Ciência Animal Brasileira
Brazilian Animal Science
e-ISSN 1809-6891

Ciência Animal Brasileira

DOI: 10.1590/1809-6891v23e-71762E

Seção: Veterinary medicine
Research article

In vitro toxicity of *Niedenzuella (Tetrapterys) multiglandulosa* on bovine embryos

Toxicidade in vitro da Niedenzuella (Tetrapterys) multiglandulosa em embriões bovinos

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Abstract
Niedenzuella (*Tetrapterys*) *multiglandulosa*, a vine plant found in Brazil, has been correlated to outbreaks of poisoning in cattle and buffaloes, generating economic losses related to the death due to heart failure, miscarriage, abortion, stillbirth, and neonatal mortality. The aim of this study was to examine the embryotoxic potential of the aqueous plant extract on *in vitro* bovine embryos. *In vitro* study was performed in five replicates of bovine embryo culture assigned in two groups: control, *in vitro* embryo culture medium without the aqueous plant extract; treated group, with addition of 2.7mg/mL of aqueous plant extract (10%) to the embryo culture on the sixth day of culture. Cleavage rate was evaluated at day 2 of the cell culture. Viability, hatchability and underdevelopment of blastocysts on the seventh, eighth, and ninth days (D7, D8, and D9, respectively) of culture were assessed under stereoscopic microscope. On day 7, blastocysts were submitted to TUNEL assay to determine apoptotic index. *In vitro* exposure of bovine embryos to of *N. multiglandulosa* resulted in reduced embryo development and survival, evaluated by dark cytoplasm indicating poor morphology and poor quality with marked reduction of hatchability. We observed a significant reduction of blastocyst production/number of cleaved embryos (60.6% *vs* 41.5%); reduction of blastocysts production/total number of matured bovine oocytes (35.1% *vs* 21.3%); and embryonic hatching rates (38.0% *vs* 10.0%). However, no effects were observed on the apoptotic rate. In conclusion, aqueous extract of *N. multiglandulosa* leaves reduces bovine embryo viability *in vitro*, suggesting possible detrimental effects on embryo development.
Key words: apoptosis; blastocyst; toxic plant.

Resumo
Niedenzuella (*Tetrapterys*) *multiglandulosa*, uma videira encontrada no Brasil, tem sido correlacionada a surtos de intoxicações em bovinos e búfalos, gerando perdas econômicas relacionadas à morte por insuficiência cardíaca, aborto, natimorto e mortalidade neonatal. O objetivo deste estudo foi examinar o potencial embriotóxico do extrato vegetal aquoso em embriões bovinos *in vitro*. O estudo *in vitro* foi realizado em cinco repetições de cultura de embriões bovinos distribuídas em dois grupos: controle, meio de cultura de embriões *in vitro* sem o extrato aquoso da planta; grupo tratado, com adição de 2,7mg / mL de extrato vegetal aquoso (10%) à cultura do embrião no sexto dia de cultivo. A taxa de clivagem foi avaliada no dia 2 da cultura de células. Viabilidade, eclodibilidade e subdesenvolvimento de blastocistos no sétimo, oitavo e nono dia (D7, D8 e D9, respectivamente) de cultura foram avaliados em microscópio estereoscópico. No dia 7, os blastocistos foram submetidos ao ensaio TUNEL para determinar o índice apoptótico. Observamos redução significativa da produção de blastocisto / número de embriões clivados (60,6% vs 41,5%); redução da produção de blastocistos / número total de oócitos bovinos maturados (35,1% vs 21,3%); e taxas de eclosão embrionária (38,0% vs 10,0%). No entanto, nenhum efeito foi observado na taxa de apoptose. Em conclusão, o extrato aquoso das folhas de *N. multiglandulosa* reduz a viabilidade do embrião bovino *in vitro*, sugerindo possíveis efeitos prejudiciais no desenvolvimento embrionário.
Palavras-chave: apoptose; blastocisto; planta tóxica.

Received: February 4, 2022. Accepted: May 11, 2022. Published: May 27, 2022.

