 1. Desenvolvimento embrionário inicial em ruminantes.



Fonte: (Spencer et al., 2007).

A secreção de IFNT no conceito ovino ocorre entre os dias 10 e 25, com pico de secreção entre os dias 14 e 16 da gestação (Roberts et al., 1996). Já em bovinos, a secreção ocorre entre os dias 12 e 26, com pico entre os dias 18 e 20 (Farin et al., 1990; Roberts, 1991; Hirayama et al., 2014). O IFNT é a principal citocina secretada pelas células do trofoblasto embrionário, sendo responsável pela sinalização durante esse período (Roberts et al., 1999). O mecanismo clássico de ação do IFNT consiste no controle da transcrição de receptores de estrógenos (ESR1) e conseqüentemente receptores de ocitocina (OXTR) no epitélio luminal endometrial (Spencer and Bazer, 1996). Esse controle inibe os pulsos luteolíticos de prostaglandina F2 alfa (PGF), evitando o retorno à ciclicidade.

2.3.1 O interferon tau

Na década de 1960, estudos identificaram que havia alguma substância produzida durante a fase inicial da gestação que adia a manifestação do estro (Moor and Rowson, 1966). Já na década de 1970, foi comprovado que essa substância era uma proteína produzida pelo embrião, que então foi nomeada de trofoblastina (Martal et al., 1979). Anos depois, foi demonstrado que essa proteína era produzida pelas células do trofoblasto e então mudaram sua nomenclatura para "Proteína X" (Godkin et al., 1982); e foi renomeada em proteína do trofoblasto ovino (ovine trophoblastic protein-1 oTP-1) (Godkin et al., 1984). A oTP-1 foi sequenciada e foi identificado que sua estrutura era muito semelhante aos interferons tipo I, e por essa razão foi, mais uma vez, alterada sua nomenclatura para interferon tau (Imakawa et al., 1987).

Aparentemente, o IFNT evoluiu do interferon ômega, essa evolução é caracterizada pela inserção de um promotor específico no trofoblasto. Bovinos, ovinos e caprinos possuem diversas formas polimórficas de IFNT (Roberts et al., 2003; Ealy and Wooldridge, 2017). Fatores de transcrição que possuem papel importante na regulação da expressão do gene do IFNT. Dentre eles, o fator de transcrição ETS2 e AP1 possuem um papel fundamental na regulação da transcrição de IFNT durante o início da gestação (Ezashi et al., 1998; Ezashi and Imakawa, 2017).

O IFNT é uma proteína secretada em grandes quantidades pelas células do trofoblasto do embrião de ruminantes, antes da implantação (Farin et al., 1989). O RNAm começa a ser expresso a partir do quarto dia do desenvolvimento embrionário *in vitro* (Yao et al., 2009; Talukder et al., 2018) e a sua sinalização já é detectada no endométrio no sétimo dia do

desenvolvimento embrionário (Sponchiado et al., 2017). O início da expressão de IFNT é programada geneticamente independente do ambiente uterino, visto que ele é expresso em sistemas *in vivo* e *in vitro*. No entanto, a produção de IFNT é influenciada pelo ambiente uterino, pois a produção *in vitro* aumenta na presença de endométrio (Kerbler et al., 1997). A expressão termina com a implantação (Demmers et al., 2001).

2.3.2 Receptores de interferon tipo I e genes estimulados por interferon tau (ISGs)

O IFNT liga-se a receptores de interferon tipo I (IFNAR1 e IFNAR2) e induz sua resposta por meio da sinalização via JAK/STAT (Binelli et al., 2001). Os receptores IFNAR1 e IFNAR2 são expressos em todos os tecidos corporais e têm como principal função interceder respostas antivirais. Também estão localizados no endométrio para interceder respostas maternas em função do IFNT produzido pelo embrião (Johnson et al., 1999a; Johnson et al., 1999b).

O IFNT se liga a esses receptores para exercer sua ação pela via de transdução de sinais JAK/STAT, onde as proteínas tirosino-quinases fosforilam proteínas STAT formando complexos multiméricos que agem como fatores de transcrição (Binelli et al., 2001). Esses complexos atuam basicamente em 2 vias: a primeira consiste no controle da transcrição de ESR1 e conseqüentemente OXTR no epitélio luminal endometrial (Spencer and Bazer, 1996); e a segunda ocorre quando os complexos se ligam a regiões definidas no DNA, chamadas de elementos responsivos à estimulação por interferons, que regulam a expressão de ISGs (Hansen et al., 1998; Antoniazzi et al., 2010).

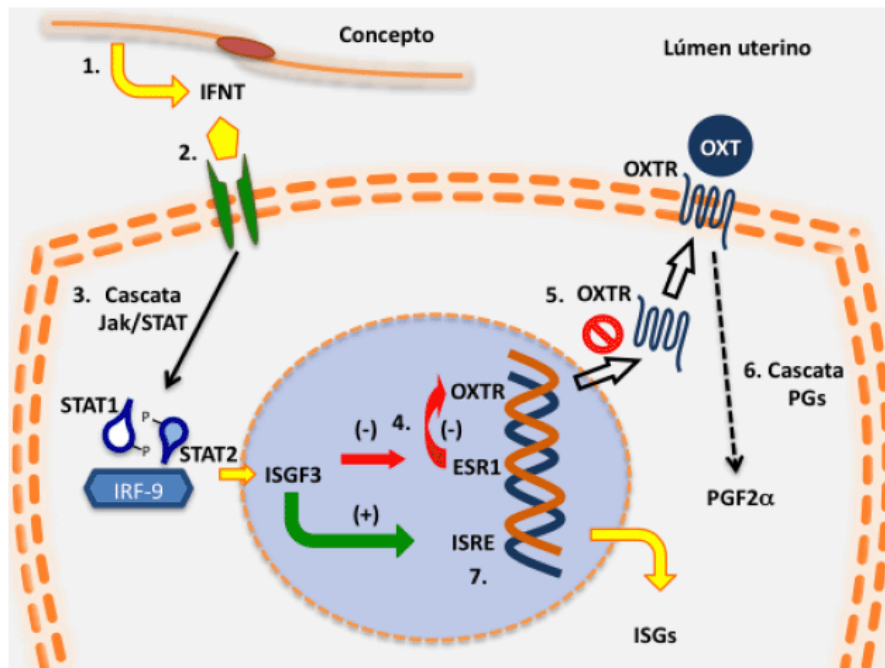
Dentre os ISGs que aumentam a expressão de RNAm durante o início da gestação em resposta ao IFNT, estão os genes 2',5' oligoadenilato sintetase (OAS1) (Mirando et al., 1991; Schmitt et al., 1993), o gene de resistência ao myxovirus 1 (MX1) (Ott et al., 1998) e o gene estimulado por interferon 15 (ISG15) (Austin et al., 1996). Estudos demonstraram a expressão de ISGs em células do sangue (Han et al., 2006; Oliveira et al., 2008) e células luteais (Oliveira et al., 2008) logo após a sinalização por IFNT no início da gestação em ruminantes.

2.3.3 Ações do interferon tau

Atualmente, sabe-se que o IFNT atua mediante 3 vias de sinalização: parácrina, endócrina e autócrina. O mecanismo de sinalização parácrino, conhecido como via clássica de

sinização do reconhecimento materno da gestação em ruminantes, consiste na ligação do IFNT aos seus receptores presentes no endométrio, ativação da cascata JAK/STAT e inibição da expressão de ESR1 e de OXTR no epitélio luminal do endométrio (Spencer and Bazer, 1996). A supressão dos receptores ESR1 e OXTR evita a liberação de pulsos luteolíticos de PGF (Spencer and Bazer, 1996), hormônio responsável pelo início da luteólise (McCracken et al., 1999).

Figura 2. Via de sinalização parácrina do reconhecimento materno da gestação em ruminantes.



