

Senecionine from *Senecio brasiliensis* reduces the expression of interferon-induced genes in corpus luteum cells *in vitro*

Caroline Raquel Poglia Lasta^{1,2}  Suzana Rossato Feltrin^{1,2}  Carlos Miguel Staudt² 
Alfredo Quites Antoniazzi^{1,2}  Valério Marques Portela^{1,2*} 

¹Programa de Pós-graduação em Medicina Veterinária, Centro de Ciências Rurais (CCR), Universidade Federal de Santa Maria (UFSM), Santa Maria, RS, Brasil.

²Laboratório de Biotecnologia e Reprodução Animal (BioRep), Departamento de Fisiologia e Farmacologia, Centro de Ciências da Saúde (CCS), Universidade Federal de Santa Maria (UFSM), 97105-900, Santa Maria, RS, Brasil. E-mail: valerio.portela@ufsm.br. *Corresponding author.

ABSTRACT: This study investigated the effects of senecionine, a pyrrolizidine alkaloid found in plants of the genus *Senecio* spp., particularly *Senecio brasiliensis*, which causes significant damage in dairy and beef cattle production. Bovine luteal cells cultured *in vitro* were used as an experimental model to explore this. These cells were exposed to two concentrations of senecionine (10 and 100 ng/mL) for 24 hours, followed by the addition of interferon tau (IFNT), a precursor protein involved in maternal recognition of pregnancy in ruminants. Gene expression analyses were performed using the RT-qPCR technique, focusing on genes responsive to IFNT, such as ISG15, MX1, MX2, and OAS1. Genes related to cellular processes were also evaluated, including GADD45B, CCDN2, and CTGF, an important precursor in the Hippo signaling pathway. The integrity of the cell culture model was confirmed by analyzing the expression of the 3B-HSD gene and measuring progesterone concentration. This study provided evidence of the effects of senecionine on bovine luteal cells, revealing its ability to interfere with the response of these cells to IFNT. These findings contributed to understanding the underlying mechanisms of senecionine's toxic effects in animals. They may assist in developing prevention and control strategies for the damage caused by this plant in production animals.

Key words: senecionine, luteal cells, bovine, interferon tau, pyrrolizidine alkaloid.

Senecionina do *Senecio brasiliensis* reduz a expressão de genes induzidos por interferon em células do corpo lúteo *in vitro*

RESUMO: Este estudo investiga os efeitos da senecionina, um alcaloide pirrolizidínico encontrado em plantas do gênero *Senecio* spp., particularmente *Senecio brasiliensis*, que causa danos significativos na produção de gado leiteiro e de corte. Para explorar isso, foram utilizadas células luteais bovinas cultivadas *in vitro* como modelo experimental. Essas células foram expostas a duas concentrações de senecionina (10 e 100 ng/mL) por 24 horas, seguidas pela adição de interferon tau (IFNT), uma proteína precursora envolvida no reconhecimento materno da gestação em ruminantes. Análises de expressão gênica foram realizadas utilizando a técnica de RT-qPCR, focando em genes responsivos ao IFNT, como ISG15, MX1, MX2 e OAS1. Adicionalmente, foram avaliados genes relacionados a processos celulares, incluindo GADD45B, CCDN2 e CTGF, um importante precursor na via de sinalização Hippo. A integridade do modelo de cultura celular foi confirmada pela análise da expressão do gene 3B-HSD e pela medição da concentração de progesterona. Este estudo fornece evidências dos efeitos da senecionina em células luteais bovinas, revelando sua capacidade de interferir na resposta dessas células ao IFNT. Esses achados contribuem para a compreensão dos mecanismos subjacentes aos efeitos tóxicos da senecionina em animais e podem auxiliar no desenvolvimento de estratégias de prevenção e controle dos danos causados por essa planta em animais de produção.

Palavras-chave: senecionina, células luteais, bovinos, interferon tau, alcaloide pirrolizidínico.

