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ANDRÉ LUCIO FONTANA GOETTEN

EFEITOS DOS ALCALÓIDES PIRROLIZIDÍNICOS PRESENTES EM *Senecio brasiliensis* SOBRE OS GENES DA CASCATA PERIOVULATÓRIA EM CÉLULAS DA GRANULOSA E SOBRE A PRODUÇÃO DE EMBRIÕES BOVINOS IN VITRO

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Orientador: Prof. Dr. Alceu Mezzalira
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Lages, 28 de abril de 2022

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“Explicar toda a natureza é uma tarefa difícil demais para qualquer homem ou qualquer época. É muito melhor fazer um pouco com certeza e deixar o resto para outros que vêm depois de você.” (ISAAC NEWTON)

RESUMO

As plantas contendo alcaloides pirrolizidínicos (APs) são amplamente distribuídas no mundo e provavelmente são as que mais causam intoxicação em animais de produção, animais silvestres e em humanos. No sul do Brasil o gênero *Senecio*, especialmente o *S. brasiliensis*, é responsável por mais de 50% das mortes de bovinos intoxicados por plantas. A intoxicação, que causa primariamente dano hepático, depende da biotransformação dos APs pela enzima citocromo P450 presente no fígado, mas que tem isoformas em outros órgãos. O curso da doença pode ser agudo ou crônico dependendo da frequência e da quantidade ingerida. Em vacas com seneciose crônica já foram descritas lesões ovarianas microscópicas semelhantes as lesões hepáticas e, em ovários suínos, uma isoforma de citocromo P450 foi identificada. A hipótese desse trabalho é que concentrações de APs insuficientes para causar seneciose atuam diretamente sobre as células ovarianas e/ou sobre os embriões, afetando a expressão dos genes da cascata ovulatória e o desenvolvimento embrionário inicial em bovinos. Os objetivos desse estudo foram avaliar se concentrações de alcaloides pirrolizidínicos, insuficientes para causar seneciose, atuam diretamente sobre: a) a expressão dos genes da cascata ovulatória nas células da granulosa bovinas, b) na expansão das células do cumulus-oócito e c) no desenvolvimento embrionário inicial em bovinos. Senecionina e seneciofilina, os dois PAs mais prevalentes no *S. brasiliensis*, foram testados nas concentrações de 0,03 µM e 0,3 µM em todos os experimentos. Células da granulosa foram cultivadas utilizando um modelo bem estabelecido no qual respondem ativamente ao LH in vitro. A concentração mais alta de ambos os APs reduziu a expressão de mRNA de *EREG*, *PTGS2* e *CTGF* 12 horas após o tratamento e de *AREG* as 24 horas após o tratamento. As concentrações de 0,03 µM senecionina as 12 horas, e 0,03 µM seneciofilina as 24 horas pós-tratamento também reduziram a expressão de *PTGS2*. Esses resultados sugerem que os APs podem prejudicar a ovulação. Utilizando um sistema de produção in vitro de embriões foram avaliadas: a expansão das células do complexo cumulus-oócito, a produção embrionária e a expressão de mRNA de genes marcadores de qualidade embrionária. A expansão do cumulus não foi impedida por nenhuma das doses de APs testadas. As taxas de clivagem e blastocisto também não diferiram do grupo controle quando os APs foram adicionados ao meio de maturação. Quando o tratamento ocorreu 18-20horas após a inseminação, as taxas de clivagem e de blastocisto foram reduzidas por ambos os APs em todas as concentrações testadas. O risco de um suposto zigoto tratado com 0.03 µM de senecionina ou de seneciofilina chegar ao estágio de blastocisto foi reduzido em 29,7% e 38,5% comparados ao grupo não tratado, respectivamente. Para os grupos tratados com 0.3 µM de senecionina e seneciofilina esse rico foi reduzido em 54,1% e 47,3%, respectivamente. Em blastocistos, a senecifilina reduziu a expressão o gene *TP53BP1* durante a maturação in vitro e aumentou a expressão do gene *RAD51* durante o cultivo in vitro, sugerindo que os APs poderiam determinar a quebra da fita dupla do DNA e a ativação de reparo via recombinação homóloga. A senecionina reduziu a expressão de mRNA de *GLUT3* e *BCL2*, sugerindo que os APs podem prejudicar o metabolismo da glicose e reduzir a atividade antiapoptótica em blastocistos bovinos. Em resumo, o presente estudo demonstra que a senecionina e a senecifilina têm efeitos toxicológicos significativos em células da granulosa bovina, bem como em embriões bovinos produzidos in vitro, sugerindo que os PAs, mesmo em concentrações insuficientes para causar a seneciose, têm o potencial de afetar diretamente a função reprodutiva em bovinos.

Palavras-chave: senecionina, seneciofilina, células da granulosa, ovário, embrião.

ABSTRACT

Pyrrolizidine alkaloids (PAs)-containing plants are widely distributed in the world and are probably the most common poisonous plants affecting livestock, wildlife, and humans. In southern Brazil, the genus *Senecio*, especially *S. brasiliensis*, is responsible for more than 50% of the deaths of cattle poisoned by plants. Intoxication, which mainly causes liver damage, depends on the biotransformation of PAs by the enzyme cytochrome P450, which is present in the liver but has isoforms in other organs. The course of the disease can be acute or chronic, depending on the frequency and amount ingested. In cows with chronic seneciosis, microscopic ovarian lesions similar to liver lesions have already been described, in porcine ovaries, an isoform of cytochrome P450 has been identified. This work hypothesizes that concentrations of PAs insufficient to cause seneciosis act directly on ovarian cells and/or on embryos, affecting the expression of genes of the periovulatory cascade and early embryonic development in cattle. The objectives of this study were to evaluate whether concentrations of pyrrolizidine alkaloids, insufficient to cause seneciosis, act directly on: a) the expression of the genes of the periovulatory cascade in bovine granulosa cells, b) the expansion of cumulus-oocyte cells, and c) early embryonic development in cattle. Senecionine and seneciphylline, the two most prevalent PAs in *S. brasiliensis*, were tested at concentrations of 0.03 µM and 0.3 µM in all experiments. Granulosa cells were cultured using a well-established culture model in which they actively respond to LH. The higher concentration of both PAs reduced the mRNA expression of *EREG*, *PTGS2*, and *CTGF* 12 hours after treatment and *AREG* 24 hours after treatment. Concentrations of 0.03 µM senecionine at 12 hours and 0.03 µM seneciphylline at 24 hours post-treatment also reduced *PTGS2* expression. These results suggest that PAs can impair ovulation. Using the in vitro embryo production system, cumulus cell expansion, embryo production, and mRNA expression of embryonic quality marker genes were evaluated. Cumulus expansion was not impeded by any of the PAs tested. Cleavage and blastocyst rates also did not differ from the control group when PAs were added to the maturation medium. When treatment was added 18-20 hours post-insemination, cleavage and blastocyst rates were reduced by both PAs at all concentrations tested. The risk of a putative zygote treated with 0.03 µM senecionine or seneciphylline reaching the blastocyst stage was reduced by 29.7% and 38.5% compared to the untreated group, respectively. For the groups treated with 0.3 µM of senecionine and seneciphylline, this risk was reduced by 54.1% and 47.3%, respectively. In blastocysts, seneciphylline downregulated the *TP53BP1* gene during in vitro maturation and the *RAD51* gene was upregulated during in vitro culture, suggesting that PAs could determine DNA double-strand breaks and activation of the homolog recombination repair pathway. Senecionine reduced mRNA expression of *GLUT3* and *BCL2*, suggesting that PAs may impair glucose metabolism and reduce antiapoptotic activity in bovine blastocysts. In summary, the present study demonstrates that senecionine and seneciphylline have significant toxicological effects on bovine granulosa cells as well as in vitro produced bovine embryos, suggesting that PAs, even at concentrations insufficient to cause seneciosis, have the potential to directly affect the reproductive function in cattle.

Keywords: senecionine, seneciphylline, granulosa cells, ovary, embryo.

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LISTA DE ABREVIATURAS E SIGLAS

AP	Alcaloides pirrolizidínicos
AREG	Anfiregulina
ATM	*Ataxia-telangiectasia mutated*
ATR	*ATM and RAD3 related*
BMP15	Proteína Morfogenética Óssea 15
BRCA1	*Breast Cancer 1 DNA repair associated*
BRCA2	*Breast Cancer 2 DNA repair associated*
BSA	Albumina Sérica Bovina
cAMP / AMPc	Monofosfato de Adenosina Cíclico
CCO / COC	Complexo cumulus-oócito
cGMP / GMPc	Monofosfato de Guanosina Cíclico
CI	*Intervalo de confiança*
CTIP	Proteína *CtBP-interacting*
DDR	Resposta ao dano no DNA
DNAPK	Proteína Quinase dependente de DNA
DNAPK-cs	Proteína Quinase dependente de DNA, subunidade catalítica
DSB	Quebra de fita dupla no DNA
EGF	Fator de Crescimento Epidermal
EGF-Like	Fator de Crescimento semelhante ao EGF
EGFR	Receptor de EGF
EREG	Epiregulina
FCS	Soro Fetal Bovino
FGF	Fatores de Crescimento Fibroblásticos
FSH	Hormônio Folículo Estimulante
GADD45B	*Growth Arrest and DNA Damage Inducible Protein, Beta*
GDF9	Fator de Diferenciação de Crescimento 9

H2AFZ	Família de histonas H2A, membro Z
HAS2	Hialurona Sintetase 2
HPI	Horas pós inseminação
HR	Recombinação Homóloga
IVM	Maturação *in vitro*
IVP	Produção de embriões *in vitro*
LH	Hormônio Luteinizante
LHR	Receptor de Hormônio Luteinizante
LIG4	DNA ligase IV
MMEJ	União Terminal Mediada por Micro-homologia
MRN	Complexo Mre11-Rad50-Nbs1
NHEJ	União Terminal Não Homóloga
PARP	Poli(ADP-ribose) polimerase
PDE	Fosfodiesterase
PGE2	Prostaglandina E2
PIKK	Proteína quinase semelhante a fosfatidilinositol-3 quinase
PPIB	Ciclofilina B
PTGS2	Prostaglandina Sintetase 2
PTIP	*PAX-interacting protein 1*
PTX3	Pentraxina 3
PV	Peso Vivo
RAD50	*DNA repair protein RAD50*
RAD51	*DNA repair protein RAD51*
RAD52	*DNA repair protein RAD52*
RIF1	*Rap1-interacting factor 1*
RPA	Proteína de Replicação A
S.E.M.	Erro padrão da média
SSA	Anelamento de Fitas Simples

SSB	Quebra de fita simples no DNA
ssDNA	Fita Simples de DNA
TBP	Proteína de Ligação TATA
TNAFAIP6	Proteína Indutora do Fator de Necrose Tumoral 6
TP53BP1	Proteína de Ligação de Proteína Tumoral P53 1
XRCC4	Proteína 4 de complementação cruzada de reparo de raios X
XRCC5	Proteína 5 de complementação cruzada de reparo de raios-X
XRCC6	Proteína 6 de complementação cruzada de reparo de raios-X
YAP	*Yes-Associated Protein*

LISTA DE SÍMBOLOS

%	Porcento
ºC	Graus Celsius
H	Horas
IU	Unidades Internacionais
mL	Mililitro
µg	Micrograma
Mm	Milímetro
µL	Microlitro
CO2	Dióxido de Carbono
Ng	Nanograma
µM	Micromolar

SUMÁRIO

1 INTRODUÇÃO

A intoxicação por plantas ainda hoje é uma das causas mais comuns de morte de animais de produção adultos, determinando expressivas perdas econômicas, diretas e indiretas, no sistema produtivo pecuário. Contudo, a avaliação precisa do prejuízo decorrente das intoxicações por plantas não é tarefa fácil devido a escassez de informações confiáveis já que nem todos os surtos são notificados e, principalmente, por conta da falta de dados a respeito dos casos nos quais a intoxicação não provoca a morte imediata, mas determina queda no desempenho dos animais.

Falhas de manejo nas áreas de pastagem ou de lavouras favorecem o aparecimento de plantas invasoras, muitas das quais tem potencial tóxico para várias espécies animais, inclusive o homem. Das plantas invasoras do sul do Brasil, aquelas pertencentes ao gênero *Senecio* spp. são as que determinam as maiores perdas econômicas na criação de bovinos, dentre elas o *Senecio brasiliensis* tem lugar de destaque. Por ser altamente resistente e com grande capacidade de adaptação às condições edafoclimáticas de onde se estabelece, o *S. brasiliensis* é uma planta amplamente difundida pela América do Sul e em especial, na região sul do Brasil (PEDROSO et al., 2007; RISSI et al., 2007; GRECCO et al., 2010). Nesta região a maioria dos bovinos é criada em sistemas extensivos o que facilita o aparecimento dos quadros de intoxicação por *Senecio* spp., especialmente em épocas em que a pastagem é escassa. Entretanto a seneciose, intoxicação por plantas do gênero *Senecio*, também tem sido descrita em sistemas intensivos e semi-intensivos quando os animais ingerem a planta involuntariamente seja através de volumoso contaminado ou pelo hábito de pastejo particular dos bovinos que dificulta a seleção do alimento durante o bocado (KARAM et al., 2004; BIFFI et al., 2019).

Os princípios ativos tóxicos associados ao *S. brasiliensis* pertencem ao grupo dos alcaloides pirrolizidínicos (APs) que, quando biotransformados em pirróis pelas enzimas do citocromo P-450, são capazes alterar a fisiologia celular, especialmente dos hepatócitos, já que causam lesões irreversíveis nessas células. Já se sabe que uma parte dos pirróis produzidos no fígado pode atingir outros órgãos através da circulação sistêmica e causar lesão extra-hepática. As lesões extra-hepáticas também podem ocorrer pela produção de pirróis localmente, já que os complexos do citocromo P-450 são encontrados em outros órgãos (PRAKASH et al., 1999).

A redução das taxas reprodutivas de fêmeas bovinas acometidas por seneciose sempre foi relacionada ao declínio do status nutricional em decorrência da lesão hepática nesses animais. Contudo, há indícios de atuação dos APs diretamente em células ovarianas já que ovários de suínos são capazes de expressar e transcrever a enzima citocromo P450 e suas isoformas e ovários de bovinos com seneciose crônica apresentaram, em células luteais, lesões semelhantes aquelas presentes nos hepatócitos. Também há relatos de que a exposição pré-natal a APs pode prejudicar o desenvolvimento físico e comportamental em ratos (LEIGHTON et al., 1995; SANDINI et al., 2014; KRABBE et al., 2015). Quando associamos problemas de infertilidade sem causas aparentes em bovinos a essas evidências de atuação dos APs sobre órgãos reprodutivos, surge a hipótese de que, mesmo em concentrações insuficientes para causar a seneciose crônica, os APs atuam diretamente sobre as células ovarianas e/ou sobre os embriões, afetando a expressão dos genes da cascata ovulatória e o desenvolvimento embrionário inicial em bovinos. Os objetivos do primeiro estudo foram avaliar se concentrações de alcaloides pirrolizidínicos, insuficientes para causar seneciose, atuam diretamente sobre: a) a expressão dos genes da cascata ovulatória nas células da granulosa bovinas, b) na expansão das células do complexo cumulus-oócito e c) no desenvolvimento embrionário inicial em bovinos.

Essa tese contém um segundo artigo que trata do padrão da expressão de genes envolvidos no reparo da fita dupla do DNA em células do cumulus bovinas cultivadas sob influência de soro fetal bovino (FCS) ou de albumina sérica bovina (BSA), cujos resultados foram utilizados para a definição da inclusão de FCS durante a maturação in vitro nos experimentos que testaram os efeitos dos APs na produção in vitro de embriões.

Apesar de seu uso generalizado, a eficácia da maturação in vitro (IVM) é menor que a observada em oócitos maturados in vivo. Portanto, informações mais detalhadas sobre as vias moleculares que controlam a expansão das células do cumulus e a maturação do oócito são necessárias para melhorar os protocolos de IVM (CAIXETA et al., 2013a; DE VOS et al.,2021). A regulação da maturação do oócito e da expansão das células do cumulus depende da comunicação entre esses dois compartimentos do complexo cumulus-oócito (CCO) e envolve trocas bidirecionais por meio de junções comunicantes e sinalização parácrina. As junções comunicantes dos CCOs permitem a troca de pequenas moléculas que dão suporte nutricional e regulam a progressão meiótica do oócito o qual, por sua vez, secreta fatores que modulam a proliferação, expressão gênica e função das células do cumulus (TANGHE et al., 2002; SUGIMURA; RICHANI; GILCHRIST, 2018).

As condições de cultura durante a produção de embriões in vitro (PIV) podem afetar os padrões de expressão gênica e, consequentemente, o potencial de desenvolvimento embrionário, indicando que o ambiente adverso gerado pelas condições in vitro prejudica a comunicação bidirecional dos CCOs. Assim, diversas alterações nas condições ou nos meios de cultura foram propostas na expectativa de minimizar essas adversidades. A escolha da fonte de suplementação proteica no meio de maturação, por exemplo, tem efeitos profundos nas taxas de desenvolvimento embrionário, justificando o uso de soro durante a IVM na maioria dos sistemas. No entanto, o uso de soro apresenta desvantagens científicas, sanitárias e éticas determinando a busca de outras fontes proteicas que possam substituí-lo e a albumina sérica bovina é a fonte proteica mais utilizada para reposição sérica. Infelizmente, a ausência de soro no meio IVM resulta em redução da taxa de blastocisto, menor grau de expansão do cumulus e maior fragmentação de DNA nas células do cumulus (LONERGAN et al., 2003; VAN DER VALK et al., 2018).

Entre os diferentes tipos de danos ao DNA induzidos por genotoxinas ambientais, as quebras de fita dupla do DNA (DSB) são o tipo de dano mais deletério. Como CCOs maturados in vitro em meio sem soro apresentam alta taxa de apoptose e fragmentação de DNA nas células do cumulus os mecanismos de reparo das DSB podem ser comprometidos nessas condições de cultura celular. Esse reparo é coordenado principalmente por duas vias moleculares: recombinação homóloga (HR) e união terminal não homóloga (NHEJ). Apesar da presença de genes codificadores de mRNA implicados nesses processos ter sido demonstrada em oócitos de várias espécies, incluindo os bovinos, e em células do cumulus em humanos e ratos, não há informações sobre a expressão de genes envolvidos nas vias HR e NHEJ nas células do cumulus em bovinos (PANIER; BOULTON, 2014; SUN et al., 2018).

Considerando que a interação bilateral coordenada entre as células do cumulus e o oócito é essencial para a aquisição da plena capacidade de ambos os tipos celulares em promover a embriogênese inicial, as informações armazenadas nas células do cumulus podem ser marcadores diretos para o futuro desenvolvimento do oócito. Portanto, os padrões de expressão gênica em células do cumulus biopsiadas podem servir como marcadores não invasivos para avaliar a maturação do oócito. É por isso que o perfil de expressão de genes de reparo de DNA em células do cumulus bovino é uma questão intrigante que pode fornecer conhecimento para melhorar a eficiência da IVM. Assim, os objetivos desse segundo estudo foram: (1) caracterizar o perfil de expressão de transcritos de genes selecionados envolvidos nos mecanismos de reparo das quebras de fita dupla de DNA em células do cumulus bovinas

obtidas a partir de COCs maturados in vitro; e (2) investigar se a suplementação do meio de maturação in vitro com BSA ou FCS afeta a expressão da transcrição desses genes.

2 REVISÃO DE LITERATURA

2.1 *Senecio brasiliensis* E SEUS EFEITOS NOS BOVINOS

Já foram descritas mais de 1200 espécies do gênero *Senecio* espalhadas por praticamente todo o planeta, exceto nas regiões polares e na região Amazônica. De todas as espécies identificadas apenas cerca de 25 a 30 demonstraram ser tóxicas para animais domésticos (TOKARNIA; DOBEREINER; PEIXOTO, 2012). No Brasil, o número de espécies do gênero *Senecio* é divergente, variando entre 67 e 128 de acordo com o critério adotado para a classificação (HIND, 1993; MÉNDEZ; RIET-CORREA, 2008). *Senecio brasiliensis* é a espécie mais abundante no Brasil, amplamente distribuída no Sul e em pequenas áreas altas e frias da região Sudeste do país (PEDROSO et al., 2007; RISSI et al., 2007; GRECCO et al., 2010). Esta planta apresenta características adaptativas tais como a dispersão de sementes pelo vento, a independência de polinizador específico, a competição por alelopatia e a capacidade de crescimento mesmo em solos inférteis as quais lhe conferem grande facilidade de propagação, principalmente sob condições climáticas ideais (DANA et al., 2021).

S. brasiliensis é uma planta invasora de pastagens, mas de pouca palatabilidade. Portanto, é consumida naturalmente pelos bovinos somente sob determinadas condições, normalmente associadas à escassez de alimentos (KARAM et al., 2004; BARROS et al., 2007; MÉNDEZ; RIET-CORREA, 2008; TOKARNIA; DOBEREINER; PEIXOTO, 2012). A ingestão ocorre principalmente a partir da segunda metade do outono até o final do inverno, quando diminui muito a disponibilidade de pastagem (BASILE et al., 2005; BARROS et al., 2007). Contudo, sob influência de precipitação, umidade do solo e cobertura vegetal favoráveis à emergência de espécies de *Senecio*, o seu desenvolvimento pode ocorrer em qualquer época do ano e, consequentemente, a ingestão e a intoxicação podem se manifestar durante todo o ano (KARAM et al., 2004). A superlotação de bovinos e a alta infestação de *Senecio* spp. nas pastagens favorecem a ingestão da planta e, se plantas novas estão estreitamente associadas ao capim, o perigo de ingestão pelos bovinos é ainda maior em função da pouca seletividade de pastejo nessa espécie (KARAM et al., 2004; RISSI et al., 2007; GRECCO et al., 2010; GIARETTA et al., 2014). Os animais também podem ser

intoxicados pela ingestão involuntária da planta quando são alimentados com feno e silagem contaminados (BASILE et al., 2005; BIFFI et al., 2019).