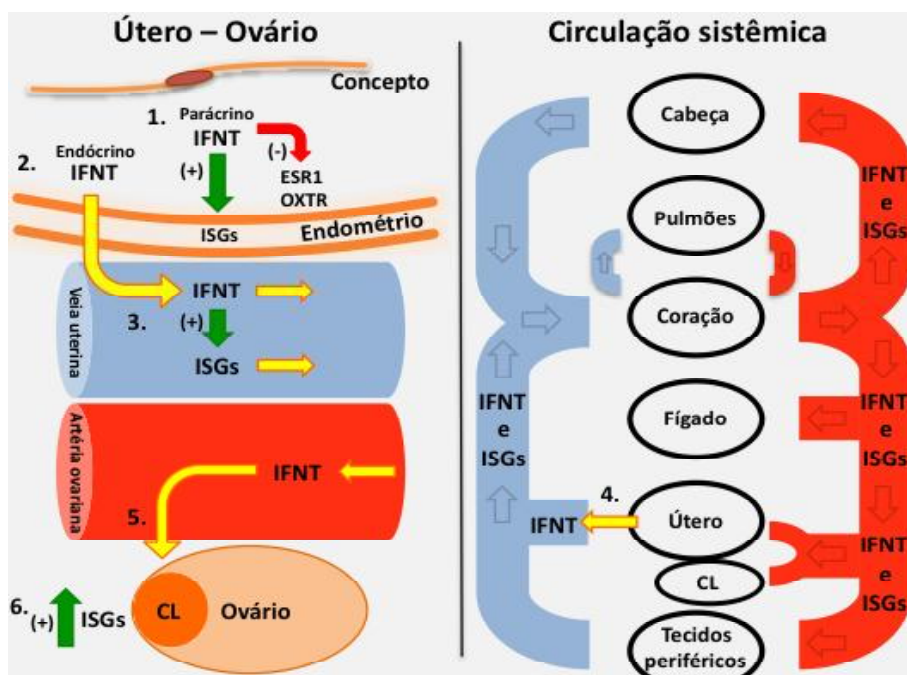
Fonte: (Antoniazzi et al., 2010).

Além do mecanismo parácrino do reconhecimento materno da gestação em ruminantes, existe a via de sinalização endócrina. Foi observado que alguns animais gestantes possuíam expressão de genes estimulados por interferons na corrente circulatória, mais especificamente o ISG15 (Han et al., 2006). Inicialmente foi pensado na presença de um mediador da ação do IFNT, uma interferomedina (Spencer et al., 1999). Recentemente foi avaliada a expressão de ISG15 em tecidos extrauterinos durante o início da gestação em ovinos (Oliveira et al., 2008; Bott et al., 2010). Pelas técnicas de biologia molecular de PCR em tempo real, western blot e imuno-histoquímica, identificou-se uma maior expressão de ISG15 em células luteais grandes no dia 15 da gestação, quando comparada com a expressão em células luteais grandes de ovelhas não prenhes (Oliveira et al., 2008). O ensaio antiviral mostrou maior bioatividade de interferons tipo I no dia 15 da gestação no sangue da veia uterina de ovelhas prenhes quando

comparadas com ovelhas não prenhes (Oliveira et al., 2008). Assim, sugere-se que a ação dos interferons presente no soro da veia uterina de ovelhas prenhes no dia 15 da gestação é exercida pelo IFNT, pois a utilização de anticorpo específico contra IFNT bloqueia ação antiviral (Bott et al., 2010).

A partir da comprovação da ação endócrina do IFNT na veia uterina, iniciou-se a investigação de sua ação em tecidos extrauterinos que poderiam estar envolvidos com o reconhecimento materno da gestação. Um novo modelo de estudo da ação endócrina do IFNT foi desenvolvido (Bott et al., 2010). Inicialmente, foi realizada a instalação de uma bomba osmótica para infusão contínua de rIFNT na veia uterina no dia 10 do ciclo estral, e foi verificado a expressão de ISG15 no CL (Bott et al., 2010). Outros experimentos foram realizados com infusão contínua de IFNT em diferentes dias e com diferentes concentrações de IFNT, e ambas foram capazes de induzir ISG15 em tecidos extrauterinos (Antoniazzi et al., 2013).

Figura 3. Mecanismo de ação endócrino do reconhecimento materno da gestação em ruminantes.



Fonte: (Antoniazzi et al., 2010).

Recentemente foi sugerido que o IFNT possui um mecanismo de ação autócrino. Foi demonstrado que células do trofotoderma ovino expressavam IFNAR1, indicando a existência de alguma função do IFNT em suas próprias células produtoras (Imakawa et al., 2002). A partir

desse resultado, outros estudos foram realizados na tentativa de elucidar essa via de sinalização. Wang et al. (2013) adicionaram diferentes concentrações de rbIFNT no cultivo de células de trofoblasto, e mostraram que o desenvolvimento celular e a expressão de ISGs aumentaram à medida que aumentava a concentração de rbIFNT no meio de cultivo, comprovando que o IFNT não é crucial apenas para o reconhecimento materno da gestação em ruminantes, mas também atua como um regulador autócrino de proliferação das células do trofoblasto.

3 CAPÍTULO 1

Artigo submetido para publicação:

**Interferon tau production in the blastocyst stage is impaired by heat stress
on bovine embryos produced *in vitro***

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Biology of reproduction, 2019

1 **Interferon tau production in the blastocyst stage is impaired by heat stress on bovine**
2 **embryos produced *in vitro***

3

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8

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12

13 **Short title:** IFNT production on heat stressed embryos

14 **Abstract**

15 Interferon tau is the cytokine responsible for the maternal recognition of pregnancy in
16 ruminants. Recent studies have shown IFNT plays a role modulating embryo-maternal
17 communication in the oviduct inducing a local response from immune cells. The aim of this
18 study was to investigate IFNT production under influence of heat stress during different
19 stages of bovine *in vitro* embryo production. Heat Stress (HS) was established when
20 temperature was gradually raised from 38.5°C to 40.5°C in a laboratory incubator, sustained
21 for 6h and slowly decreased back to 38.5°C. To address the HS effects on IFNT production
22 during bovine oocyte maturation, fertilization and/or zygote stage, ovaries were obtained from
23 a local slaughterhouse and the experiment was performed according to the following
24 treatments: (1) control group (38.5°C in all stages); (2) oocytes matured under HS (IVM HS);
25 (3) oocytes fertilized under HS (IVF HS); (4) zygotes cultured in the first day under HS (IVC
26 HS); and (5) cells submitted to HS at oocyte maturation, fertilization and first day of zygote
27 culture (IVM+IVF+IVC HS). The HS negatively affected cleavage and blastocyst rates
28 ($P < 0.0001$), in all HS groups. On Day 7, all the HS treated embryos showed downregulated
29 IFNT gene and protein expressions ($P = 0.02$), whereas reactive oxygen species were
30 upregulated ($P = 0.03$) in comparison to the control. In conclusion, the compromised early
31 embryo development due to higher temperatures during *in vitro* oocyte maturation,
32 fertilization and/or zygote stage is related to downregulated interferon tau expression and
33 increased reactive oxygen species in bovine.

34

35 **Keywords:** hyperthermia, maternal recognition of pregnancy, reactive oxygen species,
36 embryos, cows.

37 **Introduction**

38 Maternal recognition of pregnancy in ruminants occurs classically beyond Days 10-12
39 of pregnancy when the conceptus signals to the mother its presence [1]. Interferon tau is the
40 major cytokine responsible for the embryo-maternal interaction to avoid luteolysis and
41 establish and maintain the pregnancy [2, 3]. IFNT is produced by embryonic trophoblast at
42 pre-implantation period and acts in the uterus in a paracrine manner, inhibiting endometrial
43 estrogen and oxytocin receptors expression, avoiding prostaglandin F2 alpha (PGF) luteolytic
44 pulses [4]. Also, IFNT action has been identified in extrauterine tissues [5, 6] to protect the
45 CL against PGF pulses [7, 8].

46 Recently, IFNT has been identified to be produced by embryos on Day 4 when cultured with
47 oviduct cells [9], and this period coincides with maternal-zygote transition [10]. At this period
48 the embryo starts to secrete IFNT while in the oviduct stimulated in part by oviduct epithelial
49 cells, inducing an anti-inflammatory response in oviduct
50 cells without ISGs response [9]. However, local anti-inflammatory response in immune cells
51 caused by the the oviductal cells and the embryo interaction induces ISGs [11]. Therefore,
52 IFNT has a local action in the oviduct modulating anti-inflammatory response previously to
53 the classical maternal recognition of pregnancy signaling.