INTRODUCTION

Seneciosis is a major plant-induced poisoning and a leading cause of cattle mortality in southern Brazil (RIET-CORREA et al., 2013; TUSTIN et al., 2001). This intoxication results from the ingestion of *Senecio* spp., particularly *Senecio brasiliensis*, a plant widely distributed across the country, occurring in all Brazilian biomes and 12 states (TELES et al., 2023). The genus *Senecio*

contains pyrrolizidine alkaloids (PAs), which, upon metabolism, generate highly reactive pyrrolic derivatives that induce cytotoxicity and liver damage (RIET-CORREA et al., 2013). The economic losses attributed to *Senecio* spp. intoxication are substantial, particularly in chronically exposed cattle that may not exhibit overt clinical signs but suffer from compromised reproductive performance (PANZIERA et al., 2018). While low reproductive efficiency in intoxicated cattle has traditionally been attributed

to weight loss and hepatic dysfunction, emerging evidence suggests that PAs also have direct effects on ovarian tissue (KRABBE et al., 2015; PANZIERA et al., 2018). These findings indicated that *Senecio* spp. poisoning may contribute to subfertility by altering ovarian function and disrupting maternal-embryonic communication (SANTOS et al., 2008). Maternal-embryonic communication is a crucial physiological process that ensures the establishment and maintenance of pregnancy in ruminants. This communication relies on the secretion of interferon-tau (IFNT) by the developing embryo, which interacts with luteal cells to stimulate interferon-stimulated genes (ISGs) such as ISG15, MX1, MX2, and OAS1 (ANTONIAZZI et al., 2013; SPENCER et al., 2007). The activation of ISGs is essential for pregnancy recognition and maintenance, preventing premature luteolysis (BOTT et al., 2010). In addition to pregnancy recognition, IFNT regulates genes involved in luteal cell proliferation and survival. Specifically, GADD45B and CCDN2 play roles in apoptosis resistance and cell cycle progression, respectively (MIHM et al., 2008; SHERR & ROBERTS, 1995). Disruption of these processes can lead to reduced progesterone synthesis and increased luteal regression, compromising pregnancy maintenance (MANN & LAMMING, 2001). Furthermore, the Hippo signaling pathway, a critical regulator of cell proliferation and tissue homeostasis, is involved in luteal cell function (HUH et al., 2019). When activated, this pathway suppresses the expression of CTGF, a downstream effector of YAP1/TAZ, impairing cell proliferation (JUSTICE et al., 1995; WANG et al., 2016). Given the documented alterations in the luteal cells of cattle intoxicated by *Senecio* spp., it is crucial to determine whether the pyrrolizidine alkaloid senecionine (SN) affects IFNT-mediated gene expression and the functional integrity of luteal cells. This study investigated the impact of SN on the expression of IFNT-induced genes and key regulators of cellular health in bovine corpus luteum cells in vitro. Understanding these mechanisms will provide insights into the reproductive consequences of *Senecio* spp. intoxication and inform potential mitigation strategies in livestock management.

MATERIALS AND METHODS

Chemicals

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

Cell culture

Bovine ovaries were collected from a abattoir located in Santa Maria, Rio Grande do Sul, Brazil, and transported to the laboratory in 0.9% NaCl saline solution containing penicillin (100 IU/mL) and streptomycin (100 µg/mL) at 4 °C. The ovaries were washed in the laboratory with 0.9% NaCl saline solution and 70% ethanol. For this experiment, early and intermediate CLs were selected, measuring 15-25 mm in diameter, with luteal tissue visible on the ovarian surface, a bloodstained surface, and a pink, brown, or orange color with a compact and soft consistency. Luteal cell culture was adapted from a previous protocol published by ANTONIAZZI et al. (2013). The CLs were classified according to their morphological characteristics as early (1-6 days post-ovulation), intermediate (8-12 days post-ovulation), and late (15-17 days post-ovulation), as previously described (MIYAMOTO et al., 2000). The connective tissue adjacent to the corpus luteum was mechanically removed, cut into small pieces with a scalpel blade, and placed in a tube containing DMEM-F12 medium supplemented with 0.5% bovine serum albumin (BSA) at 37 °C for 10 minutes. The supernatant was then discarded, and the sedimented luteal tissue was dissociated in a solution of 0.1% type I collagenase and DMEM-F12 supplemented with 0.5% BSA, subjected to agitation for 1 minute every 10 minutes over 40 minutes at 37 °C. The cell suspension was then supplemented with DMEM-F12 containing 10% fetal bovine serum (FBS) to inactivate the collagenase. The cells were filtered through a 70 µm nylon filter, resuspended in 2 mL of Red Blood Cell Buffer, and centrifuged at 1,000 rpm for 10 minutes. The pellet was resuspended, washed, and centrifuged three times for 10 minutes at 1,000 rpm with DMEM-F12 solution supplemented with 10% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin. Cell viability was assessed using the Trypan Blue exclusion method, with viability exceeding 85% in all replicates. The cells were seeded in 24-well plates at a density of 2×10^5 cells/well and cultured in DMEM-F12 supplemented with 5% FBS, 100 IU/mL penicillin, and 100 µg/mL streptomycin for 12 hours at 37 °C in 5% CO₂. Initially, the cells were incubated with the following treatments: Group 1: DMEM-F12 (control), Group 2: DMEM-F12, Group 3: DMEM-F12 with 10 ng/mL SN, Group 4: DMEM-F12 with 100 ng/mL SN. After 24 hours of culture, the medium was replaced with fresh DMEM-F12 culture medium, free of FBS and supplemented with 100 IU/mL penicillin and 100 µg/mL streptomycin. The cells were cultured under