Introduction

Niedenzuella (*Tetrapterys*) *multiglandulosa* (A. Juss.) W. R. Anderson is a toxic plant of the Malpighiaceae family found in Southeastern and Midwestern Brazil[1]. Spontaneous consumption of *Niedenzuella* leaves has been associated with outbreaks of mortality, abortion, weak newborn calves, and heart failure in cattle[2,3] as poisoning often occurs in the dry season due to lack of alternative forages[3,4]. Detrimental toxicological effects are often associated with other causes, mainly infectious. Embryonic losses and abortions are a serious problem that result in considerable economic losses to the cattle industry, and the effectiveness of control measures requires accurate diagnosis. Unfortunately, only 30% of the cases reached to a definitive etiological diagnosis, since many agents, infectious or not, may be involved[5,6]. Previous experimental research determined that sheep might also be susceptible[7].

N. multiglandulosa also induces abortion at different gestational time and ingesting the plant at later pregnancy results in stillbirths or birth of weak offspring[3,4,8]. The effects of the plant during the early stages of embryo development remain unknown, since previous studies were performed in the middle third or at the end of pregnancy[4,8]. In an outbreak that occurred in 2004 in Brazil, approximately 80% of the 290 pregnant cows that had been introduced into a pasture highly infested by *N. multiglandulosa* had abortion or gave birth to weak calves that died within a few days of life, indicating that the toxic substance can cross the placental barrier[9]. Actually, abortion still the main harmful effect reported in spontaneous poisoning by *N. multiglandulosa*[3].

Despite the potential toxicity towards female reproductive system, we have yet to identify the mechanism of action of *N. multiglandulosa* compounds. All parts of the plant are toxic, as both young and mature leaves of *N. multiglandulosa* have flavonic heterosides, polyphenols, steroids, and cardiotonic glycosides. Furthermore, tannins and quaternary alkaloids were only found in mature leaves[10]. Another study isolated and identified 19 different compounds from the leaves, including alkaloids, glycosylated and non-glycosylated flavonoids, other glycosylated compounds and six steroids (orecdysteroids)[11].

The effects of a toxic agent on embryonic and fetal development depend on its' nature, dosage, developmental stage, and factors that modify toxicity, such as genotype (species/breed), maternal environment, and the placenta. The developmental period from fertilization until embryo implantation in the uterus and subsequent organogenesis is known as preimplantation, a very sensitive stage to injury that could determine embryo death or complete recovery of the embryonic development[12]. Testing the sensitivity of embryos to chemicals in the preimplantation period is essential to determine its potential to induce embryonic lethality. However, there are few experimental protocols to study the possible toxicity of chemicals in bovine embryos.

Alternatively *in vitro* models have been used as novel approaches in reproductive toxicology[13], mainly in women exposed to a determined toxic agent that may have a temporary negative effect on their oocyte quality[14,15]. During maturation, oocytes are susceptible to epigenetic alterations interfering with fertilization and early embryo development. The possibility to use bovine slaughterhouse material for *in vitro* tests related to gametes and early embryo development appears as an important option to diminish the number of *in vivo* tests[15], and the use of oocytes from cattle provide insight on the possibilities to evaluate chemicals on the *in vitro* oocyte maturation, fertilization and early embryonic development (preimplantation stage)[15,16].

Despite the number of reports on the *in vivo* poisoning effects of *N. multiglandulosa*, the toxicity towards the reproductive system remains unclear. Based on previous studies using living animals and trying to replicate the same lowest concentration of toxic plant (0.002mg of plant extract/µL of blood) that had detrimental effects on reproductive tract of pregnant goats[4] and rabbits[17], the aim of this study was to determine the embryotoxic effect of *N. multiglandulosa* leaves aqueous extract using *in vitro* bovine embryos as a model.

Material and Methods

Research was approved by the Ethics Committee in Animal Experimentation of UFMG (CEUA) under protocol 388/2016

N. mutiglandulosa (Malpighiaceae) was cultivated and harvested in the Toxic Plants Yard of the Veterinary School, Federal University of Minas Gerais, Belo Horizonte, MG, Brazil. A voucher specimen was deposited in the Herbarium of the Department of Botany (BHCB Herbarium), Institute of Biological Sciences, under number BHCB 38051.