54 Intensive genetic selection for high milk production significantly shortened the
55 thermoregulatory competence in dairy cows [12], which is often detected as negative effects
56 in reproductive parameters both *in vivo* and *in vitro* in cows and embryos under HS conditions
57 [13-16]. High temperature alters follicular development [17], steroidogenesis [18] and
58 decreases quality of female [19] and male [20] gametes. *In vitro* produced bovine embryos
59 demonstrated developmental inhibition when exposed to temperatures considered as heat-
60 stressed cows *in vivo*, reducing cleavage and blastocyst rates [16]. The production of reactive
61 oxygen species (ROS) mediates oxidative stress, and occasionally HS is responsible for ROS

62 production [21], and participates in several biological functions. However, pathological
63 processes may occur when an imbalance between ROS production and antioxidant capacity
64 takes place in the cells [21, 22], which is controlled by a complex antioxidant system
65 constituted of enzymatic and non-enzymatic antioxidants, including three important enzymes:
66 superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) [22].
67 Enhanced intracellular ROS may cause DNA and mitochondrial damage [21], leading to non-
68 specific modification of lipids and proteins [23], then activating cell autophagy mechanisms
69 [24] and apoptosis [25]. In addition to ROS as a possible consequence of HS, Heat Shock
70 Proteins (HSPs) represent a heterogeneous group of molecular chaperones induced by high
71 temperatures, protecting the cells from lethal thermal damage [21].

72 It has been reported that any source of stress, including HS, has the potential to disrupt
73 the process of interactions between the uterus and the embryo [26], compromising the
74 establishment and maintenance of pregnancy. We hypothesized that HS influences the
75 embryo production of IFNT and consequently affects embryo development. Therefore, the
76 objectives of the present study were: (1) to evaluate the effects of HS during oocyte
77 maturation, fertilization and/or zygote cleavage on embryo development under *in vitro*
78 conditions; (2) to demonstrate the effect of HS before genome activation on IFNT and ROS
79 production in embryos produced *in vitro*; (3) to investigate the effect of HS on oxidative
80 stress, cell survival and on the expression of genes associated with cellular stress.

81

82 **Materials and methods**

83 ***Chemicals***

84 Chemicals and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich, St.
85 Louis, MO, USA), unless otherwise indicated.

86

87 ***Oocyte recovery and in vitro maturation (IVM)***

88 Cow ovaries were obtained from a local slaughterhouse and transported to the
89 laboratory in saline solution (0.9% NaCl; 30°C) containing 100IU/ml penicillin and 50µg/ml
90 streptomycin sulfate. Cumulus oocyte complexes (COCs) from 3 to 8mm diameter follicles
91 were aspirated with a vacuum pump (vacuum rate of 20ml of water/minute). The COCs were
92 recovered and selected under a stereomicroscope. Grade 1 and 2 COCs [27] were randomly
93 distributed into 500µl of maturation medium in four-well plates (Nunc, Roskilde, Denmark),
94 and cultured in incubators at 38.5°C (101.3°F) or 40.5°C (104.9°F) (for 6h; see experimental
95 design section) in a saturated humidity atmosphere containing 5% CO₂ and 95% air, for 22-
96 24h. The maturation medium consisted of TCM199 containing Earle's salts and L-glutamine
97 (Gibco Labs, Grand Island, NY, USA), supplemented with 25mM HEPES, 0.2mM pyruvic
98 acid, 2.2mg/ml sodium bicarbonate, 5.0µg/ml LH (Lutropin-V[®]), 0.5µg/ml FSH (Folltropin-
99 V[®]), 10% fetal bovine serum (FBS; Gibco Labs, Grand Island, NY, USA), 100IU/ml
100 penicillin and 50µg/ml streptomycin sulfate. Culture medium pH was verified for stability
101 (pH = 7.4) throughout the different temperatures of incubation.

102

103 ***In vitro fertilization (IVF)***

104 After IVM, bovine oocytes were fertilized *in vitro* with tested frozen semen after
105 thawing and fractionating on discontinuous Percoll (GE Healthcare, São Paulo, SP, Brazil)
106 gradient. Sperm were diluted and added to the COCs plate with final concentration adjusted to
107 2×10^6 sperm/ml in Fert-TALP medium containing 10µg/ml heparin, 30µg/ml penicilinamine,
108 15mM hypotaurine and 1mM epinephrine. Fertilization was carried out by co-culture of
109 sperm and oocytes for 18-20h in four-well plates in the same atmospheric conditions used for
110 maturation. IVF day was considered as Day 0 of embryo production.

111

112 ***In vitro embryo culture (IVC)***

113 After IVF, presumptive bovine zygotes were denuded by vortexing, and then cultured
114 in groups of 50 in a culture chamber (CBS Scientific, Del Mar, CA, USA) at 38.5°C and
115 saturated humidity atmosphere of 5% CO₂, 5% O₂ and 90% N₂ in 500µl of SOF medium in
116 four-well plates (Nunc, Roskilde, Denmark). Cleavage rates were evaluated 48h after
117 fertilization and blastocyst rates were assessed on Day 7 of embryo development. Blastocysts
118 evaluated on Day 7 were rinsed three times in PBS and collected in a pool of 5 embryos
119 stored at -80°C for subsequent RNA extraction or fixed for immunofluorescence assay. Fresh
120 embryos collected on Day 7 were used for ROS detection.

121

122 ***RNA extraction, reverse transcription and real time PCR***

123 The total RNA was extracted from Blastocysts collected on Day 7 according to
124 TRIzol® instructions. Briefly, the extraction used 1000µl TRIzol® reagent (Thermo Fisher,
125 Waltham, MA, USA) and 200µl chloroform, followed by purification of the aqueous phase
126 with 20mg 1.33µl Glycoblue (Thermo Fisher, Waltham, MA, USA) and 700µl isopropyl
127 alcohol. Quantification and estimation of RNA purity was performed using Nanodrop
128 spectrophotometer (Thermo Scientific, Waltham, MA, USA; Absorbance 260/280nm ratio).
129 RNA was treated with 0.1U DNase Amplification Grade (Thermo Fisher, Waltham, MA,
130 USA) for 15 minutes at 27°C to neutralize any DNA molecules. DNase was inactivated with
131 1µl EDTA for 10 minutes at 65°C. Reverse transcription was performed adding 1U iScript
132 cDNA synthesis Kit (BioRad, Hercules, CA, USA) for 5 minutes at 25°C followed by 30
133 minutes at 42°C and 5 minutes at 85°C. Quantitative polymerase chain reaction (qPCR) was
134 conducted in a thermocycler (BioRad, Hercules, CA, USA) using 2µl of cDNA and 8µl of
135 MIX containing forward and reverse bovine specific primers (Table 1), nucleases free water
136 and GoTaq® Master Mix (Promega Corporation, Madison, USA). Amplification was

137 performed with initial denaturation at 95°C for 5 minutes followed by 40 cycles of
138 denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 30 seconds. To
139 optimize the RT-qPCR assays, serial dilutions of cDNA templates were used to generate a
140 standard curve, and efficiency between 90 and 110% and coefficient of determination (R²)
141 higher than 0.98 were considered. Samples were run in duplicate and the results of all genes
142 were expressed relative to *GAPDH* and *RPS18* reference genes, according to Pfaffl [28].

143

144 ***Immunofluorescence staining and embryo cell counting***

145 Embryos that reached the blastocyst stage were separated, rinsed in PBS containing
146 0.1% polyvinyl alcohol (PBS-PVA), fixed for 15-20 minutes in 4% paraformaldehyde, and
147 then stored in PBS containing 0.3% BSA and 0.1% Triton X-100 at 4°C until staining. Then,
148 embryos were permeabilized in 1% triton X-100 diluted in PBS for 90 minutes at 37°C
149 followed by 1h in blocking solution (3% BSA and 0.2% Tween-20 in PBS) at room
150 temperature. Subsequently, samples were incubated overnight with primary antibodies anti-
151 IFNT (kindly provided by Drs. Thomas R. Hansen, CO, USA and Fuller W. Bazer, TX, USA)
152 or anti-CDX2 (BioGenex, Fremont, CA), diluted 1:500 in blocking solution. Samples were
153 washed twice in blocking solution and incubated for 1h at room temperature in the presence
154 of AlexaFluor 488® anti-rabbit IgG (Thermo Fisher, Waltham, MA, USA) or AlexaFluor
155 555® anti-mouse IgG (Thermo Fisher, Waltham, MA, USA) secondary antibodies, diluted
156 1:1000 in blocking solution according to the primary antibody. After immunostaining, nuclei
157 were counterstained with 300nM 4',6-diamidino-2-phenylindole (DAPI; Thermo Fisher,
158 Waltham, MA, USA), for 20 minutes. Embryo samples were mounted into glass slides with
159 Mowiol. Embryos from each group were evaluated using an epifluorescence microscope
160 (Leica, DMI 4000B) and nuclei were counted using ImageJ Software.