starvation conditions for 6 hours at 37 °C in 5% CO₂. After this period, the cells received the following treatments: Group 1: DMEM-F12 (control), Groups 2, 3, and 4: DMEM-F12 with 1 ng/mL of rolFNT. The cells were incubated for 6, 12, and 24 hours at 37 °C in 5% CO₂. After incubation, cells and culture medium were collected and stored at -80 °C for subsequent analysis. Three independent replicates were performed on different days, using five corpora lutea from different cows for each *in vitro* experiment.

RNA Extraction, reverse transcription, and real-time PCR

Total mRNA from luteal cells was extracted following the protocol recommended by the manufacturer using the PureLink™ RNA Mini Kit (Thermo Fisher Scientific, MA, USA). The extracted mRNA was quantified by measuring optical density using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, MA, USA) at a wavelength of 260 nm. The purity of the mRNA was assessed by the OD260/OD280 absorption ratio, and values below 1.8 were not used. Reverse transcription was performed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, CA, USA) according to the manufacturer's recommendations. RT-qPCR was

carried out using the CFX384™ Real-Time System (Bio-Rad Laboratories, CA, USA) with GoTaq® DNA Polymerase (Promega, WI, USA) and specific primers (Table 1). After an initial denaturation step at 95 °C for 3 minutes, 40 cycles were performed at 95 °C for 10 seconds, followed by 1 minute at 60 °C to amplify each transcript. Target gene amplification Ct was normalized to the average expression level of RPL19, and GAPDH housekeeping genes, according to the ratio $R = \text{housekeeping ECt} / \text{target ECt}$, where E is the amplification efficiency for each primer pair. The abundance analysis of interferon-stimulated genes such as ISG15 (JOHNSON et al., 1999), MX1, MX2 (OTT et al., 1998), and OAS1 (MIRANDO et al., 1991) were performed, along with genes involved in proliferation and survival such as GADD45B and CCDN2 (MIHM et al., 2008), and the steroidogenic enzyme 3β-HSD. The quantification of relative abundance was carried out using the Pfaffl method (PFAFFL, 2001) (Table 1).

Analysis of progesterone

The progesterone concentration in the culture medium of luteal cells was measured in a specialized clinical analysis laboratory using an

Table 1 - Forward and reverse primers sequences of target and reference genes used for quantitative polymerase chain reaction.

Gene	Access number	Nucleotide sequence (5'-3')
3B-HSD	NM_001034034.2	F: TGACCCCTTCTTGACCTTC R: CGTTCTCTGCCTTGACTGTG
CCDN2	NM_001076372	F: CCAGACCTTCATCGCTCTGT R: GATCTTTGCCAGGAGATCCA
CTGF	NM_174030.2	F: AGCTGAGCGAGTTGTGTACC R: TCCGAAAATGTAGGGGGGCAC
GADD45B	NC_037334.1	F: TACGAGTCGGCCAAGCTGAT R: GTCCTCCTCTTCCTCGTCGAT
GAPDH	NM_001035034.2	F: GATTGTCAGCAATGCCTCCT R: GGTCATAAGTCCCTCCACGA
RPL19	NM_000981.4	F: CCGGCTGCTTAGACGATACC R: CCGCTTGTTTTGAACACGTT
ISG15	NM_174366.1	F: GGTATCCGAGCTGAAGCAGTT R: ACCTCCCTGCTGTCAAGGT
MX1	NM_173940.2	F: GTACGAGCCGAGTTCTCCAA R: ATGTCCACAGCAGGCTCTTC
MX2	NM_173941.2	F: CTTGAGAGACGCTCAGTCG R: TGAAGCAGCCAGGAATAGT
OAS1	NM_001029846.2	F: TAGCCTGGAACATCAGGTC R: TTTGGTCTGGCTGGATTACC