The extract was obtained from 77g of the plant, composed by 60% mature leaves, 24% young leaves, and 16% shoots, the same proportion of different parts of the plants previous used in *in vivo* performed studies with intoxication in goats[4] and rabbits[17]. Leaves and shoots were ground in a blender with 500mL of absolute ethanol (>99%). The resulting material was left to rest in an enclosed container for four days at room temperature with daily alcohol replacement. Alcohol solution was filtered and concentrated in a rotary evaporator at 65°C at 80rpm under vacuum to form a dry extract weighing 4.216g. Next, 100mL of distilled water were added to the

extract and taken to an ultrasonic bath for 15 minutes to increase extraction of the plant's phytochemical constituents. Subsequently, the water extract was separated by filtration using a qualitative paper filter. The water extract was fractioned with 50mL of ethyl acetate in a separation funnel. After discharging the ethyl acetate fraction, the remain water extract was fractioned two more times with 70mL of ethyl acetate. The residual ethyl acetate in the aqueous extract was removed in a rotary evaporator at 65°C at 100rpm. The water solution was then fractioned three times with 50mL of water-saturated butanol in a separation funnel. The aqueous extract was again concentrated in the rotary evaportator at 68°C at 100rpm, yielding 1.545g of dry brown residue. The temperature used in the laboratory processing to obtain the extract was based on previous published studies[18,19,20]. A total of 27mg of the plant extract was diluted in 1mL Synthetic Oviduct Fluid Medium (SOFaa) for *in vitro* testing.

Bovine ovaries (n = 403) were obtained from a local slaughterhouse and the follicles were aspirated to obtain oocytes for *in vitro* embryo production. Five *in vitro* embryo culture routines were performed, considering each routine as a repeat and for each repeat it was used five to six replicates. All procedures for *in vitro* embryo production were based on Leite et al. [21]. Briefly, small ovarian follicles containing immature oocytes were punctured to obtain *cumulus*-oocyte complexes (COCs). Immature CCOs were subjected to *in vitro* maturation (IVM) for 24 hours in TCM-199 bicarbonate base medium (Gibco® Life Technologies, Grand Island, USA). Mature oocytes surrounded by an expanded *cumulus* were subjected to *in vitro* fertilization (IVF) by co-incubating with sperm in FERT-TALP medium for about 18 hours. The day of fertilization was considered day zero (D0). After IVF, the presumptive zygotes were *in vitro* cultured in SOFaa medium until D9.

On the first day (D1), the zygotes were randomly assigned into two groups of five to six culture drops (replicates) of culture medium for each treatment, containing 15 to 20 structures in each drop. Group 1 (control) was composed of zygotes that only received embryo culture medium (SOFaa), and Group 2 received *N. multiglandulosa* aqueous extract on the sixth day (D6). A total of 7µL (10%) of *in vitro* culture medium was removed and replaced with 7µL of aqueous plant extract (comprising 0.2mg of *N. multiglandulosa* extract/ 70µL of culture drop), previously diluted in SOFaa medium. On day two (D2) of culture, the cleavage rate (number of cleaved zygotes per total number of COCs) was assessed. On day six (D6), embryo production/total number of oocyte and embryo production/total number of cleaved zygote rates and embryo viability were assessed. The number of embryos produced, viability and hatchability of all blastocysts were evaluated on the seventh, eighth and ninth days of culture (D7, D8 and D9, respectively). For viability, the kinetics of embryonic development were assessed, considering the expected stage of development on the day of the evaluation, and comparing the groups. The hatching rate was considered by the ability of the growing embryos to break through the pellucid zone during D7, D8 and D9. On D7, on average, three blastocysts (nine blastocysts per group) were removed from each treatment for fixation in a 4% formalin solution for TUNEL (Promega® Corporation, Wisconsin, USA) assay according to the manufacturer's instructions to determine apoptotic index. Samples were observed under a fluorescence microscope under 400x magnification. The total number of cells was obtained by observing the Hoechst-stained cells, which were visualized in blue with a 460nm filter. Cells (blastomeres) in apoptosis were identified by fluorescein, resulting in a green stain and observed with a 520±20nm filter. The apoptotic index was calculated from the ratio of the total number of cells and the number of apoptotic cells, using the IMAGEJ program (Version 1.42e, 2008).