161

162 ***ROS detection***

163 Embryos that reached the blastocyst stage on Day 7 from all experimental groups were
164 used to determine concentration of ROS using 2',7'-dichlorodihydrofluorescein diacetate
165 (H2DCFDA; Molecular Probe) as described by Amin, et al. [29]. Fifteen embryos per group
166 were incubated in 5 μ M H2DCFDA diluted in DMSO for 20 minutes at 37°C. Then, embryo
167 samples were washed twice in PBS and transferred to plates for spectrophotometry assay, at
168 520 nm emission and 488 nm excitation.

169

170 ***Experimental design***

171 HS was set for *in vitro* embryo production. The temperature was gradually raised (2h
172 period) from 38.5°C to 40.5°C, maintained for 6h and slowly decreased back (2h period) to
173 38.5°C during different periods of *in vitro* embryo production (Figure 1). The temperature of
174 40.5°C during 6h was established as a model to induce HS based on results showing body
175 temperature variation in lactating dairy cows during the summer [15, 30]. COCs were
176 randomly distributed into the following groups: (1) Control, *in vitro* embryo production at
177 38.5°C; (2) COCs matured under HS conditions (IVM HS); (3) COCs fertilized under HS
178 conditions (IVF HS); (4) Zygotes cultured in the first day under HS conditions (IVC HS); and
179 (5) Embryos submitted to HS during all the previously established periods (IVM+IVF+IVC
180 HS). The experiments were simultaneously performed in two different incubators, one set to
181 38.5 °C all the time and another one set to 38.5 – 40.5°C as previously described, both
182 incubators were always with the same humidity and atmosphere conditions. The study was
183 completed in seven replicates.

184 **Statistical analysis**

185 Continuous data were tested for normal distribution using Shapiro-Wilk test and
186 normalized when necessary. All data were analyzed by ANOVA followed by multiple
187 pairwise comparisons (Tukey-Kramer HSD test) using the JMP Software (13.1.0; SAS
188 Institute Inc., Cary, NC). Results are presented as mean \pm standard error of the mean (SEM)
189 and $P < 0.05$ was considered significant.

190

191 **Results**

192 ***Cleavage and blastocyst rates of bovine embryos produced in vitro under heat stress***

193 The cleavage rate was lower in all HS groups: IVM HS ($61.28\% \pm 4.81$), IVF HS
194 ($60.85\% \pm 3.39$), IVC HS ($60.42\% \pm 2.06$) and IVM+IVF+IVC HS ($28.42\% \pm 1.36$) when
195 compared to control group ($89.33\% \pm 1.0$) ($P < 0.0001$). Groups IVM HS, IVF HS and IVC HS
196 were not different from each other considering cleavage rates. The IVM+IVF+IVC HS
197 cleavage rate was significantly lower when compared to all the other groups (Figure 2A). On
198 Day 7 after IVF, all HS groups presented decreased developmental rates ($P < 0.0001$) when
199 compared to control group ($32.7\% \pm 0.74$): IVM HS group ($25.7\% \pm 1.39$), IVF HS ($20.4\% \pm$
200 0.86) and IVC HS ($17.28\% \pm 0.52$) and IVM+IVF+IVC HS group ($11.14\% \pm 0.91$; Figure
201 2B). Heat stress affected both cleavage and blastocyst rates of bovine embryos produced *in*
202 *vitro* regardless of the stress time-point, and this was most evident when the cells were
203 exposed to HS in all the three stressed stages (IVM+IVF+IVC HS group).

204

205 ***IFNT expression of bovine embryos exposed to heat stress***

206 On Day 7, interferon tau gene and protein expressions were determined by RT-qPCR
207 and immunofluorescence staining (Figure 3). Representative image of IFNT protein
208 immunofluorescence detection (Figure 3A), *IFNT2* mRNA expression (Figure 3B) and

209 quantification of IFNT fluorescence intensity (arbitrary units) (Figure 3C) showed IFNT
210 significant decrease ($P=0.02$) when the embryos were exposed to HS in all the stressed
211 groups, in comparison to the control embryos. These results show that HS during oocyte
212 maturation, fertilization and/or embryo cleavage interfere in both gene and protein
213 expressions of IFNT in the subsequent developed embryos.

214

215 ***Determination of number of total blastocyst and trophoblastic cells of bovine embryos***
216 ***produced in vitro under heat stress***

217 The number of blastocyst and trophoblastic cells on Day 7 were analyzed through
218 immunofluorescence staining (Figure 4A). Total number of cells (Figure 4B) and
219 trophoblastic cells (Figure 4C) counted on Day 7 were not different ($P=0.07$ and $P=0.78$,
220 respectively) among all groups. These results show that HS during oocyte maturation,
221 fertilization and/or embryo cleavage do not interfere in both number of blastocyst and
222 trophoblastic cells on Day 7 embryos.

223

224 ***Oxidative stress profile of bovine embryos post-heat stress***

225 ROS were quantified in bovine embryos on Day 7 using H₂DCFDA by
226 spectrophotometry (Figure 5A). ROS production was higher ($P=0.03$) in all HS groups
227 compared to control group, and the IVM+IVF+IVC HS group presented the highest ROS
228 production. The relative expression of superoxide dismutase 1 and 2 (*SOD1* and *SOD2*;
229 Figure 5B and 5C, respectively), glutathione peroxidase 1 and 4 (*GPX1* and *GPX4*; Figure 5D
230 and 5E, respectively), catalase (*CAT*; Figure 5F), nuclear factor erythroid 2-related factor 2
231 (*NRF2*; Figure 5G) indicates oxidative stress related genes. Levels of mRNA for *SOD1*
232 ($P=0.40$), *GPX4* ($P=0.51$) and *NRF2* ($P=0.07$) were not significantly different comparing the
233 stressed groups to the control, whereas *SOD2* ($P=0.0035$) and *CAT* ($P=0.0003$) were

234 downregulated in HS groups. On the contrary, *GPXI* mRNA expression was upregulated in
235 IVF HS, IVC HS and IVM+IVF+IVC HS groups ($P=0.03$), but not in IVM HS group, when
236 compared to the control. Taking together, HS exposure during early stages of embryo
237 development may negatively affect oxidative stress, suggested by the imbalance of ROS
238 production and antioxidant genes.

239

240 ***mRNA relative expression of cellular survival and cellular stress genes in bovine embryos***
241 ***exposed to heat stress***

242 The cellular survival genes AKT serine/threonine kinase 1 (*AKT*; Figure 6A) and X-
243 linked inhibitor of apoptosis (*XIAP*; Figure 6B) were not affected ($P=0.96$ and $P=0.08$,
244 respectively) by HS on Day 7 embryos. Cellular stress was assessed by mRNA relative
245 expression of heat shock protein 90 alpha family class A member 1 (*HSP90AA1*; Figure 6C)
246 and heat shock protein family A member 1A (*HSPA1A*; Figure 6D). No significant difference
247 was shown for *HSP90AA1* ($P=0.30$), however *HSPA1A* was upregulated in all the treated
248 groups ($P=0.01$) in comparison to control embryos. These results suggest that HS exposure
249 during early stages of embryo development may not affect genes related to cellular survival.
250 Inversely, the transcripts for cellular stress *HSPA1A* gene were affected in previously stressed
251 embryos evaluated on Day 7 post-IVF.