Abbreviation: 3βHSD (3β hydroxysteroid dehydrogenase); CCDN2 (Cyclin D2); CTGF (connective tissue growth factor); GADD45B (growth arrest and DNA damage inducible beta); GAPDH (glyceraldehyde-3-phosphate dehydrogenase); ISG15 (ISG15 ubiquitin-like protein); MX1 (myxovirus resistance 1); MX2 (myxovirus resistance 2); OAS1 (2'-5'-oligoadenylate synthetase).

electrochemiluminescence kit (ADVIA Centaur®, Siemens Healthcare, Munich, Germany), with a sensitivity of 0.05 ng/mL of progesterone.

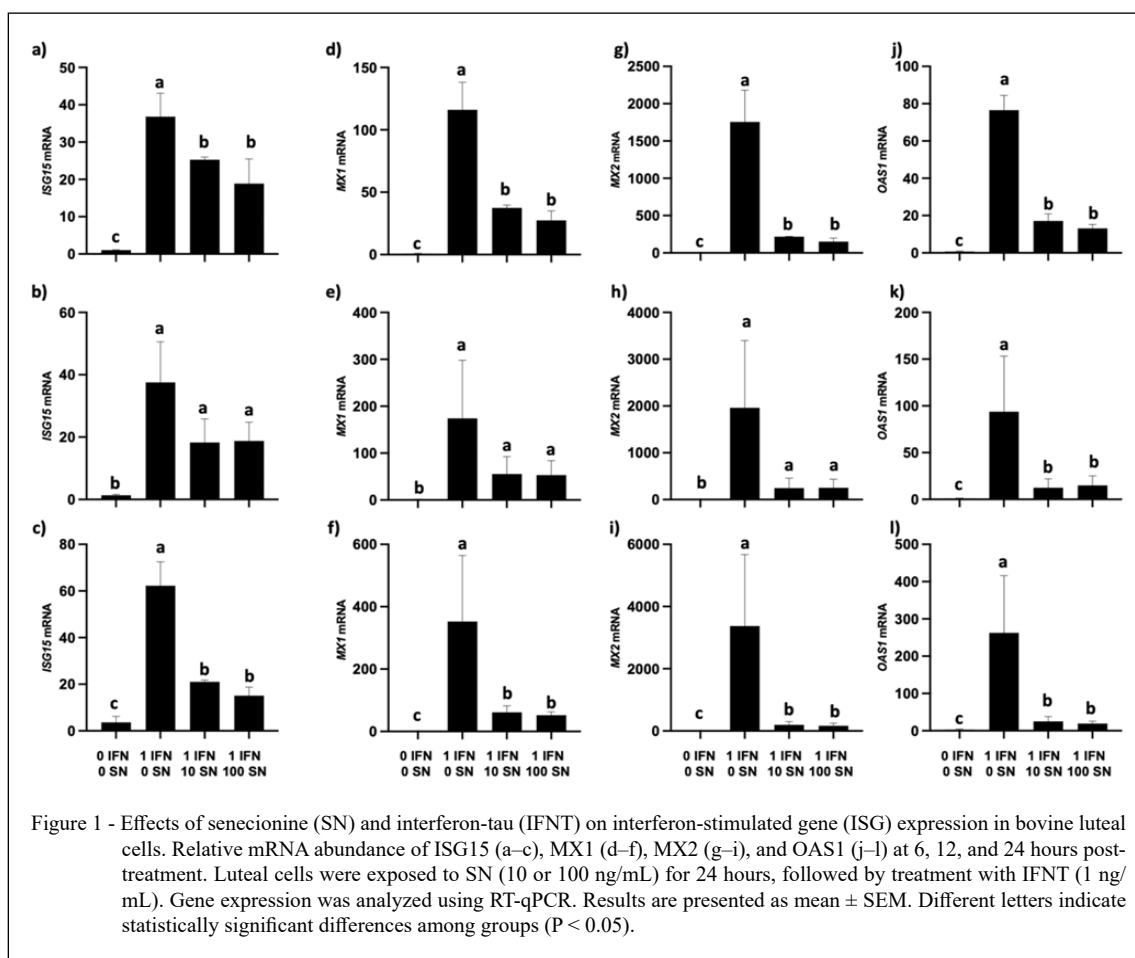
Statistical analysis