Alguns surtos de intoxicação por *Senecio* spp. foram descritos em bovinos jovens (PRAKASH et al., 1999; BASILE et al., 2005) mas, em geral, a Seneciose é observada em animais adultos (CHEEKE, 1988) por ingerirem, ao longo do tempo, maior quantidade de *Senecio* spp. já que permanecem por um maior tempo na propriedade (GORDON; COLEMAN; GRISHAM, 2000; GIARETTA et al., 2014). A morbidade é variável, entre 1 e 30%, e a letalidade é de praticamente 100% (GORDON; COLEMAN; GRISHAM, 2000).

O princípio tóxico presente nas espécies de *Senecio* pertence ao grupo dos alcaloides pirrolizidínicos cujos representantes identificados em plantas de *S. brasiliensis* são senecionina, seneciofilina, integerrimina, retrorsina, usaramina e jacobina (HARTMANN; WITTE, 1995; TRIGO et al., 2003). Destes, a senecionina e a seneciofilina são os mais prevalentes (ADAMS; GIANTURCO, 1956). Quimicamente os APs são ésteres de aminoálcoois com um núcleo pirrolizidínico (necina) e ácidos alifáticos (ácidos nécicos). A característica que determina a capacidade de um AP ser tóxico é a presença de dupla ligação entre os carbonos C1 e C2 da necina, já que aqueles que possuem a necina saturada não são tóxicos aos mamíferos (SILVA; BOLZAN; HEINZMANN, 2006).

O teor de APs varia entre as diferentes espécies de plantas e, embora sejam sintetizados nas raízes, existe variação entre as partes de uma mesma planta. O conteúdo de APs em espécies do gênero *Senecio* spp. varia muito durante o ciclo de crescimento da planta e de ano para ano (SANDINI; SAYURI; BERTO, 2013). Em plantas de *S. brasiliensis* o teor de APs apresenta variação sazonal, atingindo a máxima concentração nos meses de inverno (KARAM et al., 2004).

APs são compostos estáveis que, por si só, não apresentam toxicidade até que sejam biotransformados (BARROS et al., 2007). Após serem absorvidos no intestino, os APs são transportados até o fígado onde são primariamente metabolizados pelas enzimas do sistema citocromo P-450, originando pirróis (BARROS et al., 2007; TOKARNIA; DOBEREINER; PEIXOTO, 2012). Os pirróis são compostos altamente tóxicos, reativos e alquilantes que se ligam facilmente às moléculas proteicas e de ácidos nucleicos. No fígado atuam inibindo a mitose, promovem necrose e redução do número de hepatócitos, dando início ao quadro clínico e a morte devido à disfunção hepática (PRAKASH et al., 1999). Foi proposto que a inibição da mitose poderia ser a causa da megalocitose que precede a necrose de hepatócitos nos casos de seneciose. A inibição da citocinese sem a interrupção da síntese de DNA no

núcleo dessas células são a base desse mecanismo (SKILLETER; MATTOCKS; NEAL, 1988). Contudo, Prakash et al. (1999) sugeriram que essa megolocitose é causada por alterações no ciclo celular devido a danos no DNA dos hepatócitos.

Os efeitos hepatotóxicos dos pirróis são progressivos e irreversíveis (CHEEKE, 1988), porém quando os animais ingerem pequenas quantidades diárias de *S. brasiliensis* durante um tempo prolongado, um período de latência de várias semanas e até meses pode ocorrer até que sejam observados os primeiros sinais clínicos (KARAM et al., 2002; BASILE et al., 2005), simulando um efeito cumulativo desses xenobióticos (TOKARNIA; DOBEREINER, 1984). Quando o diagnóstico de intoxicação por *Senecio* spp. é confirmado em um rebanho bovino, provavelmente vários animais já estejam subclinicamente afetados, isto é, apresentam lesões hepáticas, mas ainda sem apresentar sinais clínicos. Como as lesões causadas pela planta são progressivas, as mortes desses bovinos subclinicamente afetados podem ocorrer bastante tempo após a ingestão da planta (MOLYNEUX; JOHNSON; STUART, 1988; TRIGO et al., 2003; CHENG et al., 2011). Existem relatos de animais que tiveram diagnóstico de lesão hepática decorrente da ingestão de *S. brasiliensis* e que depois de transcorridos mais de dois anos, tempo suficiente para duas gestações de uma fêmea bovina, não haviam sequer apresentado sinais clínicos (GUTIÉRREZ; CAMPBELL; WEBB, 1997), porém, o desempenho produtivo e reprodutivo desses animais poderia estar comprometido nesse período pois existe possibilidade de toxidade fetal após ingestão materna de *Senecio* spp. (MOLYNEUX et al., 2011).

Tanto a forma aguda quanto a forma crônica de intoxicação por *S. brasiliensis* em bovinos foram demonstradas experimentalmente. Uma única administração das partes aéreas da planta verde fresca, correspondente a 17,5 e 35 g/kg de peso vivo (PV), foi capaz de reproduzir a seneciose aguda. Doses mais baixas, de 5-10 g/kg PV, também administradas somente uma vez ou doses diárias de 0,625-5 g/kg PV, totalizando 75-150g/kg PV, produziram a forma crônica da doença. A manifestação crônica da seneciose também foi observada após administração semanal das doses de 2,18-8,75 g/kg PV, totalizando 61,25-78,75 g/kg PV, durante o período de 1 a 8 meses (TOKARNIA; DOBEREINER, 1984). Teores de seneciofilina maiores que 0.3 µM foram encontrados no sangue e no leite de bovinos durante as primeiras 18 horas depois que receberam 1 mg/kg do alcaloide por via oral. Depois de 54 horas, níveis sanguíneos de 0,033 µM ainda estavam presentes (CANDRIAN et al., 1991). Considerando que os teores dos APs em *S. brasiliensis* variaram entre 0,178% a 0,31% (KARAM et al., 2004; LUCENA et al., 2010), a ingestão de 0,3 – 0,5

g/kg PV das partes aéreas da planta, quantidade menor que a dose mínima que causar a seneciose crônica, seria suficiente para determinar níveis sanguíneos de seneciofilina equivalentes a 0.3 μM.

Embora a sensibilidade aos APs seja bastante variável entre espécies e entre os diferentes tipos de tecidos nos mamíferos, os APs in vitro determinaram efeitos citotóxicos e prejudicaram via de síntese de prostanóides em células do endotélio umbilical humano (HUVEC) em doses bem maiores que as que produziram sintomas em bovinos experimentalmente intoxicados (EBMEYER et al., 2019). Da mesma forma, a dose experimental suficiente para destruir 50% dos hepatócitos primários e células endoteliais sinusoidais do fígado (LSECs) de camundongo (HESSEL-PRAS et al., 2020) foi maior que a menor dose tóxica capaz de induzir experimentalmente a seneciose em bovinos. Além disso, a citotoxidade em ambos os modelos experimentais só se manifestou após a bioativação dos APs, sem a qual somente níveis tão altos quanto 500 μM de senecionina prejudicaram as LSECs. Por sua vez nenhum efeito foi observado em HUVECs para os APs equimidina, heliotrina, lasiocarpina, senecionina, senkirkina e platifilina na ausência de um sistema de metabolização externo, até a concentração mais alta testada de 500 μM.

Os prejuízos econômicos decorrentes de intoxicações por plantas nos rebanhos brasileiros são de difícil determinação em função dos poucos dados disponíveis a esse respeito, contudo estima-se que no mínimo 5% da população bovina morrem anualmente no Brasil (PESSOA; MEDEIROS; RIET-CORREA, 2013) e dados de laboratórios de diagnóstico mostram que entre 7,5 e 15,83% desses casos devem-se à intoxicação por plantas (RIET-CORREA; MEDEIROS, 2001; PEDROSO et al., 2007; RISSI et al., 2007). A ingestão de plantas do gênero *Senecio*, em especial de *S. brasiliensis*, é considerada a principal intoxicação por plantas e uma das principais causas de morte entre bovinos na região Sul do Brasil, respondendo por cerca de 50 – 60% dos óbitos decorrentes de intoxicações nessa espécie (KARAM; SCHILD; MELLO, 2011).

Segundo o IBGE (2020) a população de bovinos na região Sul do Brasil é de pouco mais de 24 milhões de cabeças. Considerando o valor médio de US$ 250,00 por animal pode-se inferir que as perdas anuais decorrentes de mortes causadas pela Seneciose em bovinos, apenas nos três Estados do Sul do país, possam passar de US$ 25 milhões. Presume-se que estas perdas sejam ainda maiores já que animais com intoxicação crônica e que ainda não manifestem sinais clínicos da Seneciose possam apresentar baixa taxa de produção de carne e

leite, bem como subfertilidade, em função dos danos hepáticos que prejudicam o metabolismo animal (GÓRNIAK, 2008).

Apesar de o tecido hepático ser o principal alvo das lesões causadas pela ingestão de *S. brasiliensis*, outros tecidos e órgãos, como pulmões e rins podem sofrer ação dos AP uma vez que as enzimas do citocromo P-450 também estão presentes nesses locais (PRAKASH et al., 1999). Corroborando essa informação, já foi demonstrado que alguns APs podem ser metabolizados pela isoforma CYP1A1 do citocromo P-450 presente em astrócitos de camundongos (NASCIMENTO et al., 2017). Com base nos efeitos dos APs em múltiplos tecidos foi sugerido que essa ação deletéria também seja capaz de causar danos diretos ao sistema reprodutivo feminino, bem como afetar o desenvolvimento embrionário inicial, uma vez que já foram descritos megalocitose em células luteais bovinas causada pela Seneciose (KRABBE et al., 2015), expressão do Citocromo P-450 1A1 em células da granulosa de suínos (LEIGHTON et al., 1995) e atraso no desenvolvimento físico e comportamental em ratos recém nascidos que tiveram exposição pré-natal a APs (SANDINI et al., 2014).

Boa parte dos casos de infertilidade em bovinos não tem etiologia estabelecida ou são erroneamente atribuídos a agentes infecciosos (ANTONIASSI et al., 2013), porém o desconhecimento dos mecanismos patogenéticos das fitotoxinas sobre a reprodução faz com que sua importância não seja completamente reconhecida (KRABBE et al., 2015). Além disso, até o momento, não existem informações a respeito dos efeitos de APs sobre a fisiologia ovariana ou sobre o desenvolvimento inicial em bovinos. Dessa forma, o estudo das possíveis alterações reprodutivas causadas diretamente por APs pode contribuir para elucidar a causa de uma fração dos casos de infertilidade sem causa aparente em bovinos.

2.2 A IMPORTÂNCIA DA COMUNICAÇÃO ENTRE AS CÉLULAS DO COMPLEXO CUMULUS-OÓCITO

O complexo cumulus-oócito (CCO) pode ser definido como um conjunto de células da granulosa intimamente associadas, denominadas células do cumulus, circundando o oócito em um folículo antral. Além desses dois tipos celulares, as células da granulosa mural e da teca que também compõem o folículo, desempenham suas funções específicas e interligadas para garantir que o oócito tenha atingido seu total desenvolvimento e esteja apto para ser fecundado no momento da ovulação (TANGHE et al., 2002; SUGIMURA; RICHANI; GILCHRIST, 2018). Nesse sistema, os oócitos regulam a proliferação, expressão gênica e

função das células do cumulus, enquanto as células do cumulus fornecem o suporte nutricional essencial para o crescimento e desenvolvimento do oócito (SUGIURA et al., 2008).

No CCO, o oócito e as células do cumulus estão conectados por junções comunicantes ("*gap junctions*"). Esse tipo de conexão também acontece com as células do cumulus entre si e com as células da granulosa mural. Canais de membrana intercelulares, chamados projeções transzonais, estão associados a essas junções comunicantes e permitem a transferência física de pequenos fatores reguladores e metabólitos entre o oócito, as células do cumulus e as células murais da granulosa. Além disso, o oócito secreta fatores parácrinos capazes de regular várias funções biológicas das células somáticas que o rodeiam, configurando um sistema de comunicação bidirecional dinâmico entre duas categorias de células distintas (RUSSELL et al., 2016).

A importância dessas trocas bidirecionais ficou comprovada quando oócitos bovinos desnudos maturados in vitro ou na presença de inibidores de junções comunicantes apresentaram alterações metabólicas e menor capacidade de sustentar a fertilização e o subsequente desenvolvimento embrionário, comparados com CCOs maturados nas mesmas condições (TANGHE et al., 2002; AUCLAIR et al., 2013). Da mesma forma, na ausência de um oócito, as células do cumulus não são metabolicamente competentes o que altera ou até mesmo impede a execução de vários processos metabólicos, como a expansão do cumulus (RICHANI et al., 2021).

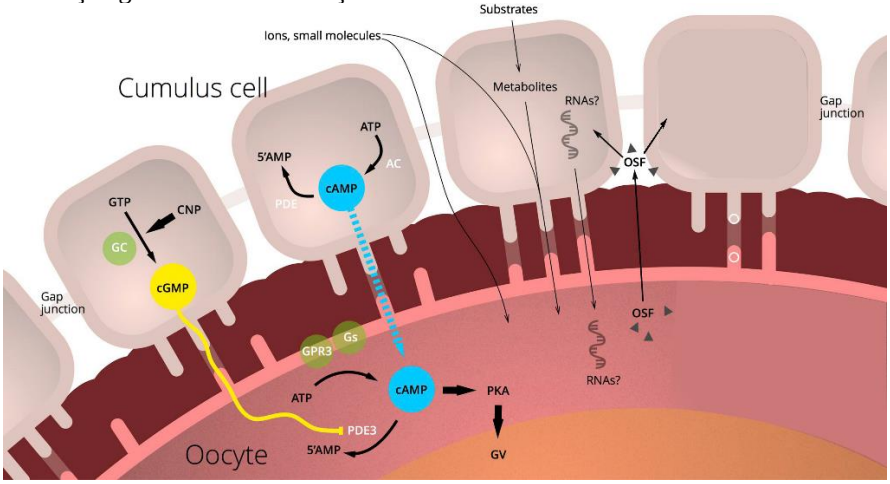
Um dos papeis preponderantes das junções comunicantes é a manutenção do bloqueio meiótico oocitário. Nos mamíferos os oócitos permanecem bloqueados como vesícula germinativa no estágio diplóteno da prófase I da meiose até o pico pré-ovulatório do hormônio luteinizante (LH) (SIRARD et al., 1989; RICHARDS et al., 2002; DEL COLLADO et al., 2018). Através da sinalização regulatória das junções comunicantes ocorre a passagem de monofosfato de adenosina cíclico (AMPc) e o monofosfato de guanosina cíclico (GMPc) para o oócito a partir das células do cumulus. Esses nucleotídeos cíclicos desempenham um papel crítico na regulação meiótica oocitária pois altos níveis intracelulares de AMPc no oócito promovem o bloqueio meiótico (CONTI et al., 2012), portanto a degradação do AMPc presente no oócito é essencial para a retomada da meiose. Essa degradação é feita pela ação da enzima AMPc-fosfodiesterase (PDE), contudo, a transferência de GMPc das células do cumulus para oócito através das junções comunicantes inibe a ativação da PDE, mantendo os

níveis de AMPc intraocitários altos e, consequentemente, o bloqueio meiótico (SELA-ABRAMOVICH et al., 2006).

O pico de LH liberado pela adenohipófise determina redução na produção GMPc e, com a retomada da meiose e expansão do cumulus, concomitantemente ocorre o desacoplamento das junções comunicantes. Em conjunto esses eventos determinam a diminuição do fluxo de GMPc para o oócito, permitindo a rápida degradação do AMPc pela PDE e a consequente retomada da meiose in vivo (SUN; MIAO; SCHATTEN, 2009). In vitro a remoção do óocito do folículo ovariano desencadeia espontaneamente a retomada da meiose (LONERGAN et al., 2003).

Apesar de depender diretamente dos íons, metabólitos, aminoácidos e pequenas moléculas reguladoras que recebe das células do cumulus, o oócito regula vários processos nas células somáticas que o rodeiam por meio da secreção dos chamados fatores secretados pelo oócito, dentre os quais o fator de diferenciação de crescimento 9 (GDF9), a proteína morfogenética óssea 15 (BMP15) e alguns membros da família dos fatores de crescimento fibroblásticos (FGF) são os mais estudados, embora não sejam os únicos conhecidos (CAIXETA et al., 2013a; RUSSELL et al., 2016). Por meio desses fatores parácrinos, o oócito é capaz de controlar: a diferenciação fenotípica das células do cumulus em relação as células da granulosa mural, a proliferação das células da granulosa e do cumulus (RUSSELL et al., 2016), a glicólise nas células do cumulus (SUGIURA et al., 2008), a captação de glicose pelo CCO (CAIXETA et al., 2013a), a aquisição da capacidade de sinalização da família EGF nas células do cumulus (SUGIMURA; RICHANI; GILCHRIST, 2018), e a mucificação e expansão das células do cumulus (CAIXETA et al., 2013a; MACHADO et al., 2015). Um esquema gráfico resumido de todo esse processo está representado na Figura 1.

Figura 1 – Representação gráfica da comunicação bidirecional entre o oócito e as células do cumulus



Fonte: Adaptado de (RUSSELL et al., 2016)

2.3 A EXPANSÃO DAS CÉLULAS DO CUMULUS

A ovulação é um processo complexo que determina alterações estruturais, endócrinas e bioquímicas no folículo pré-ovulatório a fim de liberar um gameta feminino apto para ser fecundado e que dê suporte ao futuro embrião durante o período pré-implantacional (SHIMADA et al., 2006). Assim como na retomada da meiose pelo oócito, o fator desencadeante da ovulação é o pico de LH. Esse hormônio se liga a receptores de LH (LHR) nas células da granulosa uma vez que nem o oócito, nem as células do cumulus expressam LHR, e estimula a transcrição de genes relacionados com a ovulação em si, com a expansão do cumulus, com a maturação nuclear e com a esteroidogênese (RICHARDS et al., 2002).

O pico pré-ovulatório de LH também induz a expansão in vivo dos CCOs. Durante a expansão, as células do cumulus secretam uma matriz rica em ácido hialurônico e sintetizam as proteínas estabilizadoras dessa matriz. A hidratação desse ácido promove o aumento no espaço entre as células do cumulus e mucificação da matriz que as envolve (NAGYOVA et al., 2008; YAMASHITA; HISHINUMA; SHIMADA, 2009).

Sob efeito do LH, as células da granulosa produzem fatores de crescimento semelhantes ao EGF (EGF-Like), especialmente epiregulina (EREG), anfiregulina (AREG) e betacelulina (SHIMADA et al., 2006). Esses EGF-Like, particularmente EREG e AREG nos bovinos (PORTELA et al., 2011), atuam de forma autócrina nas células da granulosa mural e de forma parácrina nas células do cumulus. Da mesma forma que o LH nas células da granulosa, o hormônio folículo estimulante (FSH) induz a expressão dos EGF-like nas células do cumulus (CAIXETA et al., 2013b). Entretanto, os EGF-Like a princípio são sintetizados como precursores ancorados à membrana e de forma subsequente são liberados da célula por proteólise mediada por ADAM17 (BEN-AMI et al., 2006). Uma vez liberados, os EGF-Like se ligam a receptores de EGF (EGFR) expressos nas células da granulosa e do cumulus onde estimulam a sua própria síntese, a produção de prostaglandina sintetase 2 (PTGS2) e prostaglandina E2 (PGE2), além de induzir a expressão de genes envolvidos com a esteroidogênese e daqueles envolvidos com a expansão do cumulus. A PGE2 também participa do processo de ovulação por induzir a síntese de EGF-like de maneira semelhante ao LH (SHIMADA et al., 2006).

Recentemente foi demonstrado que a atividade transcricional de *Yes-associated protein* (YAP), um efetor da via Hippo, regula a cascata de sinalização do EGF em células da granulosa mural e do cumulus em bovinos, interferindo diretamente nos processos de

expansão do cumulus e ovulação induzidos pelo LH (DOS SANTOS et al., 2022; KOCH et al., 2022). Dessa forma e em conjunto, os EGF-like, a YAP, a PTGS2 e a PGE2 potencializam a produção da proteína indutora do fator de necrose tumoral 6 (TNFAIP6), da hialurona sintetase 2 (HAS2) e da pentraxina 3 (PTX3) as quais, por sua vez, são responsáveis pela expansão do cumulus (CONTI et al., 2006; SHIMADA et al., 2006; DOS SANTOS et al., 2022; KOCH et al., 2022).

HAS2 promove a síntese do ácido hialurônico, principal componente da matriz extracelular do cumulus (VELHO et al., 2013). TNFAIP6 é necessário para a estabilização da matriz do cumulus expandido e sua expressão é induzida nas células murais da granulosa e nas células do cumulus (NAGYOVA et al., 2008). Essa proteína é indispensável para a fertilidade feminina visto que camundongos deficientes em TNFAIP6 não são capazes de estruturar a matriz extracelular do oócito e, por isso, são estéreis (FÜLÖP et al., 2003). A PTX3 também é essencial para a estabilidade da matriz do cumulus. Sua afinidade pela TNFAIP6 e a interação entre elas parece ser determinante para a estruturação da matriz do cumulus e fertilidade da fêmea (SALUSTRI et al., 2004).