252

253 **Discussion**

254 Interferon tau gene and protein expression are diminished in bovine embryos produced
255 *in vitro* under HS. In this study, we used an *in vitro* bovine embryo production model to stress
256 the oocyte maturation, fertilization and zygote in order to simulate the temperature increase
257 during the warmest season of the year. This environmental high temperature was identified to
258 increase the body temperature of lactating dairy cows during the warmest periods of the year

259 [15]. Initially, the incubator temperature was set to 38.5°C, and in the stressed groups the
260 temperature was increased to 40.5°C in a two hour interval, and it was sustained for 6h and
261 then decreased back to 38.5°C, again in a two hour interval. This means the embryos did not
262 receive a heat shock, they had two hours to adapt to a new higher temperature. The first
263 impact of heat stress is the decrease in cleavage and blastocyst rates. Some studies presented a
264 decrease in cleavage and blastocyst rates, however the embryos were subjected to
265 immediately change of temperature, without adapting period. [31-34]. Our results have shown
266 that HS during oocyte maturation, fertilization and/or embryo development decreases IFNT
267 expression on Day 7 embryo. There are evidences that HS decreases embryo survival *in vivo*
268 [35-37]; however, few studies have shown an impact on IFNT of *in vitro* embryo production
269 model of HS.

270 Trophoblastic cells produce IFNT, which is essential for maternal recognition of
271 pregnancy [38], it is also a good marker for embryonic health [39]. Another approach to
272 investigate embryo/trophoblast health is to evaluate Caudal-type homeobox transcription
273 factor (CDX2), which plays an essential role in trophoblast lineage emergence in ruminants
274 [40]. Therefore, the CDX2 antibody was used to assess trophoblastic cells on Day 7 embryos.
275 The number of trophoblastic cells of blastocysts collected on Day 7 after IVF were not
276 different in all groups, although the IFNT expression was downregulated. This finding
277 indicates that the reduction of IFNT was not due to the reduction of trophoblastic cell number.
278 Total number of cells per blastocyst may also be an indicative of blastocyst quality [41, 42].
279 In our study, total number of blastocyst cells were not different. A decrease of total and
280 trophoblastic number of cells per blastocyst was observed when bovine oocytes were exposed
281 to heat shock at 42°C. In contrast, bovine embryos submitted to heat shock at 41°C had no
282 difference in total number of cells and in trophoblast/inner cell mass rate [43]. The results

283 presented herein suggest the reduction of IFNT on Day 7 embryos under HS is not caused by
284 the reduction of total number of embryonic cells nor trophoblastic cells.

285 ROS production and antioxidants capacity must be a well-balanced condition in
286 physiological situations [44]. However, in some adverse conditions, like HS, cellular
287 mechanisms experience an imbalance in oxidative metabolism and the production of ROS
288 increases [45]. HS increases mainly mitochondrial superoxide anion, hydrogen peroxide and
289 hydroxyl radical levels [21]. In our experiments it was observed an increase in ROS levels on
290 Day 7 embryos followed HS during early stages of embryo production, more specifically on
291 oocytes maturation and fertilization and/or first day zygote stages. Another stress model,
292 using embryos cultured under high oxygen levels also showed increase of ROS production
293 [29].

294 Antioxidant enzymes superoxide dismutase 1 and 2 (*SOD1* and *SOD2*), glutathione
295 peroxidase 1 and 4 (*GPX1* and *GPX4*), catalase (*CAT*) and nuclear factor erythroid 2-related
296 factor 2 (*NRF2*) gene expression were investigated to understand if the antioxidant system is
297 effective on balancing ROS increase. SOD belongs to the enzymes class that catalyze
298 superoxide anion hydrolysis into oxygen and hydrogen peroxide; CAT and GPX catalyzes
299 hydrogen peroxide reduction in water, and together stabilizes the injured cell [21, 44]; and
300 NRF2 are involved in the transcriptional activation of genes that increases the expression of
301 antioxidant enzymes [29]. Every antioxidant enzymes prevents the accumulation of ROS on
302 blastocyst cells. Our data showed lower expression on *SOD2* and *CAT* genes in all HS groups
303 compared to control. Conversely, *GPX1* was higher on HS groups, except in IVM HS
304 blastocysts, which may be due to use of more maternally stored antioxidants by the embryo
305 under oxidative stress to prevent the deleterious effects of ROS. The *NRF2*, *SOD1* and *GPX4*
306 mRNA expressions did not differ in blastocysts. Our data show the imbalance between ROS
307 production and cell antioxidant capacity, characterizing oxidative stress. Thus, bovine

308 embryos submitted to HS present lower amounts of IFNT at the same time oxidative stress
309 increases, and the antioxidant system is not capable to reduce ROS.

310 Due to the low antioxidant action, we asked whether HSPs would be modulated on
311 stressed embryos. HSPs belong to the protein class named chaperones and are induced as a
312 defense mechanism in any type of cellular stress [46], including HS [21]. We assessed the
313 *HSP90AA1* (HSP90) and *HSPA1A* (HSP70) gene expression. In this study, HS treatments
314 modify only the *HSPA1A* (HSP70) expression, increased in all HS blastocysts. Dairy cows
315 under heat stress presented higher activity on HSP70 and his transcription factor HSF, but not
316 HSP90 [47]. Although stressed cells can trigger mechanisms of thermotolerance mediated by
317 the HSPs [48], we demonstrated here that the embryo capacity to express proteins responsible
318 for cellular protection mechanisms for HS is not sufficient to prevent the reduction of IFNT
319 produced by the trophoblastic cells.

320 A recent study conducted to investigate the interaction between apoptosis and HSPs
321 expression in response to HS in chicken myocardial cells demonstrated that HS caused a
322 decrease on cellular viability and an increase in apoptosis [49]. Therefore, we question
323 whether cellular survival would be affected by HS, examining *AKT* and *XIAP* genes
324 expressions. The *AKT* is important for cell growth, survival, proliferation, angiogenesis,
325 metabolism, and migration [49], whereas *XIAP* acts as an anti-apoptotic gene to inhibit the
326 activity of some caspases and other apoptotic proteins [50]. Khan, et al. [41] observed that HS
327 for 20h caused a decrease in embryo quality and increased the number of apoptotic cells in
328 blastocysts. However, we observed here that *AKT* and *XIAP* expressions were not affected by
329 HS. Therefore, these results suggest that blastocyst viability is not affected when HS is
330 maintained for 6h on oocyte maturation, fertilization and/ zygote in the first day of culture.

331 In summary, this study supports the idea that IFNT is under influence of heat stress as
332 early as oocyte maturation, fertilization and/or zygote first day of culture. Initially, HS

333 decreases the cleavage and blastocyst rates, and IFNT expression without altering total
334 blastocyst cell number and specific trophoblastic cells. Antioxidant enzymes do not avoid
335 ROS production, leading to oxidative stress. Although, HS may increase HSP, it was not
336 efficient in controlling IFNT decrease. In conclusion, HS during oocyte maturation,
337 fertilization and/or early embryonic development affects subsequent embryo developmental,
338 compromising IFNT and increasing oxidative and cellular stresses. Our data support the
339 hypothesis that HS prior to embryonic genome activation decreases IFNT expression on Day
340 7 embryos, simultaneous to ROS-mediated stress response pathway.

341

342 **Declaration of interest**

343 The authors have nothing to declare.

344

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349

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352

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501 **Figure legends**

502 **Figure 1:** Experimental design scheme, showing the temperature curve of all groups during *in*
503 *vitro* embryo production (IVP). To mimic HS conditions, the temperature was gradually
504 raised in a laboratory incubator from 38.5°C to 40.5°C, maintained for 6h and slowly
505 decreased back to 38.5°C during different periods of IVP. The IVP conditions were followed
506 as standard protocol, unless indicated the temperature increase (HS = heat stress) according to
507 the following groups (n = 50 oocytes/group): (A) Control: cells were maintained at 38.5°C
508 during all the IVP stages; (B) IVM HS: oocytes matured under heat stress conditions; (C) IVF
509 HS: oocytes fertilized under heat stress conditions; (D) IVC HS: zygotes cultured in the first
510 day under heat stress conditions; (E) IVM+IVF+IVC HS: embryos submitted to heat stress
511 during all the previous stages. All treatments were completed in seven replicates.