The analysis of variance was conducted using JMP software (SAS Institute), with treatment as the main factor and replicates as the random variable. Differences between means were evaluated using Tukey's multiple comparison test. Homogeneity of variance was assessed using the O'Brien test. When the data did not follow a normal distribution (Shapiro-Wilk test), they were transformed to a logarithmic scale. The results are presented as mean $\pm$ standard error of the mean (SEM), and a value of $P < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

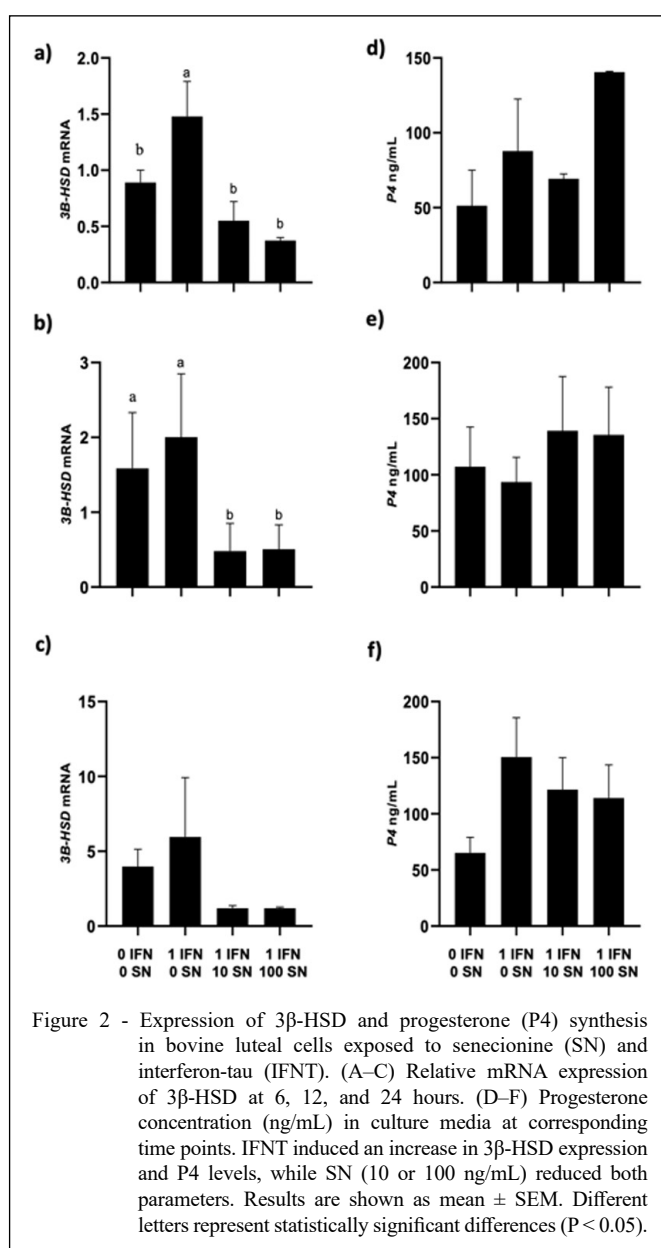
Interferon-stimulated genes (ISGs), particularly ISG15, are proteins expressed in the