Data were submitted to Kolmogorov-Smirnov and Cochran-Barttlet tests for normality and homoscedasticity, respectively. Cleavage rate, blastocyst production/cleaved, blastocyst production/total number of oocytes in culture, hatching rate and cellular apoptosis rates were assessed by Fisher's exact test using GraphPad Instat 3.0 software. The significance level was set at 5%.

Results

The effects of *N. multiglandulosa* on blastocysts production at D7 are shown in Table 1. The cleavage rate did not differ between experimental groups (58% *vs* 51%), indicating that the oocytes had similar quality prior to the beginning of embryo production. However, embryo production, according to the total number of oocytes and the number of cleaved embryos, were significantly reduced after *N. multiglandulosa* treatment. Hatching rate was also significantly lower in the treated group. Embryonic hatching rates were 38.0% (38/100) and 10.0% (6/60), for the control and *N. multiglandulosa* extract groups, respectively.

Furthermore, embryos of the *N. multiglandulosa* extract group presented poor morphology (dark cytoplasm) and poor-quality when compared to those of the control group (Figure 1).

N. multiglandulosa aqueous extract did not cause significant apoptosis of embryo cells, as the number of apoptotic cells and apoptotic rate remained unchanged, as shown in Figure 1 and Table 2.

Table 1. Production of bovine blastocysts relative to the number of cleaved embryos, and production of blastocysts relative to the total number of bovine oocytes matured *in vitro* after culture with *N. multiglandulosa* aqueous extract

Groups	Total number of oocytes	Production rate of embryos/ cleaved, n (%)	Production rate of embryos/ total number of oocytes, n (%)	Hatching rate, n (%)
Control	404	142/234[a] (60.68%)	142/404[a] (35.14%)	38/100[a] (38.0%)
N. multiglandulosa extract	427	91/219[b] (41.55%)	91/427[b] (21.31%)	6/60[b] (10.0%)

[a,b] Values in the same column followed by superscript letters differ from each other by Fisher's exact test with 5% significance.