A expressão de mRNA de *HAS2*, *PTGS2*, *TNFAIP6* e *PTX3* foi positivamente associada com aumento na qualidade embrionária e por isso esses genes foram sugeridos como marcadores de competência oocitária nas células do cumulus (NAGYOVA et al., 2008; TESFAYE et al., 2009), assim como a expressão de mRNA dos genes envolvidos na cascata periovulatória (EGF-Likes, *PTGS2*, *YAP*) em células da granulosa é crítica para ruptura da parede do folículo e a liberação do CCO (PORTELA et al., 2011; DOS SANTOS et al., 2022). Assim, a alteração no padrão de expressão de qualquer desses genes sugere que parte das funções ovarianas pode estar comprometida.

2.4 VIAS DE SINALIZAÇÃO E REPARO DOS DANOS DE DNA

A replicação e transmissão precisas do material genético são essenciais para a homeostase celular e viabilidade dos organismos. No entanto, as células estão continuamente expostas a fatores ambientais e agentes endógenos deletérios que prejudicam a integridade do DNA e ameaçam a integridade genômica (PANIER; BOULTON, 2014).

A hidrólise que leva à depurinação espontânea do DNA, as espécies reativas de oxigênio (ROS) que induzem oxidação de bases e quebras de DNA, os defeitos de replicação, e o colapso da forquilha de replicação que pode resultar em quebras de fita do DNA são exemplos de processos endógenos prejudiciais ao ácido nucleico (BRANZEI; FOIANI, 2008).

Danos ambientais/exógenos ao DNA podem ser produzidos por fontes físicas ou químicas. A radiação ionizante, tanto de origem cósmica quanto de origem hospitalar (radioterapia ou raios-X) e a luz ultravioleta solar são agentes genotóxicos físicos que podem induzir a oxidação de bases de DNA e gerar quebras de DNA de fita simples e de fita dupla. Os agentes usados na quimioterapia do câncer, os inibidores da topoisomerase que induzem a formação quebras das fitas do DNA, o tabagismo que causa uma grande variedade de adutos e danos oxidativos no pulmão e em outros tecidos, e os APs que também formam adutos com DNA e proteínas, são exemplos de danos de origem química que acometem o DNA (FU et al., 2004; BRANZEI; FOIANI, 2008; LORD; ASHWORTH, 2012). A maturação in vitro (IVM) de CCOs bovinos, especialmente em meio não suplementado com soro fetal bovino (FCS), assim como a vitrificação de embriões murinos determinaram aumento na expressão de genes relacionados com o reparo de DNA (IKEDA; IMAI; YAMADA, 2003; CHANG et al., 2019). Da mesma forma, a exposição a luz ultravioleta induziu a quebra da fita dupla do DNA (DSB) de embriões bovinos produzidos in vitro (BARRETA et al., 2012).

Para proteger apropriadamente o genoma, todos os tipos de alterações estruturais do DNA precisam ser detectadas e corretamente reparadas. A resposta ao dano de DNA (DDR) é executada por uma série de vias de transdução de sinal entrelaçadas que detectam o dano no DNA e, após a ativação dos pontos de verificação do ciclo celular (*cell-cycle checkpoints*), determinam dois desfechos principais: o próprio mecanismo de reparo e/ou apoptose (MAGNUSON; BEDI; LJUNGMAN, 2016). Por meio de uma cascata de sinalização firmemente coordenada, os pontos de verificação do ciclo celular coordenam a parada do ciclo celular e o recrutamento de proteínas de reparo que modificam quimicamente o DNA a fim de proteger o genoma. Entre as proteínas de reparo estão nucleases, helicases, polimerases, topoisomerases, recombinases, ligases, glicosilases, demetilases, quinases e fosfatases, as quais devem ser precisamente reguladas já que cada uma, por si só, tem potencial de alterar a integridade do DNA se acionada no momento inapropriado ou no segmento incorreto do ácido nucleico. Uma vez que o DNA tenha sido reparado o ciclo celular é retomado, caso contrário, a DDR encaminha para a senescência ou apoptose celular (CICCIA; ELLEDGE, 2010).

Como outras vias de transdução de sinal, a DDR consiste em sensores de sinal, transdutores e efetores. Os sensores desta via são proteínas que reconhecem estruturas aberrantes, induzidas por danos e/ou estresse de replicação do DNA, e ativam os transdutores. Os transdutores são representados por uma cascata de proteínas quinases e proteínas

mediadoras que facilitam eventos de fosforilação dentro da rede de reparo ao dano no DNA. Os efetores são substratos das quinases transdutoras que participam de um amplo espectro de processos celulares importantes para a estabilidade genômica, como replicação de DNA, reparo de DNA e controle do ciclo celular (MARÉCHAL; ZOU, 2013).

A DDR é mediada principalmente por proteínas (transdutores) da família das proteínas quinases semelhantes a fosfatidilinositol-3 quinase (PIKK) e pelos membros da família poli(ADP-ribose) polimerase (PARP). Nos mamíferos, três quinases da família PIKK atuam como transdutoras: *ataxia telangiectasia mutated* (ATM), *ATM and RAD3 related* (ATR) e subunidade catalítica da proteína-quinase DNA-dependente (DNAPK-cs). A família PARP é composta por 16 membros, porém apenas PARP1 e PARP2 estão envolvidos na DDR (CICCIA; ELLEDGE, 2010).

Em resposta ao dano do DNA, centenas de proteínas podem ser fosforiladas por ATM ou ATR, enquanto a DNAPK parece regular um número menor de substratos. ATM e DNAPK são ativados por agentes que determinam DSBs. Por sua vez, ATR responde a um amplo espectro de danos ao DNA, incluindo DSBs e uma gama de lesões de DNA que interferem na replicação. PARP1 e PARP2 são ativados tanto por quebras de fita simples no DNA (SSB)s quanto por DSBs e catalisam a adição de cadeias de poli(ADP-ribose) em proteínas para recrutar fatores para a DDR (MARÉCHAL; ZOU, 2013).

A proteína de replicação A (RPA) tem alta afinidade por regiões de DNA de fita simples e, quando ocorrem SSBs, sua ligação a esses substratos parece ser o sinal para o recrutamento das quinases transdutoras. Por sua vez, ATM é recrutada poucos minutos após a ocorrência de DSB e fosforila várias proteínas que são necessárias para sinalizar danos e reparos no DNA. A histona H2AX é um alvo proximal de atividade nessa cascata de sinalização e, após ser fosforilada por ATM, DNAPK ou ATR no local do dano, age no recrutamento de proteínas que iniciam o processo de remodelação da cromatina. ATM e ATR também atuam reduzindo a atividade da quinase dependente de ciclina, pela ativação da transcrição de p53. Isso causa um atraso na progressão do ciclo celular, aumentando o tempo disponível para que ocorra o reparo das lesões do DNA antes da replicação ou divisão celular (CICCIA; ELLEDGE, 2010; CECCALDI; RONDINELLI; D'ANDREA, 2016).

A seleção de qual sistema de reparo será ativado depende do tipo de lesão no DNA e da fase do ciclo celular que o dano foi detectado (BRANZEI; FOIANI, 2008). A via de reparo por excisão de nucleotídeos (*nucleotide excision repair*) atua no reparo a danos que causam a distorção da hélice do DNA e que interferem no pareamento das bases. Dessa forma, quando

esses erros não são reparados ocorre a interrupção da transcrição e a replicação normais. A via do reparo por excisão de bases (*base excision repair*) é responsável por corrigir pequenas alterações nas bases nitrogenadas. Essas alterações geralmente não impedem a transcrição ou a replicação apesar de muitas vezes gerarem erros de codificação. A via do reparo a erros de pareamento (*mismatch repair*) é responsável pela correção de erros de pareamento das bases nitrogenadas e pequenas inserções ou deleções que ocorrem durante a replicação celular. As lesões que causam quebra física de uma ou de ambas as fitas de DNA são alvo de dois tipos de sistemas de reparo. O primeiro independe de homologia entre as fitas do DNA e é representado pela via da união terminal não homóloga (NHEJ - *non-homologous end-joining*); o segundo é representado por três vias dependentes de homologia: recombinação homóloga (HR - *homologous recombination*), união terminal mediada por micro-homologia (MMEJ – *microhomology-mediated end joining*) e anelamento de fitas simples (SSA – *single-strand annealing*) (CICCIA; ELLEDGE, 2010; LORD; ASHWORTH, 2012).

2.4.1 Reparos de quebras na fita dupla do DNA

Dos muitos tipos de lesões de DNA, as DSBs são consideradas as mais prejudiciais já que apenas uma DSB, caso não seja devidamente reparada, é suficiente para desencadear a parada permanente do ciclo e a morte celular. Além disso, as DSBs são potentes indutores de rearranjos cromossômicos, tais como deleções, translocações e amplificações. Esses rearranjos do genoma podem resultar na ativação de oncogenes e/ou na perda de supressores de tumor, o que, por sua vez, alimenta a transformação carcinogênica (PANIER; BOULTON, 2014; SUN et al., 2018).

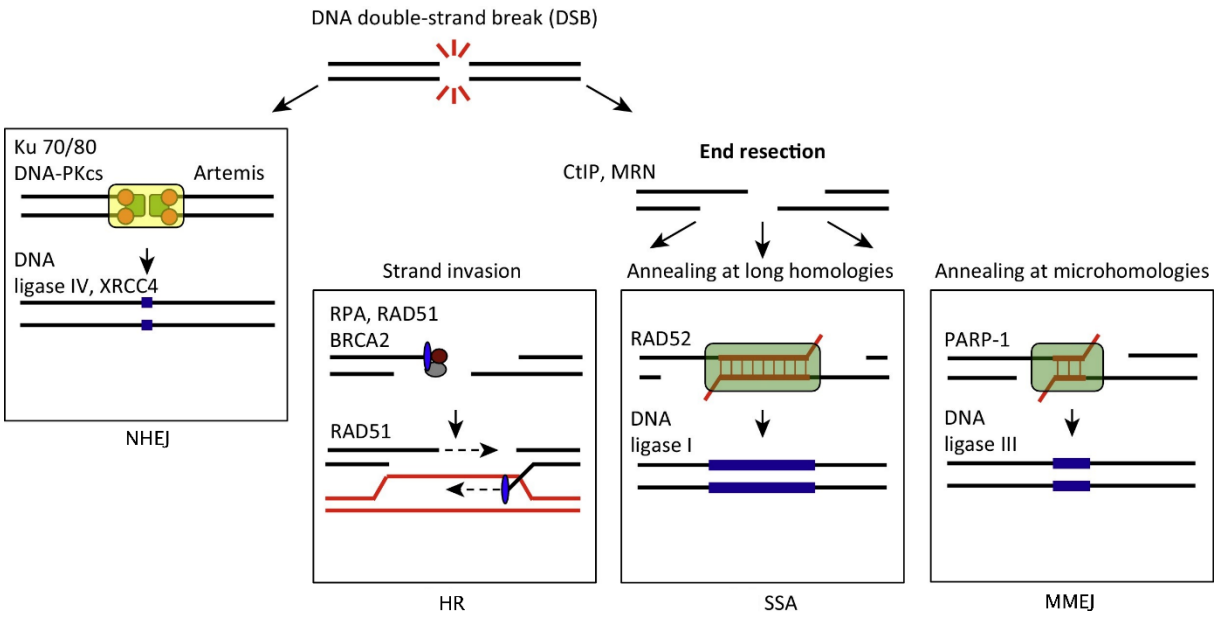
As DSB são caracterizadas pela quebra simultânea de ligações fosfodiéster na mesma região ou em regiões próximas de ambas as fitas de DNA. Como resultado dessa dupla ruptura, a integridade sequencial da informação genética é fisicamente desconectada, assim como a perda da referência precisa que é utilizada em muitas vias onde apenas uma das fitas está comprometida. Quando esse tipo de lesão é comparado com os demais, fica claro seu nível de complexidade e potencial de dano (JACKSON; BARTEK, 2009; LIEBER, 2010).

Frente a grande diversidade de fatores determinantes de DSBs, algumas vias de reparo especializadas evoluíram para manter a integridade do genoma. As células respondem as DSBs montando uma rede de sinalização complexa que coordena as reações de reparo do DNA com a ativação do ponto de verificação de danos no DNA e a reorganização da cromatina. A maioria desses processos de reparo envolve uma nuclease para remover o DNA

danificado, uma DNA polimerase para preencher o novo DNA e uma ligase para restaurar a integridade de cada fita do duplex (LIEBER et al., 2010). Além disso, pelo menos quatro sensores parcialmente independentes podem detectar DSBs: PARP, Ku70/Ku80, complexo Mre11-Rad50-Nbs1 (MRN) e RPA (CICCIA; ELLEDGE, 2010; CECCALDI; RONDINELLI; D'ANDREA, 2016).

Embora os mecanismos celulares que determinam a escolha da via para o reparo das DSBs ainda não estejam totalmente elucidados, parece que a extensão do processamento das pontas de DNA desempenha papel fundamental nessa escolha (PANIER; BOULTON, 2014). NHEJ não requer ressecção das pontas das fitas de DNA enquanto as outras três vias dependem dessa ressecção, que é mais restritiva (5 – 25 nucleotídeos) para MMEJ e mais extensiva para HR e SSA (CICCIA; ELLEDGE, 2010; CECCALDI; RONDINELLI; D'ANDREA, 2016). A Figura 2 representa estas quatro abordagens para o reparo das DSB.

Figura 2 – Quatro abordagens para o reparo das quebras na fita dupla do DNA



Fonte: Adaptado de (CECCALDI; RONDINELLI; D'ANDREA, 2016)

Por serem as duas principais vias de reparo de DSB (BRANZEI; FOIANI, 2008; JACKSON; BARTEK, 2009; LORD; ASHWORTH, 2012; PANIER; BOULTON, 2014; BOHRER et al., 2018), NHEJ e HR serão descritas a seguir.

2.4.1.1 União terminal não homóloga (NHEJ)

A NHEJ representa o mecanismo mais simples e rápido para reparar DSBs, portanto, é a via de reparo predominante na maioria das células de mamíferos, embora possa ocasionalmente levar à perda de informação genética (VÍTOR et al., 2020). NHEJ promove a ligação entre as pontas das fitas de DNA rompidas independente de homologia e/ou grande ressecção, portanto, essa via está mais propensa a erros que a HR. Pode ser acionada durante qualquer uma das fases do ciclo celular (LIEBER, 2010; LORD; ASHWORTH, 2012) mas a alta compactação da cromatina e a ausência de cromátides irmãs disponíveis são importantes fatores que tornam a NHEJ a via de reparo predominante durante a fase G1 (WARD et al., 2004; BRANZEI; FOIANI, 2008; FENG et al., 2015; VÍTOR et al., 2020), contudo, para Ceccaldi, Rondinelli e D'Andrea (2016) essa é a via dominante nas fases G0/G1 e G2 do ciclo celular.

O ponto de partida do processo NHEJ se dá com o reconhecimento da DSB e ligação das extremidades do DNA de fita dupla pelo heterodímero KU, formado pelas proteínas KU70 e KU80 (produtos dos genes *XRCC6* e *XRCC5*, respectivamente), o que ocorre de maneira extraordinariamente rápida e eficiente devido à sua abundância e forte atração por esse tipo de substrato de DNA. O heterodímero KU ligado ao DNA, por sua vez, recruta DNAPK-cs para formar a holoenzima DNA-PK, de modo que as duas moléculas de DNAPK-cs ligadas a lados opostos do DSB possam interagir entre si, contribuindo para a sinapse das extremidades quebradas do DNA e impedindo a ressecção das extremidades das fitas rompidas. O complexo DNA-PK é o principal regulador do processo NHEJ, coordenando o recrutamento de fatores acessórios, como a proteína 4 de complementação cruzada de reparo de raios X (*XRCC4*) e a DNA ligase IV (*LIG4*), que contribuem para o adequado pareamento das extremidades DSB e realizam a ligação final das fitas de DNA (CICCIA; ELLEDGE, 2010; SERRANO-BENÍTEZ; CORTÉS-LEDESMA; RUIZ, 2020).

Após a ligação da DSB, a auto fosforilação de DNAPK-cs resulta na desestabilização de sua interação com as extremidades do DNA, possibilitando acesso a enzimas de processamento terminal, como ARTEMIS. Essa auto fosforilação da DNAPK-cs, em seu cluster PQR, previne o processamento terminal excessivo e tem efeito inibitório sobre a HR pois impede a ressecção excessiva da dupla fita (CICCIA; ELLEDGE, 2010).

2.4.1.2 Recombinação homóloga (HR)

A HR requer uma sequência de DNA homóloga para servir de molde para o reparo das DSB e envolve extensivo processamento do DNA. Como esperado, a HR é extremamente precisa, pois leva ao reparo do locus danificado usando sequências de DNA homólogas não danificadas às extremidades quebradas. Há fortes indícios de que a ressecção das extremidades das fitas de DNA no sentido 5' – 3', promovendo a extensão da extremidade 3'ssDNA (fita simples de DNA), é um passo crítico e determinante para escolha da HR como via de reparo para as DSBs. Esses trechos de 3'ssDNA estendidos são utilizados para busca da região de homologia na cromátide irmã que servirá de molde para o reparo (MARINI et al., 2019; VÍTOR et al., 2020). HR acontece principalmente nas fases S e G2 do ciclo celular, momentos nos quais a replicação do DNA é mais intensa e existe uma cromátide irmã disponível (BRANZEI; FOIANI, 2008; LORD; ASHWORTH, 2012; FENG et al., 2015; VÍTOR et al., 2020).

A via HR pode ser dividida didaticamente em três etapas: pré-sinapse, sinapse e pós-sinapse. Na pré-sinapse, ocorre o reconhecimento das quebras de fita dupla e posterior processamento das pontas do DNA (ressecção), gerando filamentos de ssDNA em cada uma das fitas quebradas. Na fase sináptica ocorre a invasão da fita de DNA a ser reparada em seu molde de reparo na cromátide homóloga. Esta invasão resulta em uma estrutura chamada de D-loop. A seguir inicia-se a fase pós sinapse quando ocorre a síntese da porção danificada e a separação das cromátides homólogas (RENKAWITZ; LADEMANN; JENTSCH, 2014).

A HR é iniciada pelo reconhecimento das DSBs pelo complexo MRE11-RAD50-NBS1 (MRN), que promove a ativação de ATM e a preparação de DNA para o reparo. RAD50 contém domínios ATPase que interagem com MRE11 e se associam com as extremidades rompidas de DNA. MRE11 também possui atividades de endonuclease e exonuclease que atuam nas etapas iniciais de ressecção das fitas danificadas. A terceira subunidade do complexo MRN, NBS1, associa-se com ATM através de sua região C-terminal, que promove o recrutamento de ATM para DSBs, onde ATM é ativado pelo complexo MRN (CICCIA; ELLEDGE, 2010).

A ressecção final do DNA é regulada por ATM através da proteína *CtBP-interacting* (CtIP), que interage com BRCA1 e MRN. Durante este processo, a formação de DNA de fita simples (ssDNA) é continuada pela ação da exonuclease 1 (Exo1). Em S e G2, CtIP associa-se com BRCA1 que facilita sua ligação com locais de dano. Na fase G1 CtIP atua de maneira independente de BRCA1 e, dessa forma, promove a reparação por MMEJ que é mediada pelo

emparelhamento de regiões de microhomologia de ssDNA, seguido por ligação terminal de DNA dependente de Ligase 3 (CICCIA; ELLEDGE, 2010; RENKAWITZ; LADEMANN; JENTSCH, 2014)

Na sequência do processo a RPA recobre a extremidade da ssDNA protegendo-a da formação de estruturas secundárias (GUDMUNDSDOTTIR; ASHWORTH, 2006; MARINI et al., 2019). Além da estabilidade das extremidades do DNA, RPA atua garantindo que haja a correta incorporação de filamentos RAD51 (*DNA repair protein RAD51*), mediada por BRCA2 (*Breast Cancer 2 DNA repair associated*) e RAD52 (*DNA repair protein RAD52*) (CICCIA; ELLEDGE, 2010). Por sua vez, durante a fase sináptica, o filamento RAD51 invade a cadeia de DNA não danificada e pareia com um duplex homólogo, se uma sequência doadora tiver sido encontrada durante o processo de pesquisa de homologia. A troca de fitas de DNA entre o DNA alvo e o filamento RAD51 gera uma estrutura conhecida como loop de deslocamento (D-loop) que contém o novo DNA heteroduplex e a fita deslocada do DNA doador. Por fim a síntese de DNA é iniciada a partir da extremidade 3', a segunda extremidade de DSB alinha-se com o D-loop estendido para formar a dupla junção *Holliday* e as estruturas resultantes são resolvidas. Após a síntese as junções *Holldays* são clivadas e as fitas de DNA são ligadas para produzir duas moléculas intactas de DNA (CICCIA; ELLEDGE, 2010; RENKAWITZ; LADEMANN; JENTSCH, 2014; CECCALDI; RONDINELLI; D'ANDREA, 2016).

A Proteína de Ligação de Proteína Tumoral P53 1 (TP53BP1) é um importante regulador da resposta celular a DSB. Apresenta marcado antagonismo com BRCA1 e, portanto, o equilíbrio entre BRCA1 e TP53BP1 é o modulador para determinação da via de reparo que será utilizada. Como TP53BP1 bloqueia a ressecção de DNA, impedindo que o CtIP e ATM acessem as extremidades do ácido nucleico durante a fase G1 do ciclo celular, sua atuação promove a via NHEJ em detrimento a HR (CECCALDI; RONDINELLI; D'ANDREA, 2016; MARINI et al., 2019). Dessa forma, TP53BP1 bloqueia a via HR e promove a NHEJ. Contudo, essa proteção das extremidades do DNA depende da fosforilação da TP53BP1 por ATM que promove o recrutamento de fatores de interação *Rap1-interacting factor 1* (RIF1) e *PAX-interacting protein 1* (PTIP) (SERRANO-BENÍTEZ; CORTÉS-LEDESMA; RUIZ, 2020). Vale ressaltar que nas fases S/G2 do ciclo celular, BRCA1 promove a desfosforilação de 53BP1 e a liberação de RIF1, determinando a ressecção das extremidades das fitas do DNA e direcionando o reparo para HR (MARINI et al., 2019).