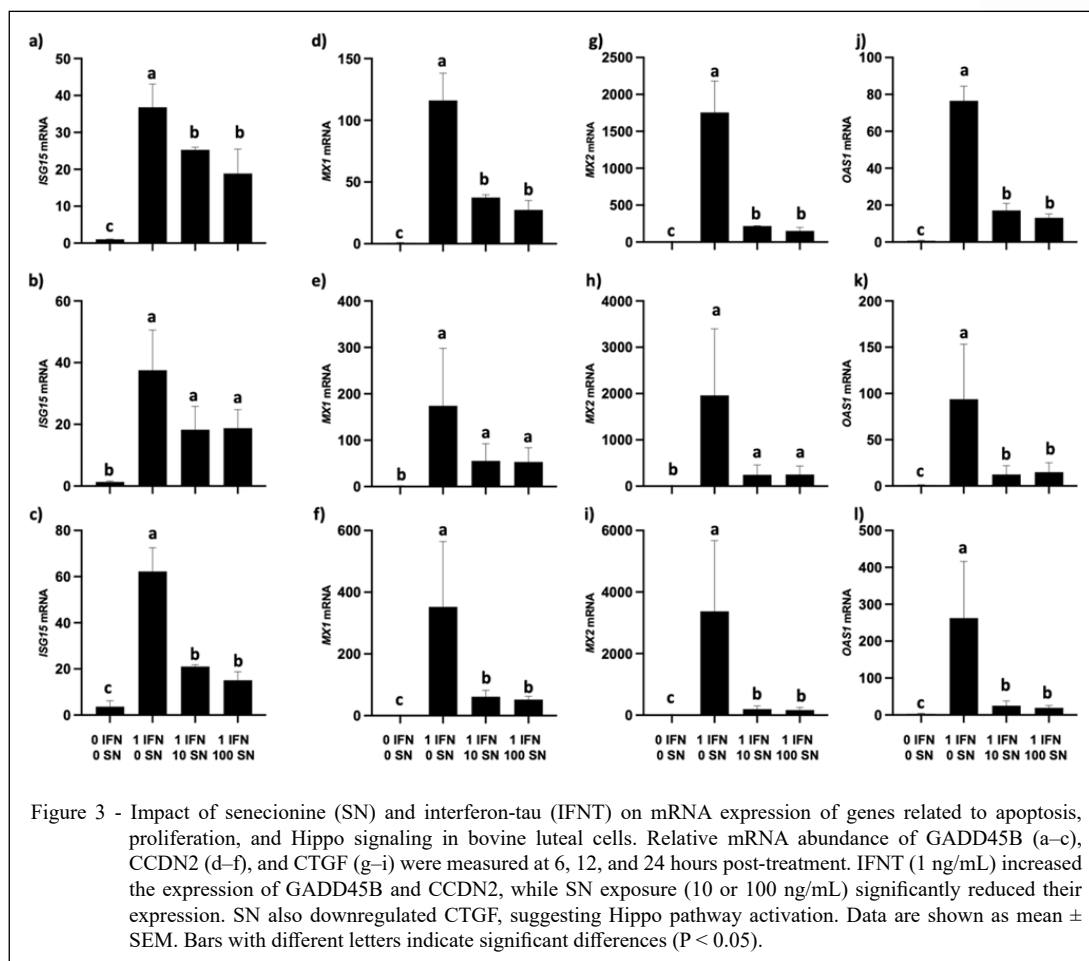
uterine endometrium during early pregnancy in response to the paracrine action of IFNT derived from the embryo in ruminants (BOTT et al., 2010). When IFNT was added to the corpus luteum (CL) culture, a dose-dependent response was observed in ISG15, MX1, MX2, and OAS1. This response is consistent with studies suggesting that the abundance of ISG15 in luteal cells increases dose-dependently in response to recombinant ovine or bovine IFNT up to 1 ng/mL (ROBERTS et al., 2008). Our treatment with one ng/mL of IFNT increased the mRNA abundance of these ISGs at all time points in cells without SN (Figure 1, panels a-l). Conversely, groups intoxicated with 10 or 100 ng/mL of SN showed significant decreases in ISG15 and other ISGs compared to groups without SN, indicating that SN can significantly reduce the mRNA abundance of ISGs in CL and negatively impact the maternal recognition mechanism of pregnancy. Recent studies have associated GADD45B and CCDN2 with ovarian function and dominant follicle development (MIHM et al., 2008; SHERR & ROBERTS, 1995).