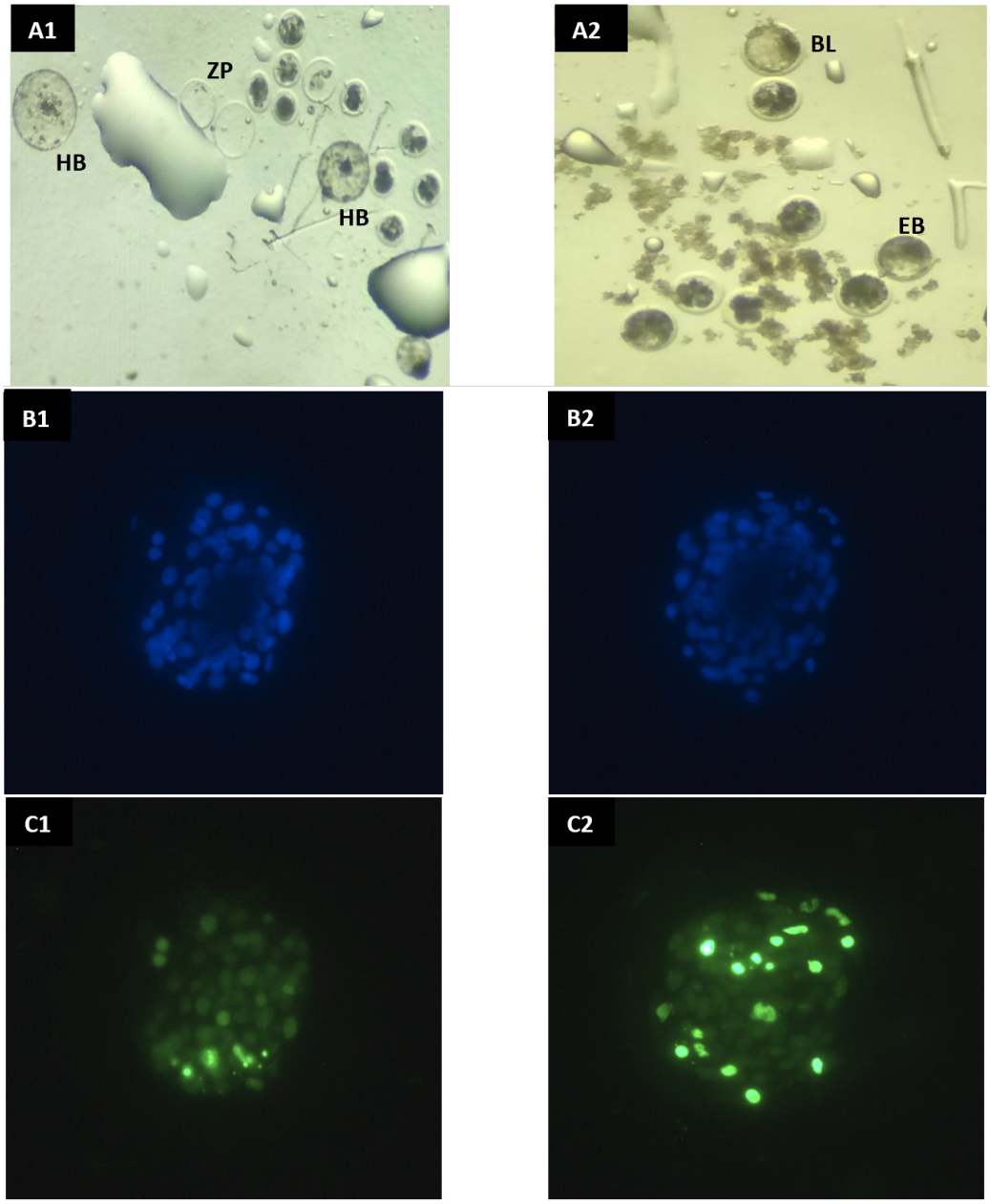

Figure 1. Bovine *in vitro* produced embryos at day 9 of culture. A1, B1 and C1: Control Group; A2, B2 and C2: *N. multiglandulosa* aqueous extract; HB: hatched blastocyst; EB: early blastocyst; BL: blastocyst; ZP: *zona pellucida* after hatching the embryo (stereomicroscopic at a magnification of 40X). B and C: Apoptosis evaluation of *in vitro* bovine embryos (fluorescence microscope at a magnification of 400X). B1 and B1: Total of bovine embryo cells stained with Hoechst (blue) (460nm filter). B2 and C2: bright green cells (TUNEL) in apoptosis (520± 20nm filter).

Table 2. Total number and number of cells in apoptosis ± standard deviation, and apoptotic rate (%) of *in vitro*-produced bovine embryo cells in control and *N. multiglandulosa* aqueous extract groups

Groups	Total number of cells	Number of cells in apoptosis	Frequency of apoptosis (%)
Control	46.66 ± 14.41	3.33 ± 3.00	7.22 ± 7.20
N. multiglandulosa extract	65.20 ± 26.03	4.80 ± 4.10	7.80 ± 7.12

Means of total number and number of cells in apoptosis did not differ. Apoptotic rate did not show significant difference. Fisher's exact test with 5% significance.

Discussion

Based on previous studies using living animals of our group, and trying to replicate the same lowest concentration of toxic plant (0.002mg of plant extract/µL of blood) that had detrimental effects on reproductive tract of pregnant goats[4] and rabbits[17], we proposed to test a single dose of plant extract (0.002mg of plant extract/µL of *in vitro* culture media) to replicate the same concentration used in those *in vivo* trials. To reach the exact concentration of toxic plant we based on methods of Melo et al.[4] that tested two different concentrations (10 and 20g/kg of body weight) of *N. multiglandulosa* leaves. The final concentration of 0.2mg of *N. multiglandulosa* aqueous extract/70µL of embryo media culture drop was related to the lowest dose (10mg/Kg BW or 380g of green leaves/goat/day) that had reproductive negative effects in Saanen goats (average of 38kg BW). *N. multiglandulosa* had 35% of dry matter and animals ingested, on average, 133g/day of dry matter (DM). 1g of DM of plant is equals to 57.3mg of extract, and 133g/day x 57.3mg = 7,620.9mg of extract (daily intake of extract by the goats). For goats with average of 38kg of BW (goat = 38,000,000g) = 7,620.9mg of extract *per* 38,000,000mg of BW= 0.0002 x 100 = ingesting of 0.02% of live weight in extract. Goats have 8 to 10% of BW in blood (3.04 to 3.80L of blood) and 7,620.9mg of extract in 3,000mL of blood (3,000,000µL of blood) = 0.002mg of plant extract/µL of blood. The same calculations were performed by Bull[17] using pregnant rabbits (average of 3kg of BW; ingesting 10.5g/day of DM of plants; 240 to 300mL of blood; 0.002mg of plant extract/µL of blood).