512

513 **Figure 2:** Percentage of cleavage (A) and blastocyst (B) rates of bovine embryos produced *in*
514 *vitro* (n = 50 oocytes/group) under heat stress (HS) during oocyte maturation (IVM HS),
515 fertilization (IVF HS), first day of embryo culture (IVC HS) or combining both treatments
516 (IVM+IVF+IVC HS). Bars represent the group mean \pm SEM for seven replicates. Different
517 letters indicate statistical significance among groups (P<0.05).

518

519 **Figure 3:** IFNT production from all groups. (A) Representative image of bovine embryos
520 immunofluorescence detection of IFNT. Nuclei were counterstained with DAPI. IFNT is
521 located in embryo cytoplasm. Scale bars 50 μ m. (B) Relative mRNA expression of *IFNT2* (n =
522 5 embryos/group) on bovine embryos from all groups. (C) IFNT protein quantification
523 (arbitrary units) on bovine embryos from all groups. Bars represent the group mean \pm SEM
524 for seven replicates. Different letters indicate statistical significance among groups (P<0.05).

525

526 **Figure 4:** Determination of number of blastocyst cells and trophoblastic cells of bovine
 527 embryos from all groups. (A) Representative image of bovine embryos immunofluorescence
 528 detection of CDX2. Nuclei were counterstained with DAPI. CDX2 is located in embryo
 529 nuclei. Scale bars 50µm. (B) Total number of cells counted on bovine embryos. (C)
 530 Trophoblastic cells counted on bovine embryos. Bars represent the group mean ± SEM for
 531 seven replicates. Different letters indicate statistical significance among groups (P<0.05).

532

533 **Figure 5:** (A) Results of spectrophotometry assay (arbitrary units) using H2DCFDA as an
 534 indicator for ROS production in bovine embryos (day 7; n = 15 embryos/group) produced *in*
 535 *vitro* under heat stress (HS) during oocyte maturation (IVM HS), fertilization (IVF HS), first
 536 day of embryo culture (IVC HS) or combining both treatments (IVM+IVF+IVC HS). (B-G)
 537 Relative mRNA expression of genes associated with oxidative stress on bovine embryos
 538 produced *in vitro* (n = 5 embryos/group) under heat stress (HS) during oocyte maturation
 539 (IVM HS), fertilization (IVF HS), first day of embryo culture (IVC HS) or combining both
 540 treatments (IVM+IVF+IVC HS). (B) *SOD1*, (C) *SOD2*, (D) *GPX1*, (E) *GPX4*, (F) *CAT* and
 541 (G) *NRF2*. Bars represent the group mean ± SEM for seven replicates. Different letters
 542 indicate statistical significance (P<0.05) among groups.

543

544 **Figure 6:** Relative mRNA expression of genes associated with cellular survival (A: *AKT* and
 545 B: *XIAP*) and cellular stress (C: *HSP90AA1* and D: *HSPA1A*) on bovine embryos produced *in*
 546 *vitro* (n = 5 embryos/group) under heat stress (HS) during oocyte maturation (IVM HS),
 547 fertilization (IVF HS), first day of embryo culture (IVC HS) or combining both treatments
 548 (IVM+IVF+IVC HS). Bars represent the group mean ± SEM for seven replicates. Different
 549 letters indicate statistical significance among groups (P<0.05).

550

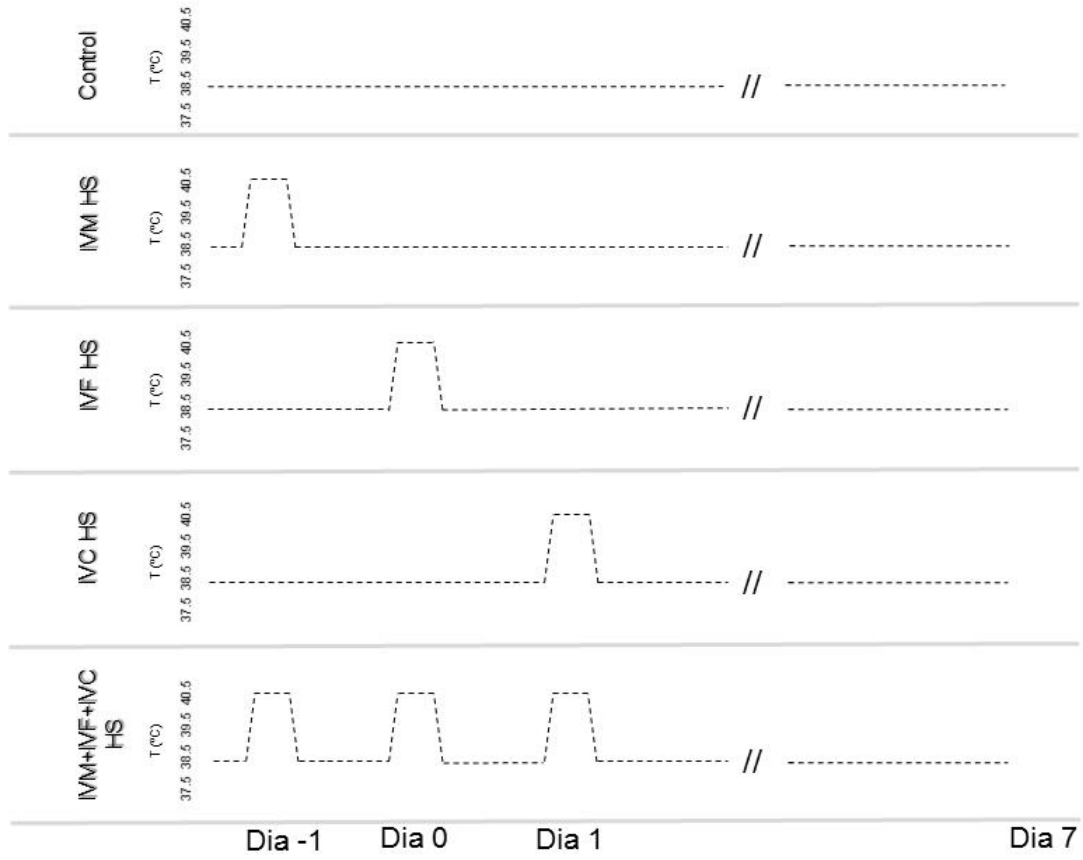
551 **Tables**552 **Table 1.** Primers designed for quantitative real-time PCR analysis.

Target	Accession number	Primer sequence
<i>IFNT2</i>	NM_001015511.3	F: TCTGAGGACCACATGCTAGG R: GATCCTTCTGGAGCTGGTTG
<i>HSP90AA1</i>	NM_001012670.2	F: AGTCCTTCAGCCAAGATGCC R: GACAGCCAAGTGATCCTCCC
<i>HSPA1A</i>	NM_203322.3	F: CTTCAACATGAAGAGCGCCG R: TGATGGGGTTACACACCTGC
<i>SOD1</i>	NM_174615.2	F: AAGGCCGTCTGCGTGCGAA R: CAGGTCACCAACATGCCTCT
<i>SOD2</i>	NM_201527.2	F: CCCATGAAGCCTTTCTAATCCTG R: TTCAGAGGCGCTACTATTCCTTC
<i>GPX1</i>	NM_174076.3	F: TTGGGCATCAGGAAAACGCC R: TTCTCGCCATTCACCTCGCA
<i>GPX4</i>	NM_001346430.1	F: CGCCGAGTGTGGTTTAC R: AGGTCCTTCTCTATCACCAG
<i>CAT</i>	NM_001035386.2	F: GTTCGCTTCTCCACTGTT R: GGCCATAGTCAGGATCTT
<i>NRF2</i>	NM_001011678.2	F: AACTACTCCCAGGTTGCCAC R: AGTGACTGAAACGTAGCCGAAG
<i>AKT</i>	NM_173986.2	F: GATTCTTCGCCAGCATCGTG R: GGCCGTGAACTCCTCATCAA
<i>XIAP</i>	NM_001205592.1	F: GAAGCACGGATCATTACATTTGG R: CCTTCACCTAAAGCATAAAATCCAG
<i>GAPDH</i>	NM_001034034.2	F: GATTGTCAGCAATGCCTCCT R: GGTCCATAAGTCCCTCCACGA
<i>RPS18</i>	NM_001033614.2	F: CCTTCCGCGAGGATCCATTG R: CGCTCCCAAGATCCAACACTAC