GADD45B, a protein rapidly induced by stress factors, possesses anti-apoptotic properties (DE SMAELE et al., 2001). When IFNT was added to the CL, higher GADD45B mRNA abundance was observed compared to the group without any addition, suggesting that IFNT acts like a protector of the CL. This protection is likely due to GADD45B's role in DNA damage repair and cell survival (Figure 2, panels a-c) (SHEIKH et al., 2000). In groups intoxicated with 10 or 100 ng/mL of SN, a reduced abundance of GADD45B mRNA was observed, supporting the hypothesis that GADD45B helps protect cells against oxidative damage-induced

cell death (YU et al., 2013). CCDN2 regulates the G1-to-S transition in the cell cycle, promoting proliferation (SHERR & ROBERTS, 1995). The CL treated with IFNT showed higher CCDN2 mRNA abundance compared to the untreated group, indicating that IFNT may enhance cell cycle functionality (Figure 3, panels d-f). However, groups treated with 10 or 100 ng/mL of SN exhibited significant decreases in CCDN2 mRNA, suggesting that SN might induce apoptosis by disrupting the cell cycle. The drastic reduction in proliferative and pro-survival factors observed in SN-treated groups raises questions regarding cell





viability. Although, initial viability assessed by Trypan Blue exclusion was high, the possibility remains that prolonged exposure to SN may lead to increased cell death. However, it is important to highlight that the morphology of the cultured cells remained unchanged across all groups without evidence of corpus luteum cell cluster disaggregation. This suggested that the treatment did not cause widespread cell death. Thus, the observed decrease in proliferation and survival gene expression could be attributed more to transcriptional regulation by SN rather than to direct cytotoxic effects. Future studies should include additional viability assessments, such as annexin V/propidium iodide staining or caspase activity assays, to clarify if SN induces apoptosis or necrosis in luteal cells (BRABSON et al., 2021; LEE et al., 2018; XU & OUYANG, 2022). The Hippo pathway, which regulates tissue growth, differentiation, proliferation, and apoptosis, primarily targets CTGF (connective tissue growth factor) (JUSTICE et al., 1995; SHOME et al., 2020). In the group treated only with IFNT, there was no statistical difference in CTGF mRNA abundance

compared to the untreated group, suggesting an inactive Hippo pathway. However, groups treated with 10 or 100 ng/mL of SN showed significantly reduced CTGF mRNA, indicating Hippo pathway activation and the YAP1/TAZ-TEAD complex's inability to regulate CTGF mRNA. This suggested that SN inhibits cell proliferation by activating the Hippo pathway. Interferon-tau (IFNT) increases the mRNA abundance of ISGs, GADD45B, and CCDN2 in the corpus luteum (CL), enhancing cell survival and proliferation. However, SN significantly reduces these gene expressions, negatively impacting cell function and the maternal recognition of pregnancy. SN activates the Hippo pathway, inhibiting cell proliferation and decreasing the abundance of 3 β -HSD mRNA, which may impair progesterone synthesis; although, it was not detected in this experiment. Future research should focus on determining whether SN-induced toxicity directly leads to apoptosis or necrosis and assess the broader implications of SN exposure on reproductive health in cattle.

CONCLUSION

In summary, senecionine from *Senecio brasiliensis* impairs the response of bovine luteal cells to interferon-tau, reducing the expression of interferon-stimulated genes, as well as markers of cell survival, proliferation, and steroidogenesis. These effects likely involve activation of the Hippo pathway and occur without evident cytotoxicity, suggesting transcriptional modulation. The findings highlighted a potential mechanism by which pyrrolizidine alkaloids compromise reproductive function in cattle, underscoring the need for further studies on their long-term effects and mitigation strategies.

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DECLARATION OF CONFLICT OF INTEREST

The authors declare there are no conflicts of interest.

AUTHORS' CONTRIBUTION

All authors contributed equally to the conception and writing of the manuscript. All authors critically reviewed the manuscript and approved the final version.

DATA AVAILABILITY STATEMENT

The raw data is available directly with the author.

DECLARATION OF USE OF ARTIFICIAL INTELLIGENCE

For the linguistic review of the manuscript, we used artificial intelligence tools with the purpose of enhancing the clarity and coherence of the text. We emphasize that the authors carefully supervised all modifications to ensure that the scientific content remained unchanged. This use is declared to ensure full transparency.

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