Os principais genes que controlam as vias HR e NHEJ de reparo de DNA são expressos em embriões bovinos (BARRETA et al., 2012) e oócitos e blastocistos humanos (JAROUDI et al., 2009). Embriões bovinos com baixa competência mostraram maior expressão de *TP53BP1* e *RAD52* quando comparados aos embriões com alta competência. Quando DSBs foram induzidas experimentalmente por radiação ultravioleta em blastocistos bovinos, a expressão de *TP53BP1*, *RAD51* e *KU70* diminuiu 72 horas pós inseminação e depois ficou maior que a do grupo não irradiado as 168 horas pós inseminação (BARRETA et al., 2012). Em conjunto esses resultados indicam que o perfil de expressão dos genes envolvidos no reparo das DSBs em embriões com baixa competência está alterado e, portanto, podem atuar como indicadores de viabilidade e saúde dos embriões.

ARTIGO I

ARTIGO A SER SUBMETIDO PARA PUBLICAÇÃO

Senecio brasiliensis-DERIVED PYRROLIZIDINE ALKALOIDS DECREASE OVULATORY CASCADE GENE EXPRESSION AND BOVINE EMBRYOS PRODUCTION

André Lucio Fontana Goetten, Carolina dos Santos Amaral, Daniele Missio, Vitor Braga Rissi, Alceu Mezzalira, Christopher Allan Price, Marcos Henrique Barreta &Valério Marques Portela

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Senecio brasiliensis-derived pyrrolizidine alkaloids decrease ovulatory cascade gene expression and bovine embryos production

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ABSTRACT

Pyrrolizidine alkaloids (PAs)-containing plants are widely distributed in the world and probably the most common poisonous plants affecting livestock, wildlife, and humans. Livestock poisoning, mainly liver damage, depends on PAs metabolization by cytochrome P450, whose isoforms can occur in other organs, including ovaries. This study aimed to assess whether low PAs concentration have a direct effect in vitro on bovine granulosa cells and embryos. Low concentrations (0.03 or 0.3 µM) of senecionine or seneciphylline, PAs present in the *Senecio* genus, were added to the culture medium. Senecionine and seneciphylline downregulated mRNA expression of *AREG*, *EREG*, *PTGS2*, and *CTGF* genes in granulosa cells, suggesting that PAs could impair bovine ovulation. Both tested PAs do not impair cumulus expansion. Cleavage and blastocyst rates did not differ from the control group when PAs were added to in vitro maturation medium. Cleavage and blastocyst rates were reduced when senecionine or seneciphylline were added to the medium 18–20 hours post-insemination (hpi). Seneciphylline added to maturation medium downregulated the *TP53BP1* gene in blastocysts but upregulated the *RAD51* gene when added at 18–20 hpi, suggesting that PAs could determine DNA double-strand break and homologous repair pathway activation. Senecionine reduced *GLUT3* and *BCL2* mRNA expression, suggesting that PAs could impair glucose metabolism and reduce antiapoptotic activity in bovine blastocysts. In summary, this study demonstrates that senecionine and seneciphylline have significant toxicological effects on bovine granulosa cells as well as in in vitro produced bovine embryos, suggesting that PAs, even at low concentrations, have the potential to directly impact cattle reproductive physiology.

Keywords: Senecionine, seneciphylline, granulosa cells, ovary, embryo.

3.1 INTRODUCTION

Plant intoxication is still one of the most common causes of death in adult production animals, resulting in significant direct and indirect economic losses in the livestock production system [1–3]. Management failures in pasture or crop areas favor the appearance of invasive plants, many of which have toxic potential for various animal species, including humans [4]. The toxic plants in southern Brazil that generate the most economic losses in livestock are those belonging to the genus *Senecio* spp., which cause 50% of all cattle deaths by phytotoxins [2,4,5]. *Senecio brasiliensis* is one of the most dangerous species in the genus.

It can cause hepatotoxicity and poisoning in horses and cattle, which can lead to their death [6].

The toxic principles present in *Senecio* spp. belongs to the group of pyrrolizidine alkaloids (PAs). PAs described in *S. brasiliensis* include senecionine, seneciphylline, integerrimine, retrorsine, usaramine, and jacobine [7,8], with senecionine and seneciphylline being the most prevalent [9]. There is considerable variation in the content of PAs in different plant species or in different parts of the same plant, however, PAs only become toxic when biotransformed into pyrroles, mainly by hepatic cytochrome P-450 enzymes [10]. Pyrroles cause irreversible damage to hepatocytes by inhibiting mitosis and causing necrosis, resulting in hepatic dysfunction [11]. Megalocytosis is one of the most common findings in liver biopsies of cattle that have been intoxicated with *S. brasiliensis*. It could be caused by inhibition of mitosis [12] or by DNA damage [10]. It is already known that some of the pyrroles produced in the liver can reach other organs through the systemic circulation and cause extrahepatic damage [4,13].

Both the acute and chronic forms of intoxication by *S. brasiliensis* in cattle have been experimentally demonstrated. Single doses of 5 to 10 g/kg resulted in chronic poisoning, but when animals received daily repeated doses of 0.625 to 5 g/kg for 1 to 8 months, they did not show any symptoms for weeks or even months, simulating a cumulative effect of these xenobiotics [14]. During the first 18 hours after receiving 1 mg/kg of the alkaloid orally in a single dose, seneciphylline levels greater than 0.3 µM were found in the blood and milk of cattle. Blood levels of 0.033 µM were still present after 54 hours of treatment [15]. Given that the amount of PAS in *S. brasiliensis* ranged from 0.178 to 0.31 percent [16,17], an intake of 0.3–0.5 g/kg BW of aerial parts of the plant would be enough to determine blood levels of seneciphylline equivalent to 0.3 µM.

The decrease in reproductive rates of seneciosis-affected cows has traditionally been linked to a deterioration in nutritional condition caused by the liver damage. However, there are evidences that PAs act directly on ovarian cells since swine ovaries are capable of expressing and transcribing the enzyme cytochrome P-450 and its isoforms [18], and ovaries of bovines with chronic seneciosis showed lesions in large luteal cells similar to those present in hepatocytes [19]. There are also reports that prenatal exposure to APs can impair physical and behavioral development in rats [20].

When we associated cattle infertility with no apparent cause with evidence of PAs acting on reproductive organs, we hypothesized that low PAs concentrations, insufficient to

cause chronic seneciosis, could act directly on ovarian cells and/or embryos, affecting the periovulatory EGF-like cascade and early embryonic development. The aims of this study were to evaluate whether low concentration of pyrrolizidine alkaloids have a direct effect: (a) on expression of periovulatory Epidermal Growth Factor-Like (EGF-Like) cascade genes in bovine granulosa cells; (b) on bovine cumulus-oocyte cell expansion; and (c) on early embryonic development in cattle.

3.2 MATERIAL AND METHODS

All reagents were purchased from Sigma-Aldrich Co. (Rocklin, CA, USA) unless otherwise stated. Senecionine (cat. # 50351) and seneciphylline (cat. # 73913) were dissolved in ethanol, and the final concentration of ethanol never exceeded 0.01% in the media.

3.2.1 Granulosa cell culture

The granulosa cell (GC) culture system employed herein was as a previously described [21,22] in which abundance of mRNA encoding the EGF-like factors and other critical preovulatory genes is acutely upregulated by luteinizing hormone (LH). Bovine ovaries containing large follicles were collected from adult cows, a part of the estrous cycle stage, at a local abattoir and were transported to the laboratory in saline solution (0.9% NaCl; 25 ºC) containing penicillin (100 IU/mL), and streptomycin sulfate (50 μg/mL). For each experimental replicate, ten ovaries that each contained a single large follicle (≥10 mm diameter) were selected and GC were collected from each large follicle by aspiration, pooled in a single tube and were washed twice by centrifugation at 219×g for 20 min each. Cell viability was estimated with 0.4% Trypan Blue Stain. Cells were then seeded into 24-well tissue culture plates (Sarstedt, St-Leonard, QC, Canada) at a density of 1×10^6 viable cells per well in 1 mL DMEM-F12 supplemented with sodium bicarbonate (10 mM), sodium selenite (4 ng/mL), bovine serum albumin (BSA; 1 mg/mL), penicillin (100 IU/mL), streptomycin (100 μg/mL), transferrin (2.5 μg/mL), non-essential amino acid mix (1.1 mM), androstenedione (10^{-7} M), follicle-stimulating hormone (FSH; 1 ng/mL), insulin (10 ng/mL) and 2% fetal calf serum (FCS). Cultures were maintained at 38.5 ºC in 5% CO2 for 24 h. Medium was then replaced with 0.9 mL serum-free DMEM-F12 with antibiotics for 18 h, at which point LH (100 ng/mL) and the experimental treatments were added in 100 μl DMEM-F12 (with antibiotics).

3.2.2 Cumulus-oocyte complexes recovery and in vitro maturation (IVM)

Bovine ovaries were collected from adult cows, a part of estrous cycle stage, at a local abattoir and were transported to the laboratory in saline solution (0.9% NaCl; 25 ºC) containing penicillin (100 IU/mL), and streptomycin sulfate (50 µg/mL). Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 15 mL of water/minute) and pooled in a 15 mL conical tube. After sedimentation, COCs were recovered and selected under a stereomicroscope as previously described [23]. Grade 1 or 2 COCs were randomly transferred to well plates containing basic maturation medium composed by TCM199 containing Earle's salts and L-glutamine supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 µg/mL LH (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), 0.5 µg/mL FSH (Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), 100 IU/mL penicillin, 50 µg/mL streptomycin sulfate and 10% FCS. Cultures were maintained in an incubator at 38.5 ºC in 5% CO_2. The experimental treatments, number of replicates, and number of COCs/replicate varied according to the objective of each experiment.

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

After IVM, bovine oocytes were fertilized in vitro with frozen-thawed semen and fractionating on discontinuous Percoll (GE Healthcare, São Paulo, SP, Brazil) gradient. Sperm was diluted and added to the COCs plate with final concentration adjusted to 2×10^6 sperm/mL in Fert-TALP medium containing 20 mM penicilinamine, 10mM hypotaurine, 1mM epinephrine [24], 100 IU/mL penicillin, and 50 µg/mL streptomycin sulfate. Fertilization was carried out by coculture of sperm and oocytes for 18–20 hours in four-well plates (Nunc, Roskilde, Denmark) in the same atmospheric conditions used for maturation. IVF day was considered as Day 0 of embryo production.

After IVF, presumptive bovine zygotes were denuded by 2-min vortexing, and then cultured in groups of 35 – 45 in a culture chamber (CBS Scientific, Del Mar, CA) at 38.5°C and saturated humidity atmosphere of 5% CO_2,5% O_2, and 90% N_2 in 400 µL of synthetic oviduct fluid medium (SOFaaci) [25] containing 0.2mM pyruvic acid, 5% of FCS, 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate in four-well plates. Cleavage rates were evaluated 48 hours post-insemination (hpi), and blastocyst rates were assessed 168 hpi.

Blastocysts evaluated 168 hpi were rinsed three times in phosphate-buffered saline (PBS) and stored at −80°C for subsequent RNA extraction.

3.2.4 RNA extraction, reverse transcription, and real-time polymerase chain reaction

Total RNA was then extracted using PureLink™ RNA Mini Kit (Thermo Fisher Scientific, Waltham, MA, EUA) according to the manufacturer's instructions and was quantified at 260 nm wavelength using a spectrophotometer (NanoDrop1000, Thermo Scientific, Wilmington, DE, USA). Total RNA was treated with 0.1 U DNAse Amplification Grade (Thermo Fisher Scientific) for 15 min at 27°C to neutralize any DNA molecules. DNAse was inactivated with 1 µl ethylenediaminetetraacetic acid for 10 min at 65°C. Total RNA (200 ng) was reverse transcribed using the iScript™ cDNA Synthesis Kit (Bio-Rad, Des Plaines, IL, USA) at 25 °C for 5 min and 42 °C for 30 min The reaction was ended by incubation at 85 °C for 5 min.

Real-time qPCR was performed using CFX384™ Real-Time System (Bio- Rad Laboratories, Hercules, CA, USA) using 2 µl of complementary DNA (cDNA) and 8 µl of MIX containing forward and reverse bovine specific primers (Table 1), nucleases free water, and GoTaq® Master Mix (Promega Corporation, Madison). Amplification was performed with an initial denaturation at 95°C for 5 min followed by 40 cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. Samples were run in duplicate. To optimize the RT-qPCR assays, serial dilutions of cDNA templates were used to generate a standard curve, and efficiency between 90% and 110% and coefficient of determination (R^2) higher than 0.98 were considered. To select the most stable housekeeping gene H2A histone family, member Z (*H2AFZ*), cyclophilin B (*PPIB*), TATA-box binding protein (*TBP*), glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), and ribosomal protein S18 (*RPS18*) amplification profiles were compared using the geNorm software [26]. The results indicated that GAPDH was the most stable endogenous control for both blastocysts and granulosa cells. The expression of each target gene, relative to the expression of the reference genes, was calculated using the Pfaffl method with correction for amplification efficiency and normalized to a calibrator sample [27].

Table 1 – Details of primers used for gene expression analysis RT-qPCR

Gene Symbol	Primer sequence (5´ → 3´)	Accession Number
H2AFZ	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	XM_002686087.4

Gene Symbol	Primer sequence (5´ → 3´)	Accession Number
PPIB	F: GGTCATCGGTCTCTTTGGAA	NM_174152.2
	R: TCCTTGATCACACGATGGAA	
TBP	F: CAGAGAGCTCCGGGATCGT	NM_001075742.1
	R: CACCATCTTCCCAGAACTGAATAT	
GAPDH	F: GATTGTCAGCAATGCCTCCT	NM_001034034.2
	R: GGTCATAAGTCCCTCCACGA	
RPS18	F: CCTTCCGCGAGGATCCATTG	NM_001033614.2
	R: CGCTCCCAAGATCCAACTAC	
AREG	F: CTTTCGTCTCTGCCATGACCTT	NM_001099092.1
	R: CGTTCTTCAGCGACACCTTCA	
EREG	F: CTGCACAGCATTAGTTCAAACTGA	XM_010806226.3
	R: TGTCCATGCAAACAGTAGCCATT	
PTGS2	F: TGCTGAGTTTAACACGCTCTACCA	NM_174445.2
	R: TGAGACCATGTTCCAGTAAGACAGA	
CTGF	F: AGCTGAGCGAGTTGTGTACC	NM_174030.2
	R: TCCGAAAATGTAGGGGGCAC	
TNFAIP6	F: GCAAAGGAGTGTGGTGGTGTGTTT	NM_001007813.2
	R: ACTGAGGTGAATGCGCTGACCATA	
GADD45B	F: TACGAGTCGGCCAAGCTGAT	NM_001040604.1
	R: GTCCTCCTCTTCCTCGTCGAT	
TP53BP1	F: ATCAGACCAACAGCAGAATTTCC	NM_001206397.2
	R: CACCACGTCAAACACCCCTAA	
XRCC6	F: AATTGACTCCTTTTGACATGAGCAT	NM_001192246.1
	R: CCATAGAACACCACTGCCAAGA	
RAD51	F: ATGCACCGAAGAAGGAGCTAAT	NM_001046179.2
	R: GATCGCCTTTGGTGGAACTC	
BAX	F: GACATTGGACTTCCTTCGAGA	NM_173894.1
	R: AGCACTCCAGCCACAAAGAT	

Gene Symbol	Primer sequence (5´ → 3´)	Accession Number
BCL2	F: GTGGATGACCGAGTACCTGAAC	NM_001166486
	R: AGACAGCCAGGAGAAATCAAAC	
GLUT1	F: CCTTCACTGTCGTGTCGCTA	NM_174602.2
	R: GCCACAATGCTCAGGTAGGA	
GLUT3	F: GCCGCCGATAGAGGACATTT	NM_174603.3
	R: ATGGCGAAGATCAGAGGTGC	

3.3 STATISTICAL ANALYSIS

The effects of PAs on gene expression in GCs were assessed using a two-way analysis of variance (ANOVA). One-way ANOVA was used to test the effects of PAs on gene expression in blastocysts. The effects of PAs on cumulus expansion were investigated using a two-way repeated measures ANOVA analysis. Differences were significant when $P<0.05$. When a significant group effect was detected, comparisons were performed using the least-square means (LSMeans) method (R Package "emmeans" version 1.6.3) with Tukey-Kramer HSD test for multiple comparisons adjustment. The Shapiro-Wilk test was used to check the assumption of normality, and the Bartlett test was used to test the homogeneity of variances. The Box-Cox power transformation was used to transform data that did not follow a normal distribution or showed heteroscedasticity. All data are presented as means ± standard error of mean (SEM) and the level of significance was set at $P<0.05$. Cleavage and blastocyst rates were examined by a binomial generalized linear model, with log link. Risk ratios (RRs) and 95% confidence intervals (CIs) were used to present the result. All statistical analyzes were performed using the R 4.1.1 software (R Core Team, 2021, Vienna, Austria).

3.3.1 Experimental design

3.3.1.1 Experiment 1 – Effects of PAs on the periovulatory EGF-Like cascade genes in granulosa cells

To assess the effects of Senecionine and Seneciphylline in graded concentrations (0, 0.03, and 0.3 µM) on amphiregulin (AREG), epiregulin (EREG), prostaglandin-endoperoxide synthase 2 (PTGS2), and connective tissue growth factor (CTGF) mRNA expression, GCs were cultured as described in the section 3.2.1 and randomly distributed into the following

groups: Negative Control (Neg_Ctrl; without PAs and LH), Positive Control (Pos_Ctrl; without PAs), Senecionine 0.03 µM (Cion_0.03), Senecionine 0.3 µM (Cion_0.3), Seneciphylline 0.03 µM (Phyl_0.03), and Seneciphylline 0.3 µM (Phyl_0.3). At 6, 12, and 24 h post-treatment culture medium was removed and GCs were collected for total RNA extraction and RT-qPCR analyses. The main effects of group (PAs graded concentration), time (6, 12, and 24 h post-treatment), and their interaction were tested. This experiment was performed in three independent replicates, with each replicate using ovaries collected at different days.

3.3.1.2 Experiment 2 - Effect of PAs on cumulus-oocyte complexes expansion

To study the effect of senecionine or seneciphylline inclusion in graded concentrations (0, 0.03, and 0.3 µM) during in vitro maturation on COCs expansion, COCs (n=8/group/replicate) were randomly distributed into the following groups: Negative Control (Neg_Ctrl; without PAs, FSH, and LH), Positive Control (Pos_Ctr; without PAs), Senecionine 0.03 µM (Cion_0.03), Senecionine 0.3 µM (Cion_0.3), Seneciphylline 0.03 µM (Phyl_0.03), and Seneciphylline 0.3 µM (Phyl_0.3). COCs were individually (1 COC/well) matured in 50 µL of maturation medium in 96-well plates (Sarstedt, St-Leonard, QC, Canada). COCs from each treatment group were photographed using an inverted microscope (Nikon Eclipse TS-100; Nikon Instruments Inc., Melville, NY). Images of each COC were captured through Micrometrics SE Premium software (version 4.5.1, Unitron, Commack, NY, USA) at 0, 6, 12, and 24 h of maturation. Total surface area of each COC expressed in pixels was measured with ImageJ software (version 1.50, National Institutes of Health, Bethesda, MD, USA). The relative expansion (RE) of each COC at a given time point was calculated by dividing the area of the COC at that time point by the area at time 0. The main effects of group (PAs graded concentration), time (6, 12, and 24 h of IVM), and their interaction were tested. This experiment was performed in three independent replicates, with each replicate using ovaries collected at different days.

3.3.1.3 Experiment 3 - Effects of PAs added to the IVM medium on bovine embryo production and quality.

The experimental design used in IVM was similar to the experiment 2 with small modifications. COCs (n = 40 – 45/group/replicate) were matured in four-well plates (Nunc) containing 400 µL of maturation medium with graded concentrations (0, 0.03 µM, and 0.3 µM) of both PAs per well. The Neg_Ctrl group was not used.

Following IVM, oocytes were fertilized and presumptive zygotes were cultured in media free of PAs, as described in section *3.2.3*. To assess embryo production, cleavage rates were evaluated 48 hpi, and blastocyst rates were assessed 168 hpi. Blastocysts evaluated 168 hpi were rinsed three times in phosphate-buffered saline (PBS) and collected for total RNA extraction and RT-qPCR analyses. To asses embryo quality, mRNA expression of glucose-1-transporter (*GLUT1*), glucose-3-transporter (*GLUT3*), tumor protein p53 binding protein 1 (*TP53BP1*), RAD51 recombinase (*RAD51*), X-ray repair cross complementing 6 (*XRCC6*), B-cell lymphoma 2 (*BCL2*), and BCL2 associated X (*BAX*) were determined. This experiment was performed in three independent replicates, with each replicate using ovaries collected at different days.

3.3.1.4 Experiment 4 - Effects of PAs added to the IVC medium on bovine embryo production and quality.

The experimental design used in this experiment was similar to the experiment 3 with small modifications. No PAs were added to the medium during maturation or fertilization. Only after denudation (18-20 hpi), were the presumptive zygotes cultured in medium containing PAs at the same concentrations used in previous experiments. Embryo production and quality were assessed in the same manner as in experiment 3. This experiment was performed in five independent replicates, with each replicate using ovaries collected at different days.