553 F: Forward; R: Reverse.

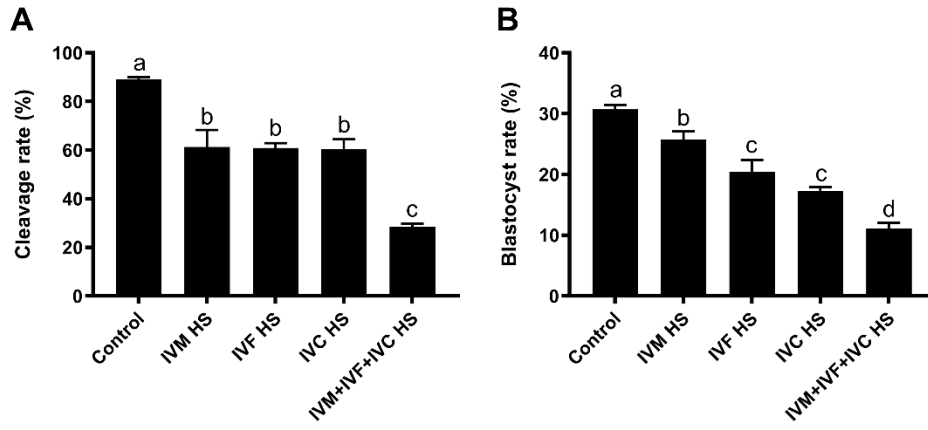
554 **Figures**

555 **Figure 1:**



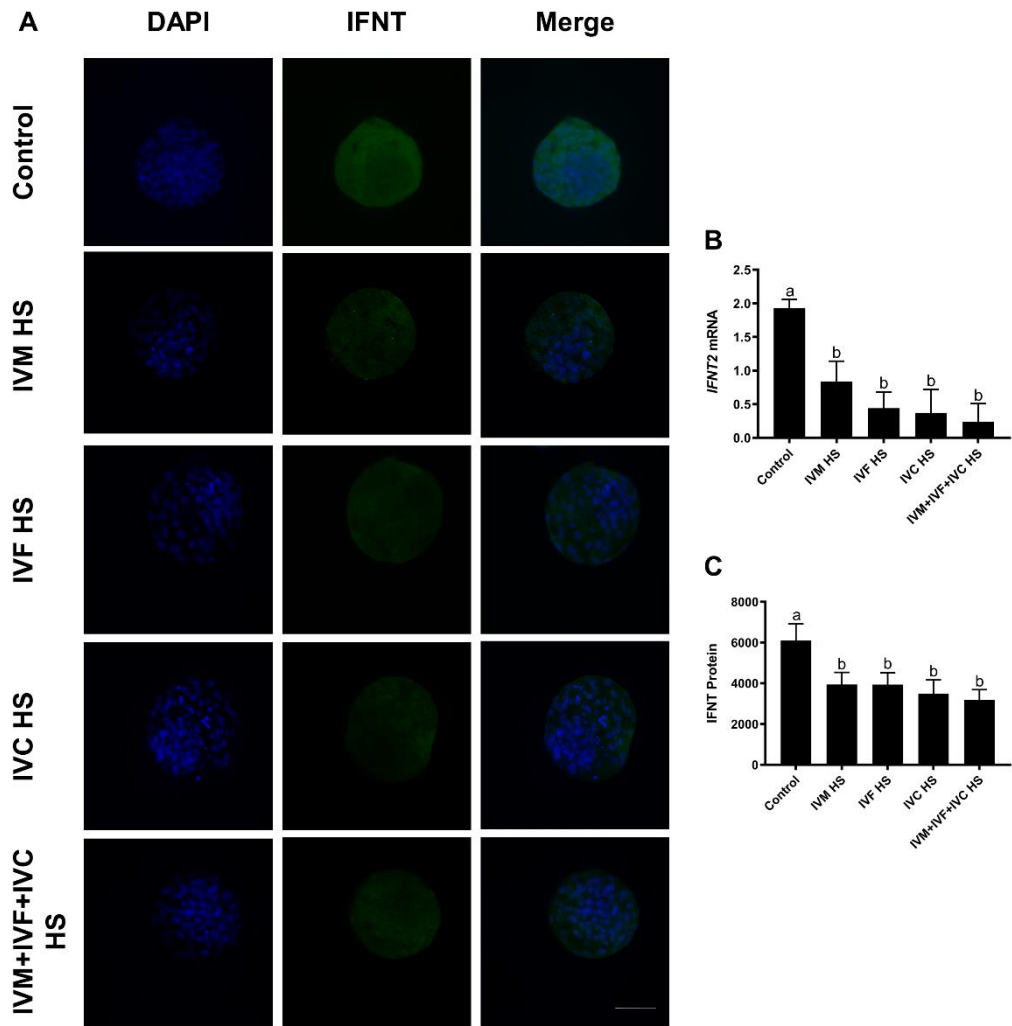
556

557 **Figure 2:**

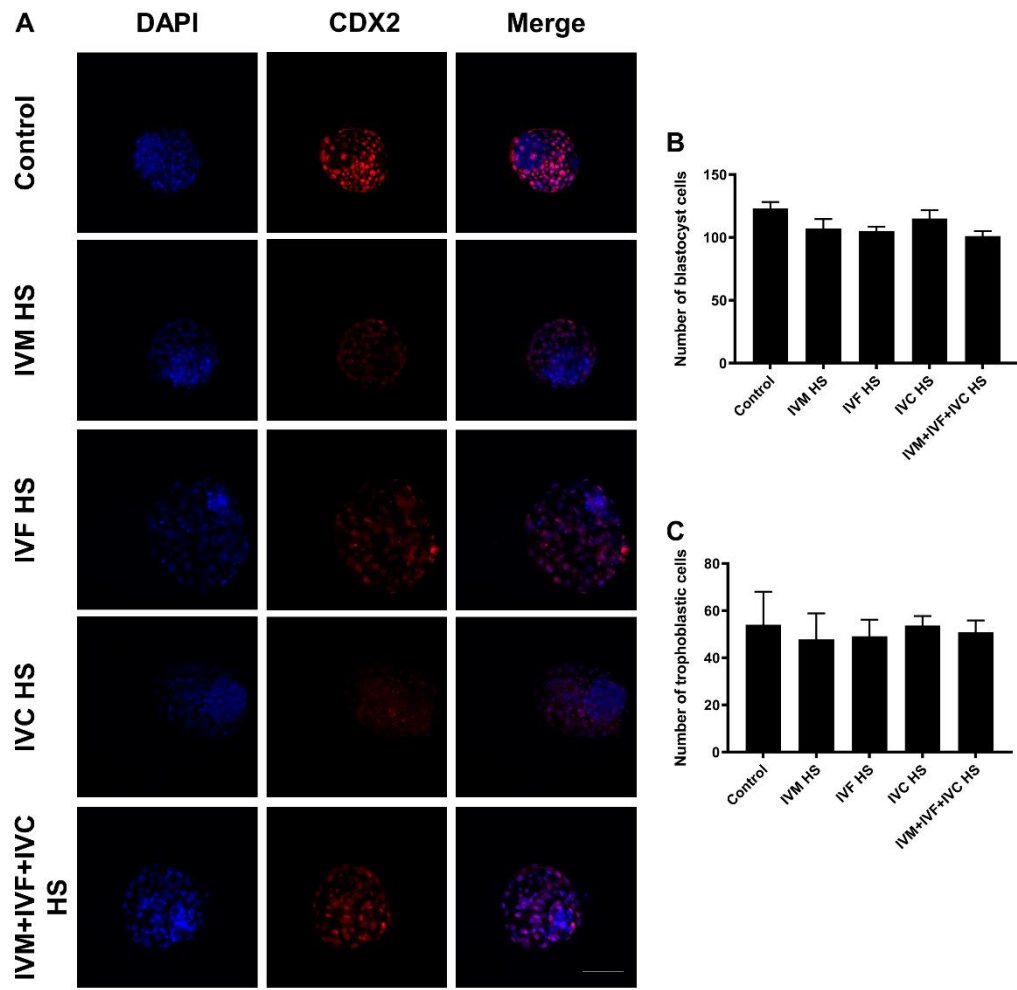


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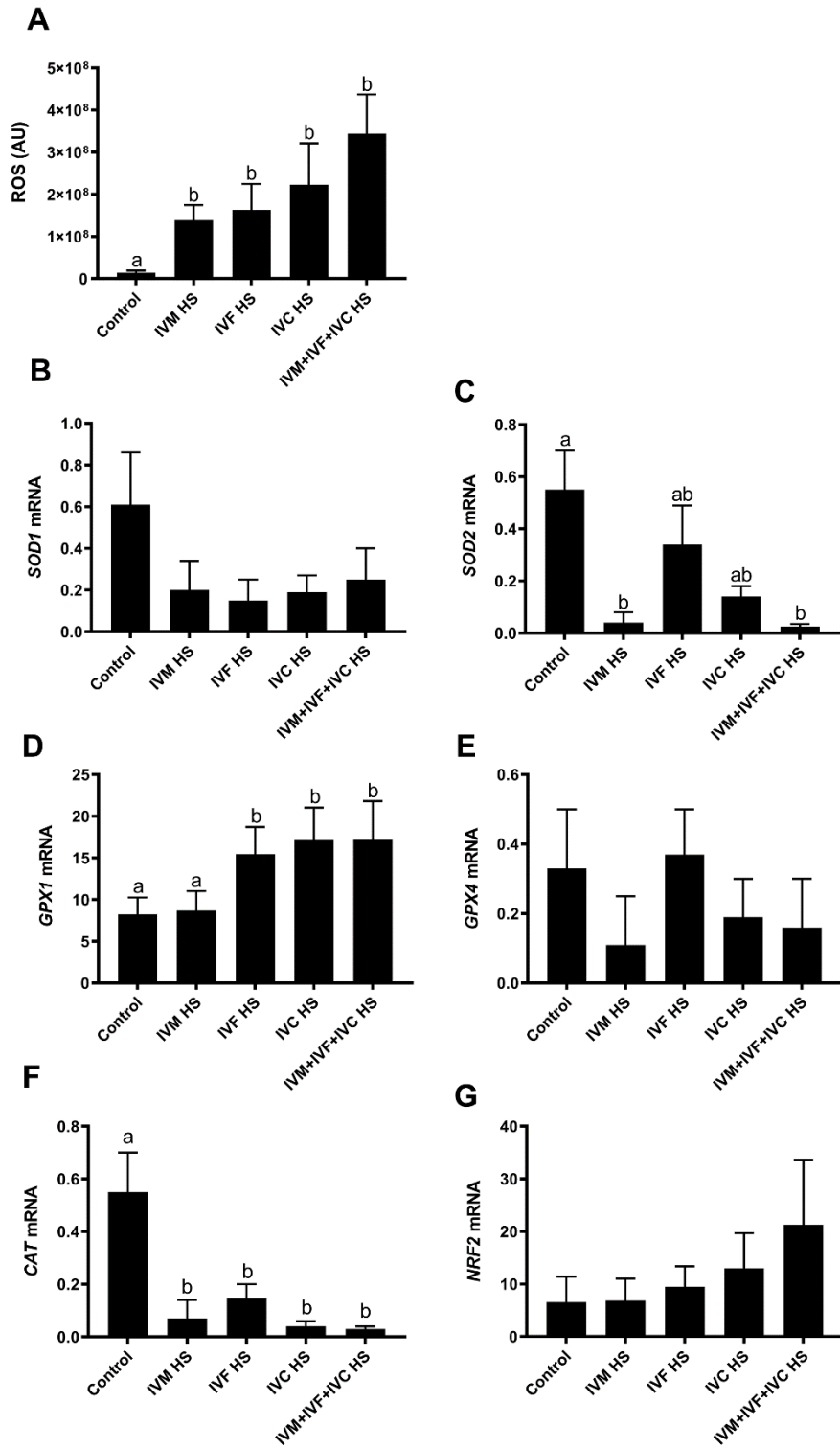
559 **Figure 3:**

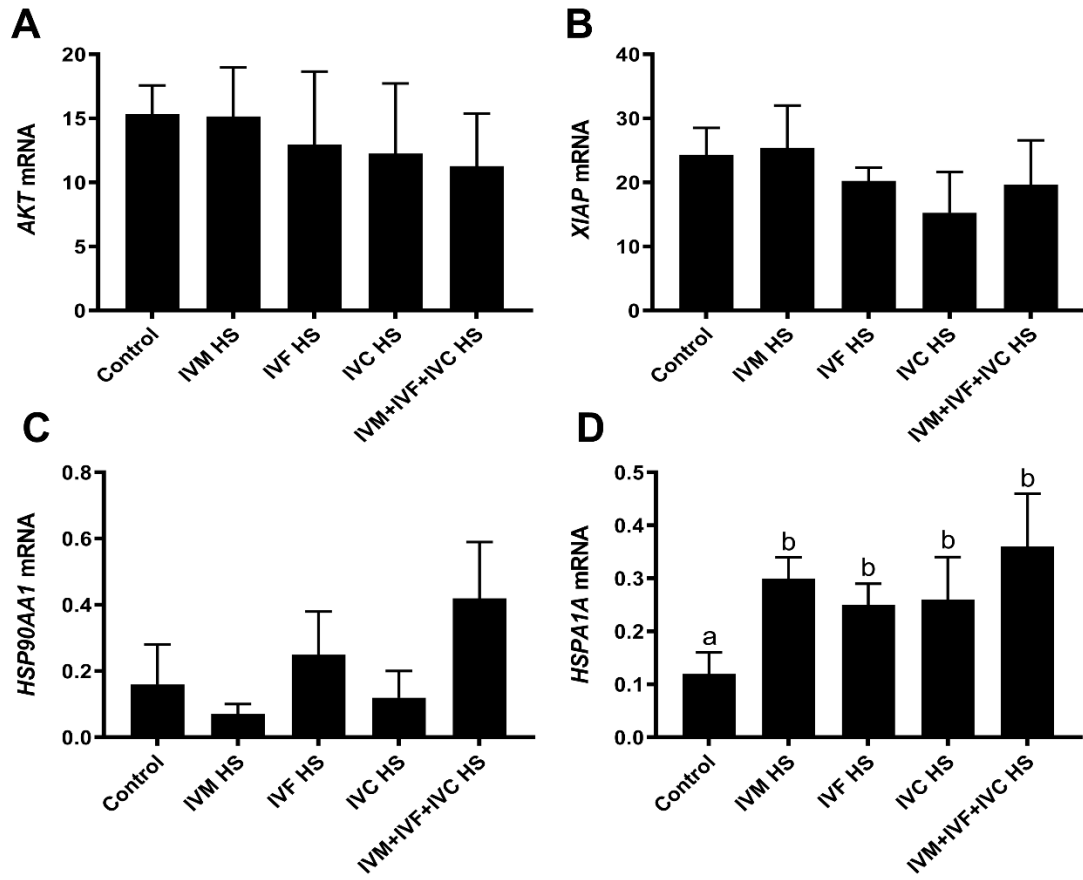


561 **Figure 4:**



562

563 **Figure 5:**

565 **Figure 6:**

566

4 CONCLUSÃO

O estresse térmico influencia na reprodução de bovinos em vários aspectos. Com base nos resultados obtidos, é possível concluir que o estresse térmico durante os primeiros dias de desenvolvimento embrionário impacta a sua viabilidade, comprovado pela diminuição das taxas de desenvolvimento embrionário e produção de IFNT, bem como o aumento do estresse oxidativo. Não houve alteração na expressão de genes de sobrevivência celular, o que pode ser correlacionado com o aumento da expressão gênica da HSP70, que tem como característica corrigir proteínas danificadas por estresse. Contudo, essa atuação não influenciou na produção de IFNT, que teve diminuição em todos os grupos submetidos ao estresse térmico. Comprovou-se que a diminuição da produção de IFNT não ocorreu devido à diminuição de suas células produtoras. Portanto, nossos resultados sugerem que a diminuição do IFNT é mediada pelo aumento do estresse oxidativo em embriões bovinos submetidos ao estresse térmico.

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