For *in vitro* study in the present trial, we used the same calculations. The extract was obtained from 77g of the plant (77g of green plant x 35% of DM= 26.95g of DM) that provides 1,545mg of extract, and it was used 27mg of extract that was diluted in 1,000 microliters of embryo culture medium = 27 mg/1,000µL = 0.027 mg/microliter of medium. 0.027mg/µL x 7µL that was removed and replaced in culture drop of culture medium (= 0.189 mg of extract in 7µL). A drop of embryonic culture medium = 7µL of the extract + 63µL of culture medium = 70µL (10 % of final volume). 0.189mg of extract in 70µL of the embryo culture medium drops = 0.002mg/µL of culture medium. Finally, the same concentration of plant extract was used for these three different trials using goats[4], rabbits[17] and bovine *in vitro* produced embryos in the present study.

Day 6 of culture was chosen to add the *N. multiglandulosa* aqueous extract based on previous study by Melo et al.[4] that found early signs of embryo toxicity that ultimately lead to decrease of heart beats, fetal dead, abortion, placentary coagulation necrosis and apoptosis of binuclear cells in the trophoblastic epithelium, placentitis and coagulation necrosis of the caruncular areas. In *in vitro* embryo production the early cell differentiation (inner cell mass and trophoblast formation) occurs in embryos around day 6 (early blastocysts), and trophoblastic differentiation, responsible for placenta formation, is found six days after *in vitro* fertilization.

The ingestion of *N. multiglandulosa* leaves induces abortion at different stages of pregnancy, also causing stillbirths or birth of weak offspring[3,4,8]. However, little is known about its possible effects on early embryonic development. The end result of abnormal early embryonic development is death and reabsorption, which usually goes undetected in the medical clinic or presents itself as estradiol-induced repeat breeder cow syndrome[22]. *N. multiglandulosa* extract was toxic during early stages of bovine embryogenesis once it causes underdevelopment and poor hatchability of embryos. These results suggest that the plant has the potential to promote embryonic death and reabsorption in cattle, similarly to reported in rats after ingestion of *Ateleia glazioviana*[23].

Under natural conditions, the embryo's internal cell mass has a higher apoptotic index, with a low proportion in the trophectoderm, which regulates cell population and removes excess damaged, unnecessary, or developmentally-compromised cells in the blastocyst phase[24]. In morphologically normal bovine embryos, apoptosis occurs starting at the 9-16 cells stage and, in the morula phase, 50% of the embryos present at least one nucleus in apoptosis during TUNEL analysis. Blastocysts with fewer than 100 cells have an apoptotic rate of 0-10%, dropping to 0-6% in those with more than 100 cells[24]. In the present study, the extract affected the early embryonic development, as verified by the lower *in vitro* production of bovine embryos. However, the relying mechanism is still uncertain. Increase in apoptosis in the pathogenesis of placental dysfunction was also studied in pregnant goats experimentally poisoned with *N. multiglandulosa.* Intense apoptosis during the early stage of pregnancy may be detrimental for the normal development and function of the placenta and may help to explain fetal death and abortion observed after *N. multiglandulosa* ingestion[25].