3.4 RESULTS

3.4.1 mRNA relative expression of periovulatory EGF-Like cascade genes in bovine granulosa cells exposed to PAs

The transcription levels of all genes tested in the positive control group increased in a time-dependent manner following LH exposure, as expected for this model, whereas in the absence of LH stimulation (negative control group), none of the genes showed a significant rise in mRNA relative expression. When compared to the positive control group, the mRNA relative abundance of *EREG*, *PTGS2*, and *CTGF* genes at 12 h and *AREG* at 24 h was lower in groups treated with 0.3 µM of both PAs, senecionine and seneciphylline ($P<0.05$). Finally, 0.03 µM senecionine reduced transcription levels of the *PTGS2* gene at 12 h, whereas 0.03 µM seneciphylline reduced transcription levels of the same gene only at 24 h in comparison with positive control group ($P<0.05$; ***Figure 1***).

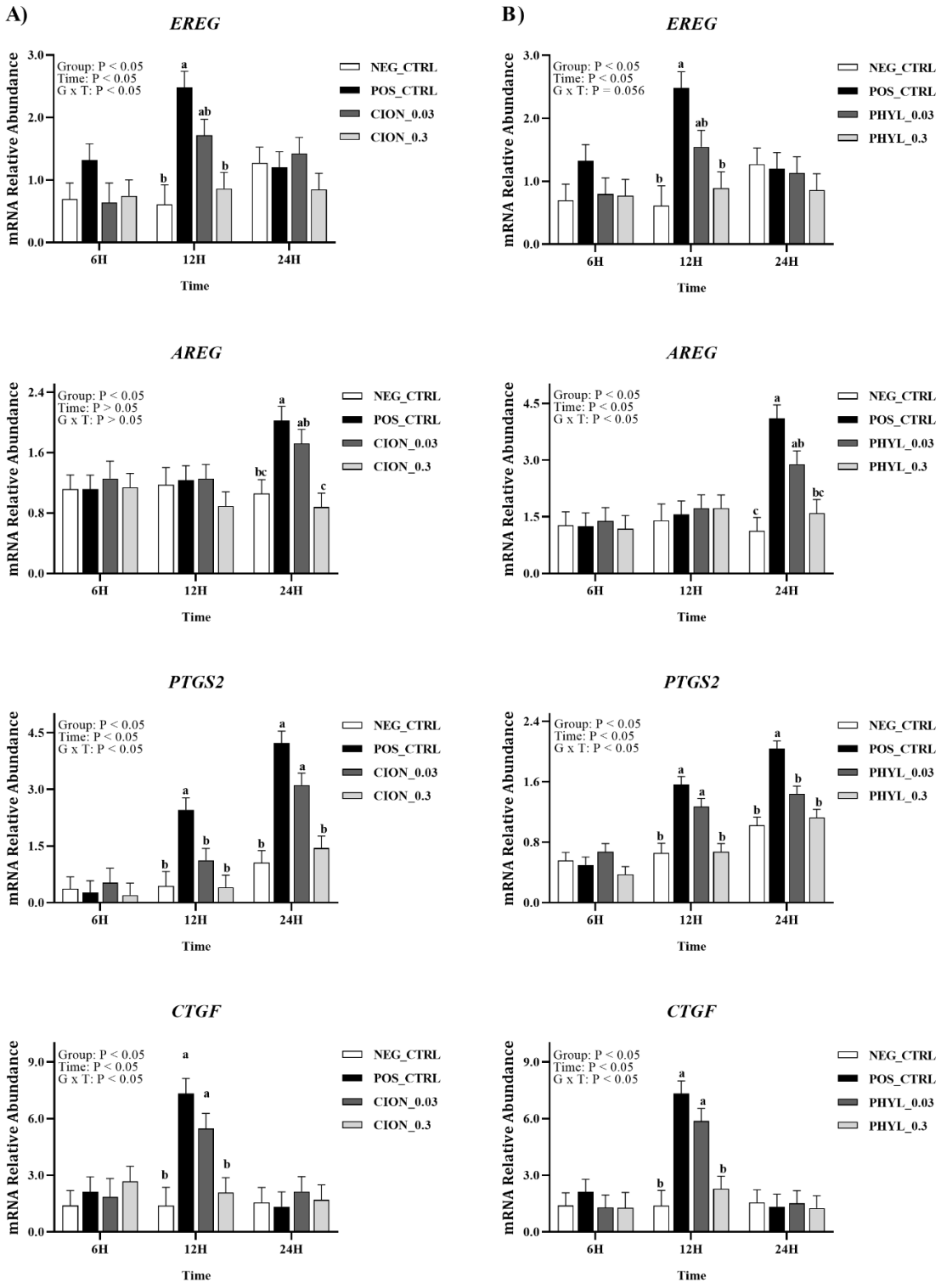

Figure 1 – Effect of Senecionine (A) or Seneciphylline (B) on expression of periovulatory EGF-Like cascade genes in bovine granulosa cells.

GC from large follicles (≥10 mm diameter) were cultured with serum for 24 h, and then in serum-free medium for another 18 h before addition of LH (100 ng/mL) and PAs (0, 0.03, and 0.3 μM) for the times given. The Neg_Ctrl group was cultured without PAs and LH. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for three independent replicate cultures. Main effects of group, time, and their interaction were determined by ANOVA and are shown in the respective figure. Different letters indicate statistical significance among groups in the same time point ($P < 0.05$; Tukey-Kamer HSD).

3.4.2 PAs have no effect on cumulus cell expansion in in vitro matured COCs.

The expansion of cumulus cells was not blocked by any of the pyrrolizidine alkaloid concentrations evaluated. Indeed, there was no significant difference in the relative expansion of any group up to 6 hours of IVM, but from 12 hours of IVM to the end of the maturation period all groups showed a greater relative expansion than the negative control group ($P < 0.05$), although they did not differ from one another (***Figure 2***).

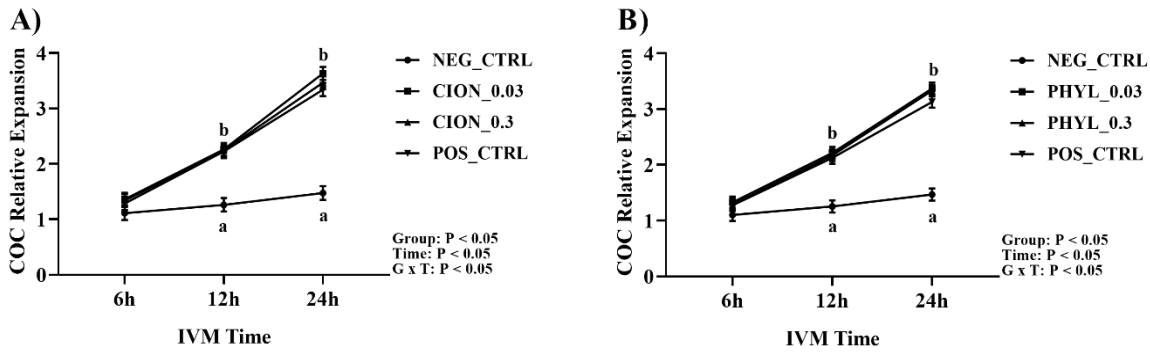

Figure 2 – Effect of graded concentrations (0, 0.03, and 0.3 µM) of A) Senecionine or B) Seneciphylline on cumulus cells expansion.
COCs (n=8/group/replicate) were individually maturated and photographed at 0, 6, 12, and 24 h of IVM. The Neg_Ctrl group was cultured without PAs, FSH, and LH whereas Pos_Ctrl group was cultured without PAs. Relative cumulus expansion was assessed by dividing total surface area of each COC at a given time by the respective area at time 0. Points represent the group mean ± SEM for three independent replicate cultures. Main effects of group, time, and their interaction were determined by ANOVA and are shown in the respective figure. Different letters show statistically significant differences between groups in the same time point ($P < 0.05$; Tukey-Kamer HSD)

3.4.3 Effects of PAs added to the IVM medium on bovine embryo production and quality.

In this experiment 759 oocytes were in vitro matured with senecionine or seneciphylline at three different concentrations (0, 0.03, and 0.3µM). After that, they were fertilized and cultivated without pyrrolizidine alkaloids. Cleavage rates were assessed 48 hpi, and blastocyst rates were assessed at 168 hpi (***Table 2***).

Table 2 – Cleavage rates at 48 hpi and blastocyst rates at 168 hpi of COCs treated with graded concentrations of pyrrolizidine alkaloids during in vitro maturation.

Maturation treatment	IVM oocytes (*N*)	Cleavage		Blastocysts	
		(*N*)	(%)	(*N*)	(%)
Control	126	106	84.13	39	30.95
Senecionine 0.03 µM	122	107	87.70	51	41.80
Senecionine 0.3 µM	123	110	89.43	36	29.27
Control	129	103	79,84	41	31,78

Maturation treatment	IVM oocytes (*N*)	Cleavage		Blastocysts	
		(*N*)	(%)	(*N*)	(%)
Seneciphylline 0.03 µM	132	109	82.58	57	43.18
Seneciphylline 0.3 µM	127	109	85.83	47	37.01

Data represents three independent replicates.

Senecionine (***Figure 3***A) and seneciphylline (***Figure 3***B) had no effect on cleavage rates (P>0.05) in oocyte groups treated during maturation, when compared to control groups. Likewise, all maturation-treated groups had no differences in blastocyst developing rates (P>0.05) on day 7 after in vitro fertilization, when compared to the control groups (***Figure 3***C; 3D).

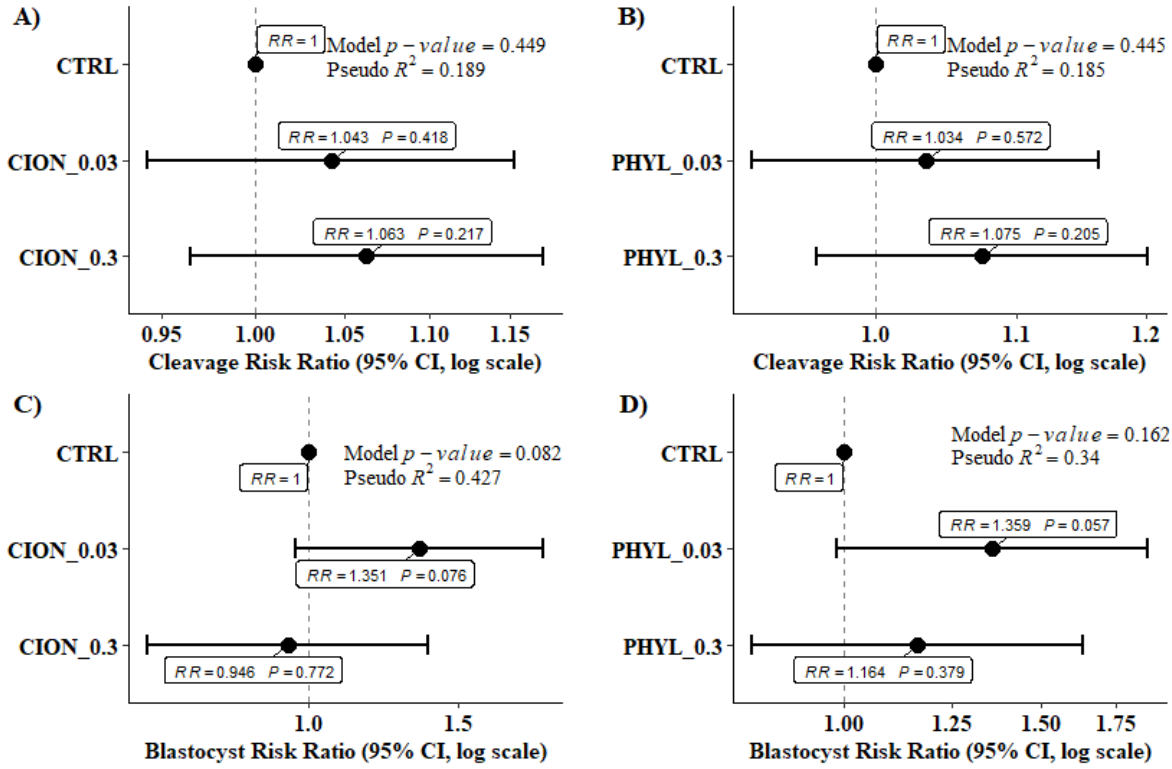

Figure 3 – Risk ratio for cleavage on day 2 (A; B) or blastocyst on day 7 (C; D) after in vitro fertilization of COCs treated with graded concentrations of pyrrolizidine alkaloids during in vitro maturation.
(A; C) Senecionine treated groups (0µM, 0.03 µM, and 0.3µM). (B; D) Seneciphylline treated groups (0µM, 0.03 µM, and 0.3µM). Dots represents RR and error bar represents 95% confidence interval of three independents replicates. RR: risk ratio. P: p-value.

Analyses of the transcription levels of genes linked to DNA repair, apoptosis, and energy metabolism were used to indirectly assess embryo quality.

Figure 4 shows the mRNA relative expression of some DNA double-strand repair related genes (*TP53BP1*, *RAD51*, and *XRCC6*) in blastocysts from groups treated with senecionine (***Figure 4***A) and seneciphylline (***Figure 4***B) during in vitro maturation. When compared to the control group, both 0.03 µM and 0.3 µM seneciphylline concentrations in the

maturation medium downregulated *TP53BP1* mRNA levels (P<0.05). The addition of seneciphylline in the maturation medium had no effect on the quantity of transcripts from other genes involved in double-stranded DNA repair. In comparison to the control group, adding senecionine to the maturation medium has no effect on the transcription levels of any of the DNA double-strand repair related genes tested.

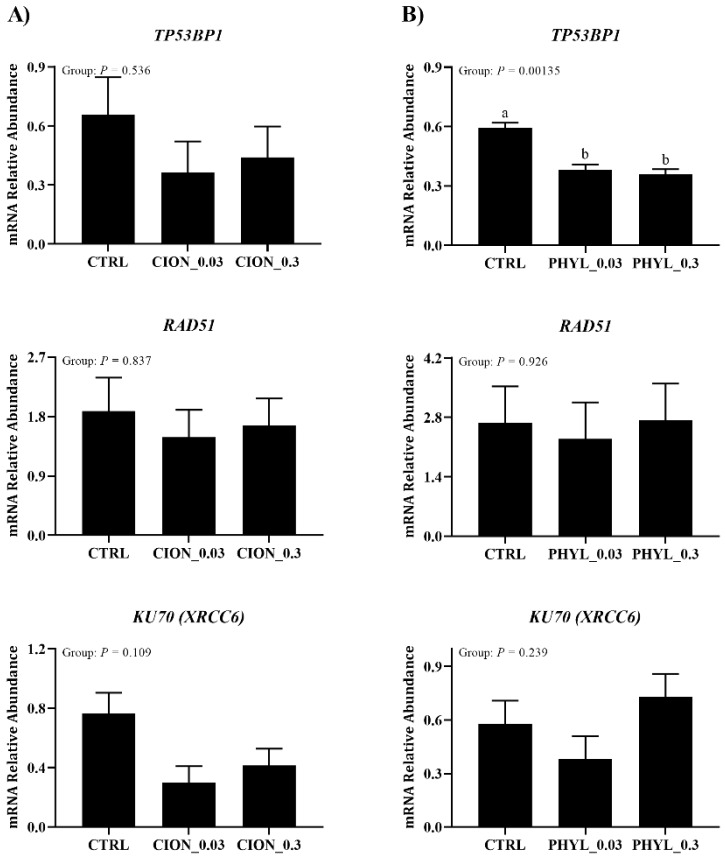

Figure 4 – Relative mRNA expression of genes associated with DNA double-strand repair in bovine embryos produced in vitro and treated with graded concentrations of pyrrolizidine alkaloids during in vitro maturation. A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for three independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups (P < 0.05; Tukey-Kamer HSD).

There was no difference in mRNA expression for BAX, BCL2, or the BAX/BCL2 ratio between groups treated with senecionine or seneciphylline during in vitro maturation and groups treated with no pyrrolizidine alkaloids (***Figure 5***).

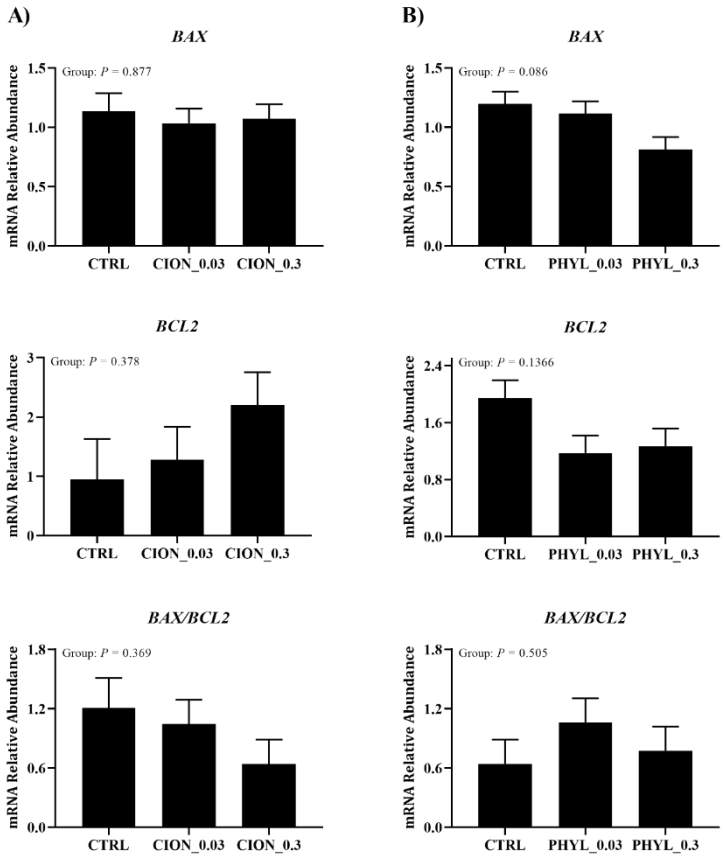

Figure 5 – Relative mRNA expression of BAX, BCL2 and BAX/BCL2 ratio in bovine embryos produced in vitro and treated with graded concentrations of pyrrolizidine alkaloids during in vitro maturation.
A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for three independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups ($P < 0.05$; Tukey-Kamer HSD).

The relative mRNA abundance of GLUT3 gene was lower in blastocysts from the group treated with 0.3 µM of senecionine (***Figure 6***A) in the maturation medium ($P < 0.05$) but neither 0.03 µM of senecionine, nor seneciphylline (***Figure 6***B) modulated mRNA expression of the GLUT1 or GLUT3 genes, when compared to their respective control groups.

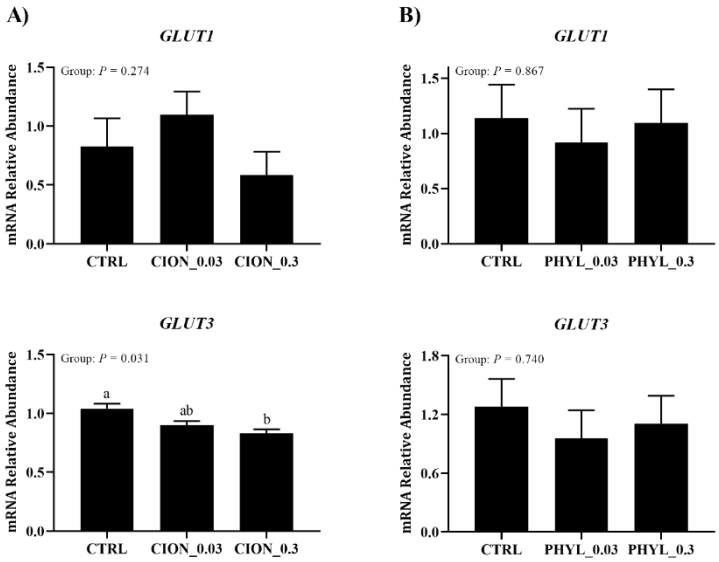

Figure 6 – Relative mRNA expression of GLUT1 and GLUT3 in bovine embryos produced in vitro and treated with graded concentrations of pyrrolizidine alkaloids during in vitro maturation.
A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for three independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups (P < 0.05; Tukey-Kamer HSD).

3.4.4 Effects of PAs added to the IVC medium on bovine embryo production and quality.

In this experiment, 1244 oocytes were in vitro matured and fertilized without pyrrolizidine alkaloids. They were then in vitro cultured at three different senecionine or seneciphylline concentrations (0, 0.03, and 0.3 µM) beginning at 18–20 hpi. Cleavage and blastocyst rates were assessed at 48 and 168 hpi, respectively. (***Table 3***).

Table 3 – Cleavage rates at 48 hpi and blastocyst rates at 168 hpi of COCs treated with graded concentrations of pyrrolizidine alkaloids added to IVC medium.

Maturation treatment	IVM oocytes (*N*)	Cleavage		Blastocysts	
		(*N*)	(%)	(*N*)	(%)
Control	209	164	78.47	63	30.14
Senecionine 0.03 µM	203	135	65.50	43	21.18
Senecionine 0.3 µM	195	118	60.51	27	13.85
Control	215	168	78.14	66	30.70
Seneciphylline 0.03 µM	212	137	64.62	40	18.87
Seneciphylline 0.3 µM	210	129	61.43	34	16.19

Data represents five replicates.