Toxins, including phytotoxins, can induce oxidative stress in cells and cause damage by compromising DNA or protein and lipid components[26,27]. Furthermore, the effects on hypoxia-induced mitochondrial respiration and changes in calcium homeostasis bring down ATP levels, compromising early embryo development and increasing *in vivo* and *in vitro* cell loss[26]. Embryos with greater cellularity and viability have a greater chance of implantation in the uterus[28]. Embryos with delayed development and less hatching power from the zona pellucida exhibit degenerative characteristics in the early stages of blastomere division, preventing their subsequent development. This result can be verified by the difference between embryos of the *N. multiglandulosa* aqueous extract group, which are morphologically darkened and of poor quality when compared with the control group, possibly impairing embryo transfer success and rendering pregnancy non-viable under *in vivo* conditions.

The chemical composition of the *N. multiglandulosa* leaves extract was previous studied by Russo et al.[11] performing a fractionation using MPLC-UV, and then a more accurate purification using preparative HPLC. Nineteen substances were isolated such as alkaloids, glycosylated and non- glycosylated flavonoids, glycosylated compounds and six steroids (also called ecdysteroids), including a new ecdysteroid that was identified and elucidated by UHPLC-TOF-HRMS and NMR. The isolated substances were identified as: trigonelline, tryptophan, 4-hydroxycinnamamide, p-Coumaric acid 4-O-β-D-glucopyranoside, icariside F2, luteoforol, (E)-4-Hydroxycinnamic acid, (Z)-4-Hydroxycinnamic acid, integristerone A, epiecdysterone, kaempferol triglucoside ecdysterone, isorhamnetin triglycoside, cinnamic acid, multiglandysterone, calonysterone and podecdysone B.

Certainly, all diagnostic tools-field studies, clinical signs, gross and microscopic pathology as well as chemical identification of plant and plant toxins in animal samples-are essential to make an accurate diagnosis, to develop intervening management strategies and to improve the reproductive performance[29,30].

Result of *in vitro* study suggests that *N. multiglandulosa* aqueous extract may impair reproduction, often silently, by damaging early-stage embryos causing under development, poor capability of *zona pellucida* hatching and possibly early embryonic death. Thus, further studies are required to investigate the detrimental effects of *N. multiglandulosa* on early stages of embryo development. This is the first preliminary study using an *in vitro* embryo production system and we know that the knowledge of this extract is important, and that further work should be done continuing this research. The present study establishes a novel and practical *in vitro* model to study the effect of toxic plants in ruminant reproduction.

Detrimental effects of chemicals and toxicants on female fertility have been tested using *in vitro* animal models mainly with oocytes from cattle and pigs[14,15,31,32]; as a possibility to study a model for human. *In vitro* models have been increasing once the use of animals for scientific purposes is decreasing in researches. Those studies are focusing on the effect of reproductive toxins and toxicants on *in vitro* oocyte maturation, fertilization and preimplantation stage of early embryos. The quality of oocytes is impaired when women are exposed to toxic agents during maturation[14], and if the oocyte does not follow an apoptotic pathway[15], there is still the risk to affect fertilization and subsequent embryo development once oocytes are susceptible to epigenetic alterations[32]. Damage to embryonic and placentary cells leads to the abnormal development and function of the placenta and may explain fetal death and abortion observed after *N. multiglandulosa* ingestion[25].

Conclusion

In conclusion, *in vitro* exposure of bovine embryos to 2.7mg/mL of *N. multiglandulosa* aqueous extract caused a marked reduction in embryo development and hatchability, suggesting the plant may cause damage to embryos when exposure occurs in the early stages of pregnancy. Furthermore, the experiment confirms that *in vitro* produced bovine embryos can be used to investigate plant toxicity.

Funding information

Authors would like to thank the FAPEMIG, CNPq and CAPES.

Declaration of conflict of interest

None of the authors of the above manuscript has declared any conflict of interest.

Author Contributions

Conceptualization: M.M. Melo, A.M.Borges; *Data curation:* V. Bull, R.H.S.da Silva, A.C. Leite, E.B.M.Silva, A.C. Nepomuceno, A.F.M. Botelho; *Project management*: M. M. Melo; *Formal analysis:* A.F.M.Botelho, B. Soto-Blanco, A.C. Nepomuceno; *Writting (original draft):* V. Bull, A.F.M.Botelho, A.M.Borges, B.Soto-Blanco, M.M.Melo; *Writting (revision and edition):* A.F.M.Botelho, A.M.Borges, M.M.Melo.

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