When pyrrolizidine alkaloids were added to the culture media at 18-20 hpi all of the treated groups had a lower rate of cleavage than the control groups (P<0.05). The cleavage

risk ratio with 0,03 µM senecionine was 0.848 [0.751 – 0.956; 95%IC] (***Figure 7***A). With 0.03 µM seneciphylline, the cleavage risk ratio was 0.827 [0.731 – 0.934; 95% IC] (***Figure 7***B). This effect was more pronounced when the highest concentrations of senecionine or seneciphylline were utilized. The cleavage risk ratio with 0,3 µM senecionine was 0.771 [0.674 – 0.881; 95%IC] (***Figure 7***A). With 0.3 µM seneciphylline, the cleavage risk ratio was 0.786 [0.691 – 0.893; 95% IC] (***Figure 7***B).

When senecionine or seneciphylline were added to the culture media 18-20 hpi, blastocyst rates on day 7 after fertilization were lowered in an even more pronounced way than the cleavage rate, as compared to groups cultured without pyrrolizidine alkaloids (P<0.05). The blastocyst risk ratio with 0,03 µM senecionine was 0.703 [0.502 – 0.983; 95%IC] (***Figure 7***C). With 0.03 µM seneciphylline, the blastocyst risk ratio was 0.615 [0.435 – 0.866; 95% IC] (***Figure 7***D). Here again, this effect was more pronounced when the highest concentrations of senecionine or seneciphylline were utilized. The cleavage risk ratio with 0,3 µM senecionine was 0.459 [0.305 – 0.689; 95%IC] (***Figure 7***C). With 0.3 µM seneciphylline, the cleavage risk ratio was 0.527 [0.365 – 0.761; 95% IC] (***Figure 7***D).

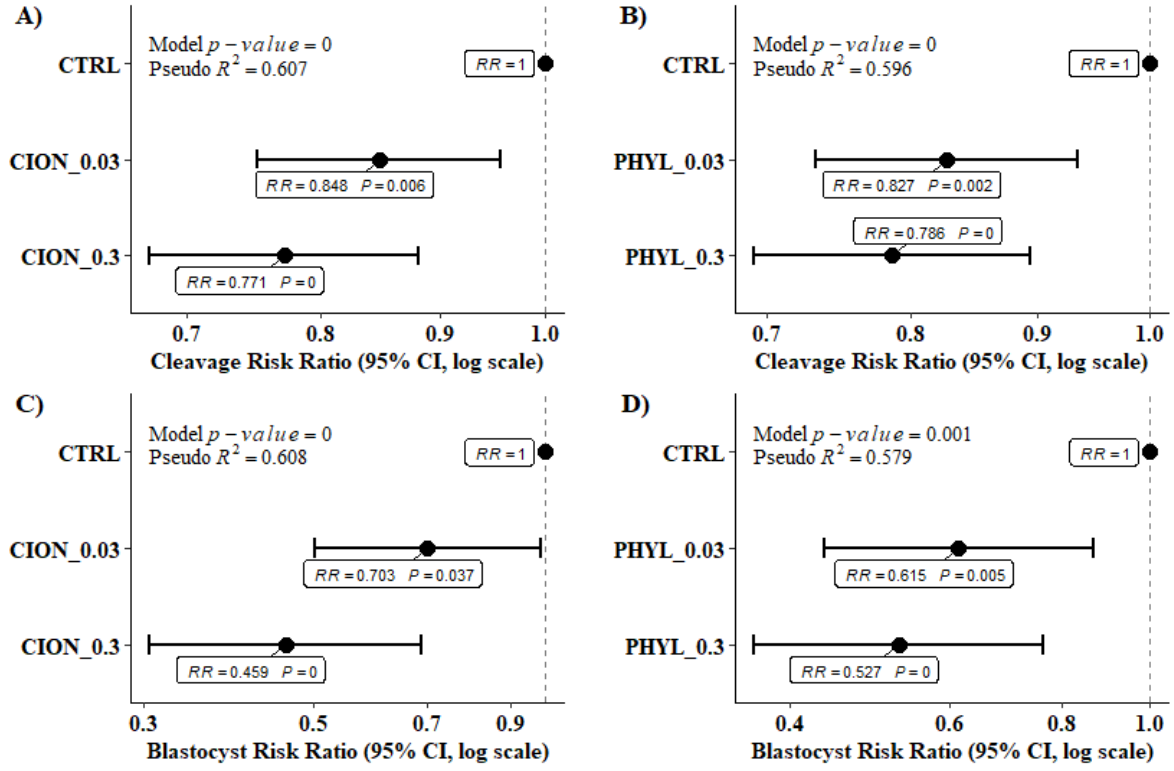

Figure 7 – Risk ratio for cleavage on day 2 (A; B) or blastocyst on day 7 (C; D) after in vitro fertilization of zygotes treated with graded concentrations of pyrrolizidine alkaloids added to the medium at 18-20 hpi.
(A; C) Senecionine treated groups (0, 0.03, and 0.3µM). (B; D) Seneciphylline treated groups (0, 0.03, and 0.3µM). Dots represents RR and error bar represents 95% confidence interval of five independents replicates. RR: risk ratio. P: p-value.

In this experiment, embryo quality was assessed using the same genes that were evaluated in the prior experiment.

Figure 8 shows the mRNA relative expression of some DNA double-strand repair related genes (*TP53BP1*, *RAD51*, and *XRCC6*) in blastocysts from groups treated with senecionine (***Figure 8***A) and seneciphylline (***Figure 8***B) 18-20 hpi. When compared to the control group, 0.3 µM seneciphylline concentration in the IVC medium, but not 0.03 µM seneciphylline concentration, upregulates *RAD51* mRNA levels ($P<0.05$). The addition of seneciphylline in the IVC medium had no effect on the quantity of transcripts from other genes involved in double-stranded DNA repair. In comparison to the control group, adding senecionine to the IVC medium has no effect on the transcription levels of any of the DNA double-strand repair related genes tested.

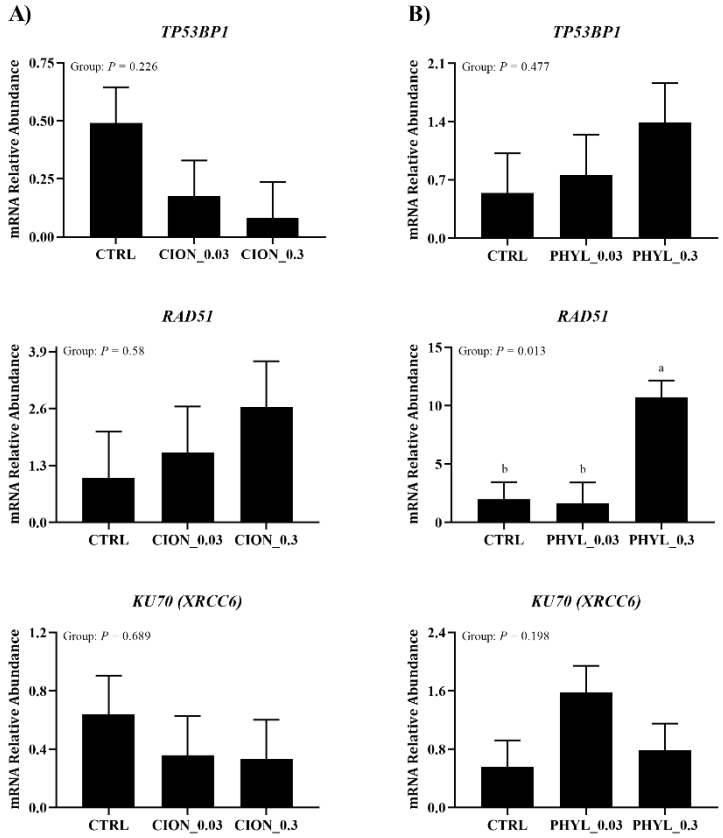

Figure 8 – Relative mRNA expression of genes associated with DNA double-strand repair in bovine in vitro produced blastocysts treated with graded concentrations of pyrrolizidine alkaloids added to the medium at 18-20 hpi.

A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for five independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups ($P < 0.05$; Tukey-Kamer HSD).

Figure 9 shows the mRNA relative expression of *BAX and BCL2* genes and the *BAX/BCL2* ratio in blastocysts from groups treated with senecionine (***Figure 9***A) and seneciphylline (***Figure 9***B) 18-20 hpi. When compared to the control group, 0.3 µM senecionine concentration in the IVC medium downregulates the anti-apoptotic gene *BCL2* (P<0.05), but 0.03 µM senecionine had no effect on the same gene expression. Senecionine showed no effect on *BAX* gene transcript levels or on the *BAX/BCL2* ratio when added to the IVC medium. Adding seneciphylline to the IVC medium had no influence on the transcription levels of *BAX* or *BCL2* genes, nor on the *BAX/BCL2* ratio, as compared to their respective control groups.

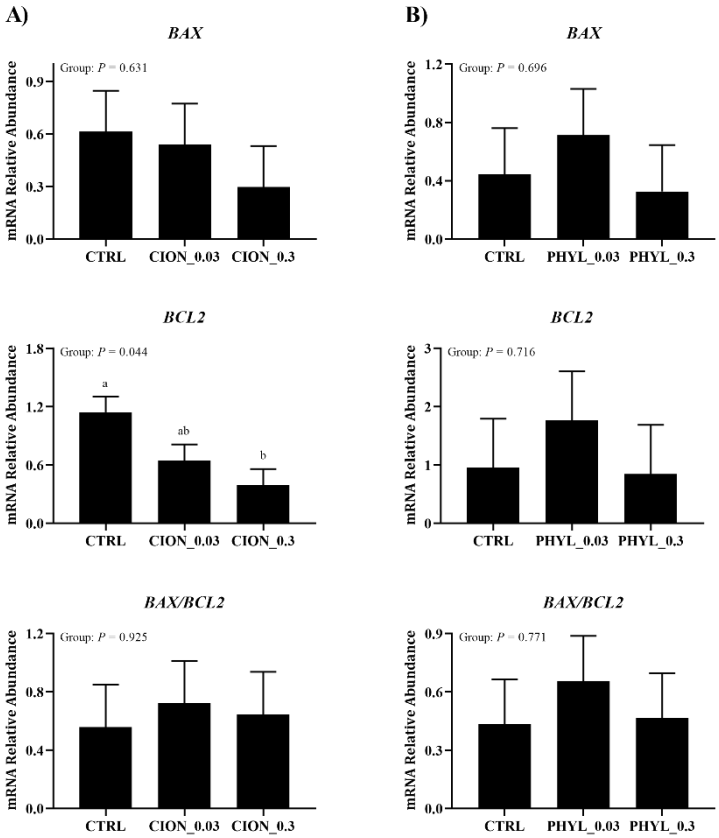

Figure 9 – Relative mRNA expression of BAX, BCL2, and bax/bcl2 ratio in bovine in vitro produced blastocysts treated with graded concentrations of pyrrolizidine alkaloids added to the medium at 18-20 hpi.
A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for five independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups (P < 0.05; Tukey-Kamer HSD).

There was no difference in mRNA expression of the *GLUT1* or *GLUT3* genes between groups treated with senecionine or seneciphylline after denudation and groups treated with no pyrrolizidine alkaloids, as shown in ***Figure 10***.

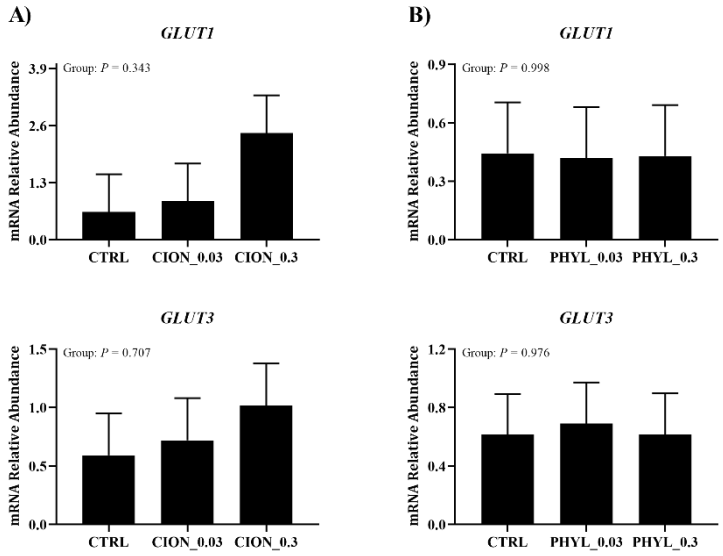

Figure 10– Relative mRNA expression of GLUT1 and GLUT3 in bovine in vitro produced blastocysts treated with graded concentrations of pyrrolizidine alkaloids added to the medium at 18-20 hpi.
A) Senecionine-treated groups (0, 0.03, and 0.3µM). B) Seneciphylline-treated groups (0, 0.03, and 0.3µM). The Control group was cultured without PAs. Messenger RNA abundance was measured by real-time qPCR and normalized to the reference gene GAPDH. Bars represent the group mean ± SEM for five independent replicate cultures. The effect of group was determined by ANOVA and is shown in the respective figure. Different letters indicate significant differences among groups ($P < 0.05$; Tukey-Kamer HSD).

3.5 DISCUSSION

This study tested the hypothesis that low concentrations of pyrrolizidine alkaloids, insufficient to cause chronic seneciosis, could act directly on ovarian cells and/or embryos, affecting the periovulatory Epidermal Growth Factor-Like (EGF-Like) cascade and early embryonic development.

Our significant findings are: (1) Senecionine as well as seneciphylline downregulates expression of periovulatory EGF-Like cascade genes in bovine granulosa cells in a time and dose dependent manner; (2) cumulus cells expansion was not blocked by any of the pyrrolizidine alkaloid concentrations evaluated; (3) when added to maturation medium PAs don’t alter embryo production, however both cleavage and blastocyst rates decrease when senecionine or seneciphylline were added to IVC medium; (4) PAs downregulates the transcript level of genes related to embryonic energy metabolism, DNA repair and apoptosis in blastocysts, suggesting that blastocysts that had contact with PAs have lower developmental competence; and (5) low concentrations of both senecionine and

seneciphylline can act directly on bovine granulosa cells and embryos, disrupting some reproductive processes in vitro.

Ovulation is a complex process that is initiated by the luteinizing hormone surge and is controlled by the temporal and spatial expression of specific genes. LH triggers a cascade of signaling processes that spread throughout the preovulatory follicle, culminating in the ovarian follicle wall rupture and the release of an oocyte into the oviduct for fertilization [28,29]. This cascade, however, requires the activation of EGF receptor (EGFR). When the EGFR is activated, the cumulus expresses *AREG* and *EREG* and increase PTGS2 levels. In mural granulosa cells, the similar loop occurs, with *AREG* and *EREG* working in an autocrine manner to intensify the cascade [21,30–32]. It has been shown that the transcriptional activity of Yes-associated protein (*YAP*), an effector of the Hippo pathway, regulates the EGF signaling cascade in bovine mural granulosa and cumulus cells and directly interferes with the processes of cumulus expansion and ovulation that are triggered by LH [22,33]. In this study, we used a previously described granulosa cell culture system [21,22] in which abundance of mRNA encoding the EGF-like factors *EREG* and *AREG*, and other critical preovulatory genes is acutely upregulated by LH. Our results clearly indicate that inclusion of 0.3 µM of senecionine or 0.3 µM of seneciphylline to the culture medium effectively blocked the effect of LH on *EREG* and *CTGF*, mRNA abundance at 12 hours, on PTGS2 transcripts from 12 hours, and *AREG* mRNA abundance at 24 hours. Similarly, *PTGS2* mRNA abundance was blocked with 0.03 µM of both PAs. In mice, disruption of the EGF-Like cascade impairs ovulation, indicating that the activation of this pathway is essential for LH-induced ovulation to occur [34]. The importance of *PTGS2* in ovulation also has been demonstrated. *PTGS2*-null mice failed to ovulate [35] whereas in cattle, the intrafollicular injection of a *PTGS2*-selective inhibitor inhibited ovulation [36]. In the same way, it has been demonstrated that *YAP* signaling is critical for expression of EGFR and downstream target genes in bovine granulosa cells in vitro [22]. Overall, our findings suggest that even low concentrations of senecionine and seneciphylline can impair ovulation in cattle.

In most mammals, before ovulation, cumulus cells synthesize a large amount of the polysaccharide hyaluronan (HA), which is organized into a highly hydrated muco-elastic matrix. This process is called cumulus expansion or mucification [37]. In vitro, the remotion of COC from the follicle is sufficient to lead to cumulus expansion, whereas in vivo, this process is triggered by LH surge. Cumulus expansion is dependent on the expression of EGF-like ligands, *PTGS2*, and *YAP* in granulosa cells, however these genes are also expressed in

484 cumulus cells when stimulated by FSH [33,38]. Intriguingly, none of the pyrrolizidine
485 alkaloid concentrations tested in our experiment prevented cumulus cells expansion. These
486 findings, indeed, contradict what we found in the granulosa cell experiment, in which we
487 demonstrated that PAs inhibit the key genes for cumulus expansion. We suppose that this
488 discrepancy could be due to the in vitro cumulus expansion models used herein, which
489 employ supraphysiological levels of FSH, LH, and fetal calf serum during the entire course of
490 the experiment, and that may be masking the effects of PAs.
491 The ability of a blastocyst to establish a pregnancy and generate a living offspring is
492 the strongest indicator of its quality. Because it is impossible to transfer every embryo, the
493 achievement of the blastocyst stage and morphological appearance are commonly employed
494 as measures of oocyte developmental capacity. For the same reason, other ways of
495 determining the quality of a blastocyst are used, such as cell counts, cryotolerance, and gene
496 expression patterns [39,40]. In this study, gene expression analyses were used to access
497 embryo quality.
498 Cell repair machinery is responsible for protecting the genome from endogenous and
499 exogenous effects that induce DNA damage, furthermore DNA repair during the early stages
500 of embryonic development has one of the most significant effects on embryonic fate [41].
501 DNA double-strand breaks (DSB) are the most deleterious type of damage and can
502 substantially alter genetic integrity [42]. The presence of DSBs can alter the kinetics of
503 embryo cell cleavage and development to the blastocyst stage, therefore only embryos with
504 less DNA damage and/or superior capacity for DNA repair are able to achieve the blastocyst
505 stage [43]. DNA repair and cell cycle checkpoint genes are up-regulated in late-cleaving
506 embryos [43,44].
507 BCL2 family members regulate the intrinsic apoptotic pathway and may be
508 considered as good markers of developmental potential. Thus, the balance between pro-
509 apoptotic and anti-apoptotic factors can determine whether and when a cell becomes
510 apoptotic. BCL2 protein is considered an anti-apoptotic agent whereas BAX protein acts as
511 pro apoptotic [45]. The expression of *BCL2* gene is increased in bovine and pig embryos with
512 good morphological quality [46,47] and in non-fragmented mouse blastocysts [45].
513 Glucose transporters (GLUT) carry glucose through COCs and embryos in a passive,
514 energy-independent manner during culture [46]. *GLUT-3* is one of the key isoforms of
515 glucose transporters and, in conjunction with *GLUT-1* are the main isoform during bovine
516 early embryo development. The association between a faster growth rate and higher rates of

517 expression of *GLUT*-1 and *GLUT-3* genes, may enhance embryo metabolism and
518 developmental competence [48]. Embryos with a higher relative abundance of *GLUT-3*
519 compared to their counterparts has significantly high levels of development to the blastocyst
520 stage [49].

521 In our experiments only four of evaluated genes were affected by senecionine or
522 seneciphylline. When PAs were added to the maturation medium, there were no differences in
523 cleavage or blastocyst rate, but the senecionine-treated group (0.3 µM) showed lower
524 blastocyst expression of *GLUT-3* mRNA, implying that these blastocysts are less competent.
525 On oocytes matured with Seneciphylline (0.3 and 0.03 µM), blastocyst *TP53BP1* mRNA
526 abundance decreases during maturation. The cellular response to DSB is regulated by
527 *TP53BP1*, which directs repair to the NHEJ pathway. The *BRCA1* modulator, on the other
528 hand, increases *TP53BP1* dephosphorylation throughout the S/G2 phases of the cell cycle,
529 directing repair to the HR pathway. Seneciphylline (0.3 µM) in IVC medium up-regulated the
530 expression of *RAD51*, another integrant of the HR pathway [50]. One of the characteristics of
531 PAs is their ability to form adducts with proteins and nucleic acids [51,52]. These findings
532 suggest that seneciphylline may have caused DNA damage to the embryo. Finally, adding 0.3
533 µM senecionine to the IVC medium reduced the amount of *BCL2* transcripts. This means that
534 there may be an imbalance between the regulators of the intrinsic apoptosis pathway and that
535 anti-apoptotic mechanisms may not operate effectively, favoring apoptosis.

536 In conventional in vitro bovine embryo production laboratory, the cleavage rate ranges
537 between 75% and 90%, whereas the blastocyst rate ranges between 30% and 40% [25,39,44].
538 In this study, the cleavage rate of the control groups ranged from 78.14% to 84.13%, whereas
539 the blastocyst rate ranged from 30.14% to 31.78%. Both PAs, senecionine and seneciphylline,
540 decreased cleavage and blastocyst rate in treated groups when added to IVC medium, but not
541 during maturation. As a result, the presumptive zygote cultured in 0.03 µM senecionine had
542 15.2% less risk of cleavage and 29.7% less risk of developing into a blastocyst when
543 compared to control group. In its turn, the putative zygote cultured in 0.3 µM senecionine had
544 22.9% less risk of cleavage and 54.1% less risk of developing into a blastocyst when
545 compared to control group. When the presumptive zygote was cultured with 0.03 µM
546 seneciphylline, it had 17.3% less risk of cleavage and 38.5% less risk of becoming a
547 blastocyst, compared to the control group. Finally, the presumptive zygote cultured in 0.3 µM
548 seneciphylline had 21.4% less risk of cleavage and 47.3% less risk of becoming a blastocyst,
549 compared to the control group. The results of the genic expression and the embryonic

production, taken together, suggest that the blastocysts formed in the treated groups are not only less numerous, but also have a lesser developmental capacity than their control counterparts.

Despite variability in pyrrolizidine alkaloids sensitivity between species or even between tissues, cytochrome P450 bioactivation is needed for toxicity. Therefore, in models where cells are unable to bioactivate PAs, exogenous pre metabolization is required to observe the effects of the PAs. Consequently, without metabolism, no relevant effects were observed up to the highest tested concentrations (500 µM) in human HUVEC, whereas cells incubated with PA after preincubation using liver homogenate showed treatment-induced responses, both cytotoxity (300 µM) and alterations of prostanoid synthesis (200 µM) [53]. In the same way, in vitro experiments showed no cytotoxicity to freshly isolated mouse LSECs up to 500 µM senecionine. However, metabolic activation of senecionine by preincubation with primary mouse hepatocytes increased the cytotoxicity to cultivated LSECs with an EC_{50} of approximately 22 µM [54]. Senecionine and seneciphylline were found to have a detrimental effect on the in vitro embryo quality and production and on granulosa cell culture groups in this investigation. These findings show that APs are metabolically activated by granulosa and embryo cells, which suggest that they can directly affects these cells even if no hepatic metabolization takes place.

In summary, this study demonstrates that both senecionine and seneciphylline have significant toxicological effects in a bovine granulosa cell model as well as in vitro produced bovine embryos, suggesting that pyrrolizidine alkaloids, even at low concentrations, have the potential to directly affect cattle reproductive physiology.

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Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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4 ARTIGO II

ARTIGO SUBMETIDO PARA PUBLICAÇÃO

EXPRESSION PROFILE OF KEY GENES INVOLVED IN DNA REPAIR MECHANISMS IN BOVINE CUMULUS CELLS CULTURED WITH BOVINE SERUM ALBUMIN OR FETAL CALF SERUM

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Expression profile of key genes involved in DNA repair mechanisms in bovine cumulus cells cultured with bovine serum albumin or fetal calf serum

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4.1 ABSTRACT

Cumulus cells from cumulus-oocyte complexes (COC) matured in vitro in serum-free medium show high incidence of apoptosis and DNA double-strand breaks (DSB). This study aimed to characterize the transcript expression profile of selected genes involved in DNA repair mechanisms in bovine cumulus cells cultured with bovine serum albumin (BSA) or fetal calf serum (FCS). Briefly, bovine cumulus-oocyte complexes were in vitro matured with either, 0,4 % BSA or 10% FCS for 3, 6, 12 or 24 hours. The total RNA of cumulus cells was used for real-time PCR analysis. The mRNA expression was regulated during the 24 h of IVM for *XRCC6, XRCC5, DNAPK, GAAD45B, TP53BP1, RAD50, RAD52, ATM* and *BRCA2* target genes ($P < 0.05$). Although, an interaction between protein source (FCS or BSA) and time was not detected ($P \geq 0.05$). Cumulus cells from COCs matured with BSA presented higher mRNA expression of two genes compared to FCS group: *TP53BP1* at 6 h and *BRCA1* at 3, 6, 12 and 24 h ($P < 0.05$). In summary, our results showed for the first time the expression profile of the key genes involved in DSB repair mechanisms in cumulus cells obtained from bovine COCs matured with FCS or BSA. The higher mRNA expression of *BRCA1* and *TP53BP1* and lower mRNA expression of *TNFAIP6* suggests an increase in apoptosis rate and DNA damage in cumulus cells cultured in BSA-supplemented medium and may explain, at least to some extent, the reduced developmental potential of bovine oocytes matured in serum-free medium.

Keywords: In vitro maturation, cumulus cells, DNA repair, apoptosis.

4.2 INTRODUCTION

In vitro maturation (IVM) of oocytes is the major approach in assisted reproductive technologies in cattle, humans and other species [1]. It was designed to obtain mature oocytes

following culture of immature cumulus–oocyte complexes (COC) collected from antral follicles [2]. Despite its widespread use, the efficacy of IVM is lower than that observed for in vivo matured oocytes. Therefore, more detailed information on the molecular pathways that control cumulus cell expansion and COC maturation are needed to improve IVM protocols [1,2].

Cumulus cells plays a fundamental role in proper oocyte maturation and acquisition of further developmental competence. Denuded oocytes when submitted to IMV showed lower competence for embryonic development than cumulus-enclosed oocytes [3]. Cumulus-oocyte communication mechanisms are established and involve bidirectional exchanges through gap junctions and paracrine signaling regulating this phenomenon. Gap junctions from COCs allow the exchange of the small molecules, such as cAMP and cGMP that fundamentally regulate the process of oocyte maturation, directing oocyte progression through meiosis. In addition, the oocyte secretes growth-differentiation factor 9 (*GDF9*) and bone morphogenetic protein 15 (*BMP15*), which are paracrine factors that regulate the biological function of cumulus cells and orchestrates many of the associated local signaling events around ovulation, which is the key to establishing oocyte competence to sustain early embryo development [3–5]. However, this communication is under stress in IVM COCs when compared to those in vivo matured [5].

The combined actions of FSH/cAMP and oocyte-secreted factors (*BMP15* and *GDF9*) induce epidermal growth factors receptors (*EGFR*) activation in COCs [6]. Activation of *EGFR* signaling in cumulus cells, together with cAMP priming, triggers oocyte nuclear maturation and acquisition of developmental competence as well as cumulus expansion, which are crucial for ovulation [7]. In cattle, granulosa cells stimulated by LH, and cumulus cells stimulated by FSH, produce epidermal growth factors *(EGFs)-Like*, mainly epiregulin (*EREG*) and amphiregulin (*AREG*). When *EGFs-Like* binds to *EGFR* expressed in granulosa

and cumulus cells, they stimulate their own synthesis, the synthesis of prostaglandin synthetase 2 (*PTGS2*) and prostaglandin E2 (*PGE2*), as well as the expression of steroidogenesis and cumulus expansion genes [8]. *PGE2* also plays a role in the ovulation process by inducing the synthesis of EGF-like proteins in a similar way as LH does [9]. *EGF-like*, *PTGS2*, and *PGE2* work together to increase the production of tumor necrosis factor 6 inducing protein (*TNFAIP6*), hyalurone synthetase 2 (HAS2), and pentraxin 3 (*PTX3*), which are responsible for cumulus expansion [7,9].

Culture conditions during in vitro embryo production (IVP) can impact on the developmental potential of the early embryo. The lower quality of these embryos is due to changes in gene expression patterns because of interaction with the adverse environment generated by in vitro conditions [10]. IVM protein supplementation can have profound effects on development rate and overall developmental efficiency, as demonstrated by morula and blastocysts yield [11]. Most IVM procedures use serum to improve blastocyst developmental rate in cattle, but this has become less attractive because it is associated with a number of problematic issues. In addition to scientific disadvantages such as unknown exact composition, seasonal and geographical variability between batches, serious safety concerns for both laboratory staff and animals due to the risk of transmitting diseases potentially present in bovine serum, as well as ethical concerns about fetal distress have been raised with regard to the use of fetal bovine serum [12]. Therefore, the goal of IVP it is to develop optimal conditions for IVM in serum-free media and bovine serum albumin is the most widely used protein source for serum replacement. Unfortunately, the absence of serum in IVM medium results in reduced blastocyst rate [13–15], lower degree of cumulus expansion [15,16] and higher incidence of apoptosis and DNA fragmentation in cumulus cells [17].

To maintain genomic integrity, DNA must be protected from damage induced by environmental agents or generated spontaneously during DNA metabolism [18]. Among the

different types of DNA damage induced by environmental genotoxins, DNA double-strand breaks (DSB) are the most deleterious type of damage and can substantially alter genetic integrity [19]. Repair of DSBs is mainly coordinated by two molecular pathways: homologous recombination (HR) and non-homologous end-joining (NHEJ). Whereas HR is known as the error-free repair pathway because it uses the sister chromatid as a template for repair, NHEJ is referred to as the error-prone pathway since it does not depend on any template [20]. Growth arrest and DNA damage inducible beta (*GADD45B*) and tumor suppressor P53-binding protein 1 (*TP53BP1*) are mediators of DSB repair which display a complex array of physical interactions with other cellular proteins and, in some cases, these systems interfere with the cell cycle and can activate specific checkpoints in order to repair the damage or induce apoptosis when cell protection is not possible [21–23]. COCs in vitro matured in serum-free media have a high rate of apoptosis in cumulus cells and DSB repair mechanisms can be compromised under these cell culture conditions. The presence of mRNA encoding genes implicated in these processes has been demonstrated in human [24], mouse [19], swine [20], and bovine [25,26] oocytes, as well as in human [27], and rat [28] cumulus cells. However, there is no information about the expression of genes involved in HR and NHEJ pathways in bovine cumulus cells.

Clearly, coordinated bilateral interaction between the cumulus cells and the oocyte is essential for the full ability of both cell types to promote early embryogenesis. Thus, the information stored in the cumulus cells may be direct markers for the further developmental fate of the oocyte. Therefore, gene expression patterns in biopsied cumulus cells might actually serve as non-invasive markers to evaluate oocyte maturation. Changes in oocyte maturation conditions are associated with modifications in the expression profile of several key genes in cumulus cells, since bovine cumulus cells isolated from in vitro matured COCs have a different transcriptomic signature than cumulus cells isolated from in vivo matured

COCs [29]. This is why the profile expression of DNA repair genes in bovine cumulus cells is an intriguing question that could provide knowledge to improve the efficiency of IVM. Thus, the aims of this study were to: (1) characterize the transcript expression profile of selected genes involved in DNA DSB repair mechanisms in bovine cumulus cells obtained from COCs in vitro matured; and (2) investigate whether supplementing IVM medium with bovine serum albumin (BSA) or fetal calf serum (FCS) affects transcript expression of these genes.

4.3 MATERIAL AND METHODS

All reagents were purchased from Sigma-Aldrich Co. (Rocklin, CA, USA) unless otherwise stated.

4.3.1 In vitro maturation of Cumulus-oocyte complexes

Cow ovaries were obtained from a local abattoir and transported to the laboratory in saline solution (0.9% NaCl) containing 100 IU/mL penicillin and 50 µg/mL streptomycin sulfate at 25ºC. Cumulus oocyte complexes (COCs) from 3 to 8 mm diameter follicles were aspirated with a vacuum pump (vacuum rate of 15mL of water/minute) and pooled in a 15 mL conical tube. After sedimentation, COCs were recovered and selected according to Leibfried & First [30] under a stereomicroscope. Three independent replicates of grade 1 or 2 COCs (n=15/group/replicate), with each replicate using ovaries collected at different days, were randomly transferred to 4-well plates into 400 µL of maturation medium with bovine serum albumin (BSA) or fetal calf serum (FCS) and cultured in an incubator at 38.5ºC in a saturated humidity atmosphere containing 5% CO2 and 95% air. The maturation medium used was TCM199 containing Earle's salts and L-glutamine supplemented with 25 mM HEPES, 0.2 mM pyruvic acid, 2.2 mg/mL sodium bicarbonate, 5.0 µg/mL LH (Lutropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), 0.1 µg/mL FSH (Folltropin-V, Bioniche Animal Health Canada Inc., Belleville, ON, Canada), 100 IU/mL penicillin, 50 µg/mL

streptomycin sulfate and 0.4% fatty acid-free BSA (BSA group) or 10% FCS (FCS group). COCs were cultured for 3, 6, 12 or 24 hours and cumulus cells were recovered by vortexing, collected into Trizol and stored at -80ºC until RNA extraction.

4.3.2 Nucleic acid extraction and qPCR

Total RNA was extracted using Trizol® (Thermo Fisher Scientific Inc. Waltham, MA, USA) according to the manufacturer's instructions. The concentration and purity of total RNA extracts were evaluated using a spectrophotometer (NanoVue, GE Healthcare, Cambridge, England). Total RNA (200 ng) was first treated with gDNA Wipeout Buffer (Qiagen, Mississauga, ON, Canada) at 42°C for 2 minutes to digest any contaminating DNA. Then RNA was reverse transcribed (RT) in the presence of Quantiscript Reverse Transcriptase and RT primer mix (QuantiTec RT Kit® - Qiagen, Mississauga, ON, Canada) in a volume of 20 µL at 42°C for 15 minutes. The reaction was stopped by incubation at 95°C for 3 minutes. The cDNA was stored at −20°C until quantitative PCR (qPCR) analysis. Quantification of specific transcripts was performed by RT-qPCR using QuantiNova SYBR Green PCR Kit® (Qiagen, Mississauga, ON, Canada), and reactions were carried out using a Stratagene MX3005P apparatus (Agilent, Santa Clara, CA, USA). Common thermal cycling parameters (3 minutes at 95°C, 40 cycles of 15 seconds at 95°C, 30 seconds at 60°C, and 30 seconds at 72°C) were used to amplify each transcript. The reaction was performed in duplicate, and melting-curves analyzes were performed to verify product identity. The selected target genes were epiregulin (*EREG*), hyaluronan synthase 2 (*HAS2*), prostaglandin-endoperoxide synthase 2 (*PTGS2*), TNF alpha induced protein 6 (*TNFAIP6*), growth arrest and DNA damage inducible beta (*GADD45B*), tumor protein p53 binding protein 1 (*TP53BP1*), X-ray repair cross complementing 5 (*XRCC5*), X-ray repair cross complementing 6 (*XRCC6*), Protein kinase DNA-activated, catalytic subunit (*DNAPK*), ATM serine/threonine kinase (*ATM*), BRCA1 DNA repair associated (*BRCA1*), BRCA2 DNA repair associated (*BRCA2*),

RAD50 double-strand break repair protein (*RAD50*), RAD51 recombinase (*RAD51*) and RAD52 homolog DNA repair protein (*RAD52*). The genes H2A histone family, member Z (*H2AFZ*), cyclophilin B (*PPIB*), and TATA-box binding protein (*TBP*) were tested as reference genes. The primers (Table 1) were previously described for cattle. The amplification data were extracted from the Stratagene MX3005P apparatus, and each sample was analyzed through LinReg PCR software [31] for baseline correction and determination of qPCR efficiency. To select reference genes, geNorm software [32] was used. *PPIB* and *TPB* were selected as the best reference genes. The expression of each target gene relative to the expression of the reference genes was calculated using the Pfaffl method with efficiency correction and normalized to a calibrator sample [33].

4.3.3 Statistical analysis

All statistical analyzes were performed using the R 4.1.1 software (R Core Team, 2021, Vienna, Austria). For the mRNA expression, the assumption of normality was checked by Shapiro-Wilk test and homogeneity of variance was tested by Bartlett test. Data that did not follow normal distribution according to the Shapiro–Wilk test, or either, exhibit homoscedasticity according to the Bartlett test were transformed by Box-Cox power transformation. Thereafter, the data were analyzed using a linear model by an ANOVA for two-way factorial design (R Package "ExpDes.pt" version 1.2.1). The main effects of protein source (BSA and FCS), time (3, 6, 12, and 24 h of IVM) and the interaction were tested. In case of significant effects in the model ($p < 0.05$), comparisons were performed using the least-square means (LSMeans) method (R Package "emmeans" version 1.6.3) with Tukey-Kramer HSD test for multiple comparisons adjustment. All data are presented as means ± standard error of mean (S.E.M.) of three independent replicates.

4.4 RESULTS

The relative expression of *PTGS2, EREG, HAS2* and *TNFAIP6* mRNA was affected over the 24 h of IVM ($P < 0.05$) (Figure 1). Only maturation time effect was found for the expression of *PTGS2, EREG* and *HAS2*, regardless of protein source supplied. The relative abundance of *PTGS2* mRNA increased gradually up to 24 h of IVM (Figure 1A). A significant increase in *EREG* transcript abundance was detected at 6 h and 12 h of IVM as compared to the beginning (3 h) and the end (24 h) of IVM (Figure 1B). *HAS2* expression was characterized by a temporary increase at 6 h of IVM, followed by a significant decrease at 12 h of IVM and then remained relatively stable up to 24 h of IVM (Figure 1C). When evaluating *TNFAIP6* expression, significant effects of protein source, time and their interaction were detected (Figure 1D). The protein source effect indicated that *TNFAIP6* expression was 1.6-fold greater in cumulus cells cultured in FCS-supplemented medium than in those cultured in BSA-supplemented medium. The interaction between protein source and time was demonstrated by a marked increase in *TNFAIP6* expression in the FCS group between 12 and 24 h of IVM. Whereas the most significant increase in *TNFAIP6* transcript in the BSA group occurred only at 24 h of IVM. In addition, the FCS group had more *TNFAIP6* transcripts than the BSA group at 12 and 24 h of IVM.

As shown in Figure 2, although a significant ($P < 0.05$) effect of maturation time was detected, there was no interaction between protein source and time in *XRCC6, XRCC5, DNAPK, GADD45B* and *TP53BP1* mRNA expression. For *TP53BP1*, significant effects of protein source and time were detected (Figure 2A). There was a 1.33-fold higher level of *TP53BP1* expression in cumulus cells cultivated in BSA-supplemented medium compared to cumulus cells cultured in FCS-supplemented medium, indicating a protein source effect. The time effect was demonstrated by relatively stable *TP53BP1* expression between 3 and 6 h of IVM, followed by a progressive increase from 12 h of IVM, regardless of protein source

supplemented. Furthermore, cells supplemented with BSA showed higher expression of TP53BP1 than cells supplemented with FCS at 6 h of IVM. The abundance of *XRCC6* transcripts (Figure 2B) showed a significant increase only at 24 h of IVM, with no changes throughout the remainder of the culture period, regardless of the use of BSA or FCS in IVM. After a sudden drop at 6 h of IVM, *XRCC5* expression (Figure 2C) increased progressively and significantly until 24 h of IVM, irrespective of the protein source in the culture medium. *DNAPK* and *GADD45* exhibited almost the same mRNA expression pattern (Figures 2D and 2E). The abundance of both *DNAPK* and *GADD45* transcripts remained relatively unchanged until 6 h of IVM, increased significantly at 12 h of IVM and then maintained relatively constant until 24 h of IVM.

Expression of the six HR pathway mediators investigated is shown in Figure 3. *RAD50*, *RAD52*, *ATM* and *BRCA2* were expressed similarly over time (Figures 3A, 3C, 3D and 3F). After a decrease at 6 h of IVM, the expression levels significantly increased up to 24 h of IVM, indicating a time effect ($P < 0.05$) for these four genes. A protein source effect ($P < 0.05$) was found for *BRCA1*, as evidenced by a 1.45-fold higher expression of *BRCA1* in cumulus cells cultivated with BSA versus FCS-supplemented (Figure 3E), but there was no significant variation in *BRCA1* transcript abundance at different time points ($P > 0.05$). The abundance of *RAD51* transcripts remained relatively constant over the course of the IVM ($P > 0.05$), regardless of the protein source supplemented (Figure 3B). There was no interaction between protein source and time for any of the HR mediators studied ($P > 0.05$).

4.5 DISCUSSION

In this study, COCs were cultured in order to characterize the transcript expression profile of selected genes involved in DNA DSB repair mechanisms in bovine cumulus cells matured in vitro and to investigate whether supplementing IVM medium with BSA or FCS affects the abundance of these transcripts. COCs were collected at four separate IVM time

247 points, corresponding to the different stages of maturation progression [34]: 3 hours -
248 germinal vesicle; 6 hours - germinal vesicle breakdown; 12 hours - metaphase-I (because
249 oocytes with DNA damage are arrested at this stage [35]; 24 hours - metaphase-II.
250 Furthermore, each of these moments is associated with the in vitro expression of key genes of
251 the ovulatory cascade that are responsible for cumulus expansion. Thus, increased abundance
252 of *HAS2* is expected between 4 and 8 hours of IVM; *EREG* until 12 hours of *IVM*; and *PTGS2*
253 and *TNFAIP6* starting at 12 hours of IVM [1,36,37]. Since the expression of *EREG*, *HAS2*,
254 *PTGS2* and *TNFAIP6* mRNA in cumulus cells was associated with an increase in oocyte
255 developmental competence and embryo quality [29,38], the levels of mRNA encoding these
256 typical genes upregulated during the preovulatory cascade were assessed to confirm that our
257 IVM system was functional. In most mammals, the EGF signaling network regulates the
258 expression of key genes involved in gap junction closure and production of a large
259 extracellular matrix by cumulus cells, as well as the downregulation of the meiotic inhibitory
260 signaling network, which leads to cumulus expansion and oocyte meiotic maturation [9]. In
261 this study, transient upregulation of the *EREG* transcript was followed by brief *HAS2*
262 upregulation and persistent *PTGS2* and *TNFAIP6* upregulation, as expected. The mRNA
263 expression patterns of these genes over time were consistent with those previously published
264 in cattle COCs [1]. Therefore, it confirms that COCs responded to the IVM protocol and that
265 the dynamic bidirectional COC communication was preserved.

266 Repair of DSBs involves two pathways, homologous recombination (HR) or non-
267 homologous end-joining (NHEJ). The HR commonly occurs during the S and G2-M phases of
268 the cell cycle [21,39]. A complex of proteins including RAD50, NSB1 and ATM generate
269 single-stranded overhangs at the point of DNA damage, which are then used to match
270 homologous sequences in the sister chromatid. The DNA repair is then affected by multiple
271 mediator proteins including RAD51, RAD52, BRCA1 and BRCA2 [40]. The tumor

272 suppressor protein BRCA1 is a crucial member of the ATM-mediated DSB repair family. It
273 plays an important role in maintaining genetic integrity via interaction with a number of other
274 proteins, including MRE11, RAD50 and BRCA2 [41,42].

275 During NHEJ, Ku70 and Ku80 (products of the *XRCC6* and *XRCC5* genes,
276 respectively) are pivotal proteins involved in DNA damage repair. Ku70 cooperates with
277 Ku80 to form Ku heterodimer, which initiates NHEJ pathways in DSBs repair. Ku
278 heterodimer bind to DNA ends and recruit the *DNAPK*. Once bound, *DNAPK* activates its
279 own catalytic subunit (*DNAPKcs*) and further enlists the endonuclease *Artemis*. At a subset of
280 DSBs, *Artemis* removes excess single-strand DNA and generates a substrate that will be
281 ligated by *DNA-ligase-IV*. NHEJ involves blunt-end ligation independent of sequence
282 homology. This pathway can occur throughout the cell cycle but is dominant in G0/G1 and
283 G2 stage of the cell cycle [20,43]. Recently, Ku70 have been proved crucial in some
284 fundamental cellular processes, metabolism, ageing, and related diseases, such as premature
285 ovarian insufficiency, cancers, diabetes, neurodegenerative and cardiovascular diseases [44–
286 47]. In hepatocarcinoma cell-lines, reduction in mRNA of these three genes coding the NHEJ-
287 initiating proteins was related to the stress caused by serum starvation compared to serum-fed
288 cells [48]. In this work, the temporal expression pattern of the DSB repair genes followed a
289 similar trend across time: a decrease or steady abundance of transcripts between 3 and 6 h of
290 IVM, followed by an increase from 6 h onwards. The highest relative abundance of RNA was
291 observed at 24 hours of IVM. The *BRCA1* expression was an exception, since it did not
292 change over the course of maturation.

293 Regarding the source of protein added to IVM medium, differences were found in the
294 expression of only three genes: *TNFAIP6*, *BRCA1* and *TP53BP1*.

295 Optimal expansion of the cumulus mass seems to be important for ovulation in mice
296 [49] and this idea was supported by an experiment in which targeted disruption of *TNFAIP6*

297 [50] led to severe infertility due to impaired cumulus expansion and a defect in its
298 organization. The presence of *TNFAIP6* protein in porcine COCs is involved in stabilizing the
299 mucoelastic extracellular matrix [38]. In cattle abundance of *TNFAIP6* mRNA was
300 significantly higher in cumulus cells from competent oocytes compared to incompetent
301 oocytes [51], and in cumulus cells derived from in vivo COCs compared with their in vitro
302 counterparts [29].

303 In the present study, *TNFAIP6* expression increased from 12 h of IVM in cumulus
304 cells cultured in FCS-supplemented medium and only at 24 h of IVM in BSA-supplemented
305 medium, with greater transcript abundance in the FCS group than in the BSA group at 12 h
306 and 24 h of IVM. These results are consistent with those reported in a previous study that
307 showed a higher degree of expansion in COCs submitted to IVM in medium supplemented
308 with FCS, compared with those submitted to IVM in medium supplemented with BSA [16],
309 and could be attributed to FCS components that act as transcription factors for genes involved
310 in both extracellular matrix expansion and mucification.

311 *BRCA1* participates in several cellular processes in response to DNA damage. The
312 expression of *BRCA1* and other genes related to the *ATM* repair pathway was significantly
313 increased in the aging and apoptotic cumulus cells of aging women [52]. Our data showed for
314 the first time that cumulus cells cultivated with BSA have higher expression of *BRCA1* and
315 *TP53BP1* than those cultivated with FCS. *TP53BP1* is a key regulator of DSB repair [53]. It
316 functions at the intersection of two major DSB repair pathways (HR and NHEJ) and
317 integrates cellular inputs to ensure the timely execution of repair in the proper cellular
318 contexts [22]. DNA damage can be induced by endogenous metabolites that can reacts with
319 macromolecules, such as lipids, proteins or nucleic acids and exogenous stimulants, such as
320 ionizing radiation, ultraviolet radiation and reactive oxygen species (ROS) [57–60]. An
321 increase in *TP53BP1* and *BRCA1* mRNA expression was observed after the induction of ROS

production in cumulus cells obtained from vitrified oocytes [54]. Although the presence of cumulus cells during in vitro fertilization protects the bovine oocyte against oxidative stress and improves the first cleavage [55], bovine COCs matured in serum-free medium produce lower progesterone, with higher rate of apoptosis and a lower capacity for embryonic development compared with their FCS-supplemented counterparts [56]. Therefore, higher levels of *BRCA1* and *TP53BP1* mRNA in cumulus cells cultivated in serum-free medium may be related with elevated rates of apoptosis. As a result, it impairs the competence of COCs for embryonic development.

In our IVM model, the HR mediator proteins *RAD50*, *RAD51*, *RAD52*, *ATM* and *BRCA2* were not differentially expressed in cumulus cells cultured with BSA or FCS and although serum suppression in culture systems has potential to reduce the expression of NHEJ-initiating proteins, this find was not observed.

In summary, our results showed for the first time the profile expression of key genes involved in the mechanism of double-strand DNA repair in bovine cumulus cells of COCs matured with FCS or BSA. The higher mRNA expression of *BRCA1* and *TP53BP1* and lower mRNA expression of *TNFAIP6* suggests an increase in apoptosis rate and DNA damage in cumulus cells cultured in BSA-supplemented medium and may explain, at least to some extent, the reduced developmental potential of bovine oocytes matured in serum-free medium.

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Conflict of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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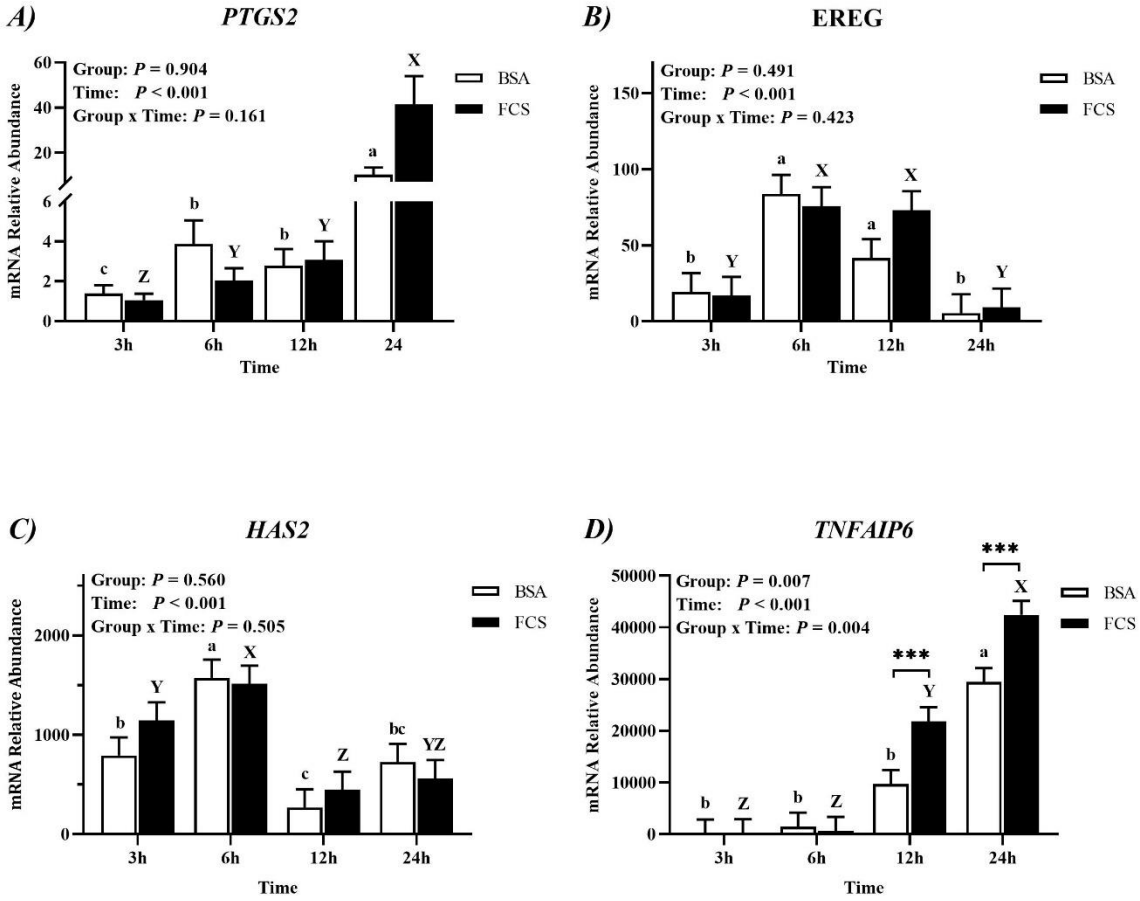
550 ***Table 1 – Information of specific primers used for amplification in real-time PCR.***

Gene Symbol	Primer sequence (5´ to 3´)	Product Size (bp)	Efficiency	Accession Number
Reference genes				
H2AFZ	F: GAGGAGCTGAACAAGCTGTTG R: TTGTGGTGGCTCTCAGTCTTC	104	2,05	XM_002686087.4
PPIB	F: GGTCATCGGTCTCTTTGGAA R: TCCTTGATCACACGATGGAA	117	2,05	NM_174152.2
TBP	F: CAGAGAGCTCCGGGATCGT R: CACCATCTTCCCAGAACTGAATAT	194	2,11	NM_001075742.1
Ovulatory cascade genes needed for cumulus expansion				
EREG	F: CTGCACAGCATTAGTTCAAACTGA R: TGTCCATGCAAACAGTAGCCATT	100	1,89	XM_010806226.3
HAS2	F: ACACAGACAGGCTGAGGACAACTT R: AAGCAGCTGTGATTCCAAGGAGGA	133	2,03	NM_174079.3
PTGS2	F: TGCTGAGTTTAACACGCTCTACCA R: TGAGACCATGTTCCAGTAAGACAGA	125	1,93	NM_174445.2
TNFAIP6	F: GCAAAGGAGTGTGGTGGTGTGTTT R: ACTGAGGTGAATGCGCTGACCATA	135	2,04	NM_001007813.2
Cell cycle checkpoint regulator				
GADD45B	F: TACGAGTCGGCCAAGCTGAT R: GTCCTCCTCTTCCTCGTCGAT	81	2,06	NM_001040604.1
TP53BP1	F: ATCAGACCAACAGCAGAATTTCC R: CACCACGTCAAACACCCCTAA	130	2,01	NM_001206397.2
Non-homologous end-joining repair				
XRCC5	F: TGGCATCTCCCTGCAGTTCT R: AGGCCCATGGTGGTCTGA	100	2,04	NM_001102141.1
XRCC6	F: AATTGACTCCTTTTGACATGAGCAT R: CCATAGAACACCACTGCCAAGA	100	2,00	NM_001192246.1
DNAPK	F: AAAGGCAATCCGTCCTCAGA R: AAGGCAGGTGCTAAACTGAGATG	100	2,05	NM_001256559.2
Homologous recombination repair				
ATM	F: CTTAGGAGGAGCTTGGGCCT R: CCGCTGTGTGGCAAACC	149	2,08	NM_001205935.1
BRCA1	F: ACAAAGCAGCAGACACAATCTCA R: TCATGGTCTCCCACACTGAAATA	110	1,87	NM_178573.1
BRCA2	F: AAATTTCACTGCACCTGGTCAA R: TCATGGGTTTGCCTATAGTTATCG	170	1,77	XM_002691807.5
RAD50	F: TGTGGAACAGGGCCGTCTA R: CAATTCTAGCTGTGTTGCCAGAGA	100	2,02	NM_001206868.1
RAD51	F: ATGCACCGAAGAAGGAGCTAAT R: GATCGCCTTTGGTGGAACTC	130	2,05	NM_001046179.2
RAD52	F: GGCCAGGAAGGAGGCAGTA R: TGACCTCAGATAGTCTTTGTCCAGAA	100	1,98	NM_001024525.1

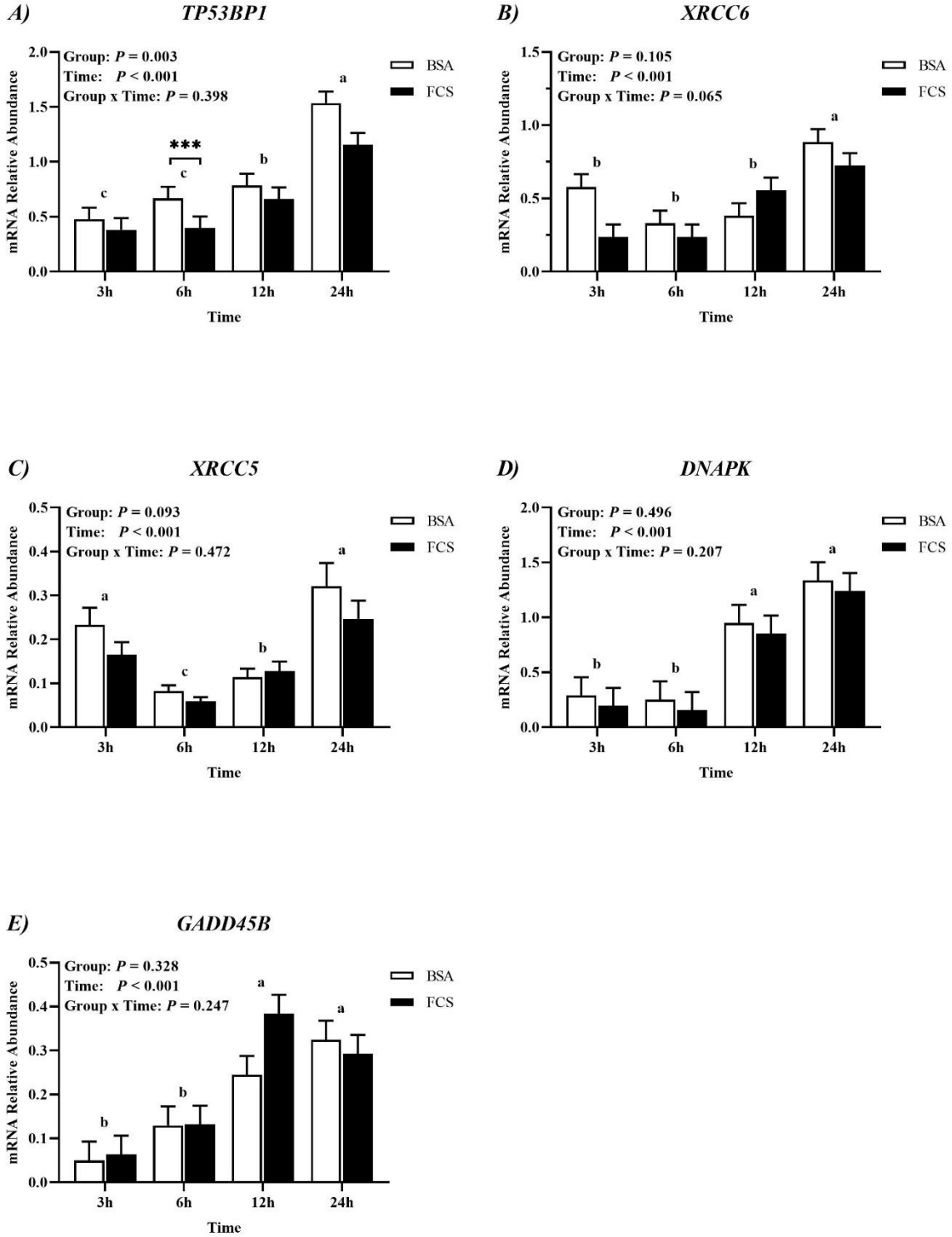
551
552

Fig. 1 – Abundance of A) Prostaglandin-endoperoxide Synthase 2 (PTGS2), B) Epiregulin (EREG), C) Hyaluronan Synthase 2 (HAS2) and D) TNF Alpha Induced Protein 6 (TNFAIP6) transcripts in bovine cumulus cells from COCs submitted to IVM for 3, 6, 12 or 24 hours in medium supplemented with bovine serum albumin (BSA) or fetal calf serum (FCS).



Data are the mean ± S.E.M of three independent replicates. Asterisks indicate significant differences ($P < 0.001$) between groups on a given time. Different lowercase letters indicate significant differences ($P < 0.05$) within the BSA group over time. Different uppercase letters indicate significant differences ($P < 0.05$) within the FCS group over time.

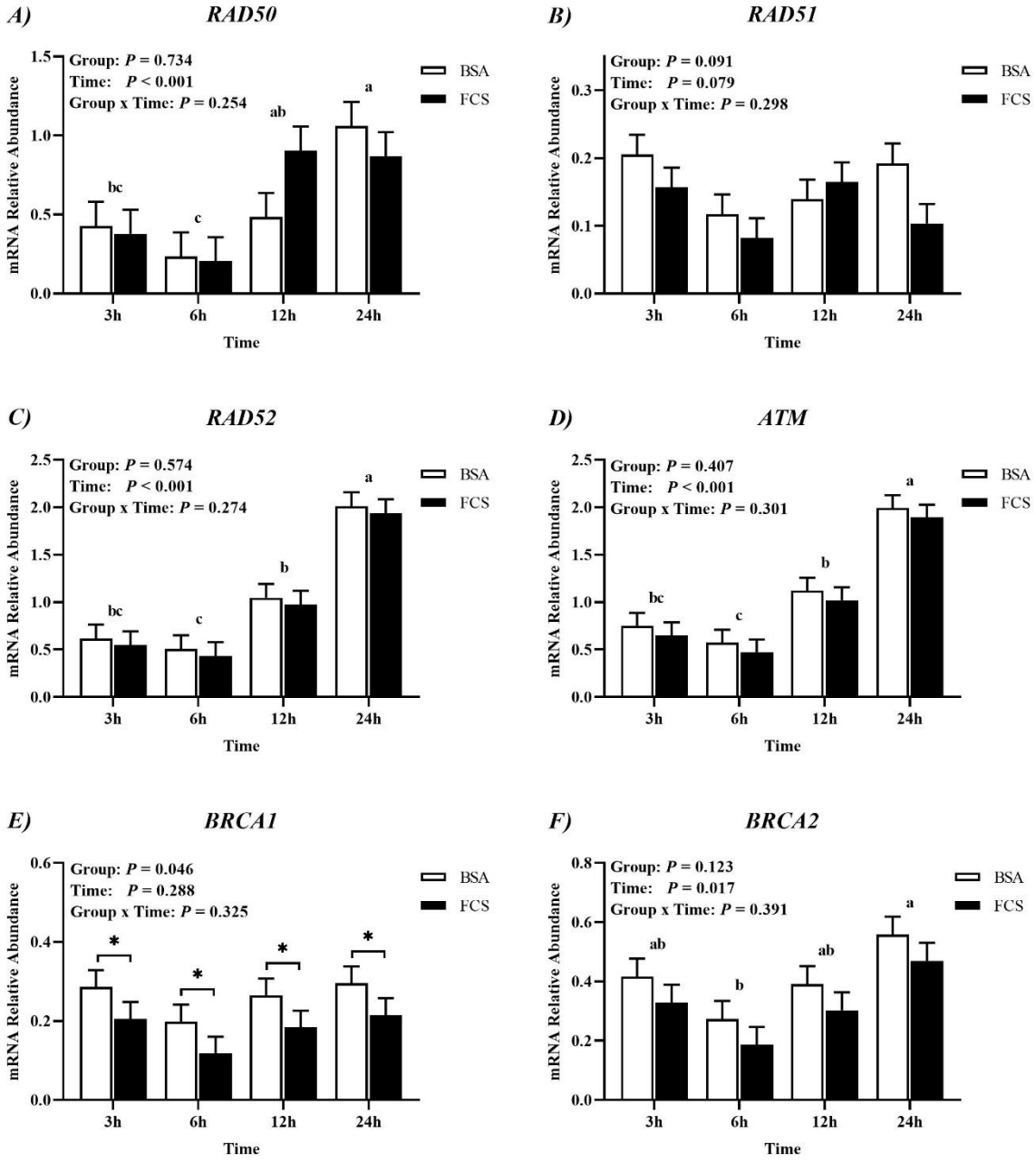
562 ***Fig. 2 – Abundance of A) X-ray repair cross complementing 6 (XRCC6), B) X-ray repair cross***
563 ***complementing 5 (XRCC5), C) Protein kinase DNA-activated, catalytic subunit (DNAPK), D) growth arrest***
564 ***and DNA damage inducible beta (GADD45B) and E) tumor protein p53 binding protein 1 (TP53BP1)***
565 ***transcripts in bovine cumulus cells from COCs submitted to IVM for 3, 6, 12 or 24 hours in medium***
566 ***supplemented with bovine serum albumin (BSA) or fetal calf serum (FCS)***



567
568 Data are the mean ± S.E.M. of three independent replicates. Asterisks indicate significant differences ($P < 0.05$)
569 between groups on a given time. Different lowercase letters indicate significant differences ($P < 0.05$) within a
570 group over time.

571

572 ***Fig. 3 – Abundance of A) RAD50 double strand break repair protein (RAD50), B) RAD51 recombinase***
573 ***(RAD51), C) RAD52 homolog DNA repair protein (RAD52), D) ATM serine/threonine kinase (ATM), E)***
574 ***BRCA1 DNA repair associated (BRCA1) and F) BRCA2 DNA repair associated (BRCA2) transcripts in***
575 ***bovine cumulus cells from COCs submitted to IVM for 3, 6, 12 or 24 hours in medium supplemented with***
576 ***bovine serum albumin (BSA) or fetal calf serum (FCS).***



577
578 Data are the mean ± S.E.M. of three independent replicates. Asterisks indicate significant differences ($P < 0.05$)
579 between groups on a given time. Different lowercase letters indicate significant differences ($P < 0.05$) within a
580 group over time.

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