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# Embryotoxic Effects of Three Natural Occurring *Veratrum* Alkaloids and One Synthetic Analog using In Vitro Produced Bovine Embryos

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# Embryotoxic Effects of Three Natural Occurring *Veratrum* Alkaloids and One Synthetic Analog using In Vitro Produced Bovine Embryos


## Abstract

Three natural occurring plant toxins from *Veratrum californicum* and one related synthetic analog were screened for embryotoxicity using *in vitro* bovine embryo production techniques. Bovine oocytes were aspirated from ovaries collected from a local abattoir and embryos were generated through *in vitro* maturation (IVM) and *in vitro* culture (IVC) procedures. The three natural steroidal alkaloids, cyclopamine, jervine and veratramine and the synthetic steroidal derivative of cyclopamine, cyclopamine-4-en-3-one, were added to IVM and IVC media at 12 µM. Oocytes were exposed to the toxins during maturation (IVM) and pre-implantation embryo during culture (IVC). Cleavage rates and embryo growth (morula and blastocyst production) and development through the hatched blastocyst stage were evaluated. Cyclopamine and cyclopamine-4-en-3-one inhibited cleavage rates and embryo growth and development of morulae and blastocysts in culture. Oocytes that were exposed to cyclopamine and cyclopamine-4-en-3-one during IVM only showed reduced cleavage rates and resulted in lower numbers of embryos that developed to the morula, blastocyst, and hatched blastocyst stages. The effects of these steroidal alkaloids on the oocyte during IVM and on the embryo during all stages of development up to and including the hatched blastocyst stage, demonstrates a dramatic cytotoxic effect on oocytes maturation and early pre-implantation embryos. This research also suggests that the Hedgehog signaling pathway may play a role in the maturing oocyte as well as the pre-implantation embryo. These *in vitro* fertilization techniques provide an economical, rapid through put and effective method to screen natural toxins, especially suspected reproductive toxins for cytotoxicity.



## Keywords

cyclopamine, sonic hedgehog, veratrum, in vitro maturation



## Cover Page Footnote

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## INTRODUCTION

*Veratrum californicum* was responsible for the induction of congenital cyclopia "monkey faced lambs" in sheep grazing high mountain ranges in central Idaho (Binns et al., 1959). The incidence of malformation in some flocks of 5,000-10,000 ewes was 25% or more (Binns et al., 1962). When open ewes from presumed *Veratrum*-induced embryo loss was included, the economic impact was even greater. It was determined that the cyclopic-type craniofacial birth defects occurred when pregnant sheep ingested *Veratrum* on day 14 of gestation (Binns et al. 1963). The teratogenic window of embryonic susceptibility to *Veratrum* also includes gestation day (GD) 13 (Welch et al., 2009). When *Veratrum californicum* root was fed to pregnant ewes at 0.88 mg cyclopamine $kg^{-1}$ body weight on GD 13 or 14 multiple craniofacial malformations including cyclopia, maxillary dysphasia and mandibular micrognathia resulted (Welch et al., 2009; Welch et al., 2011). However, *Veratrum* root fed on GD 15 resulted in normal lambs and when fed consecutively from GD 13-15 all ewes were open and *Veratrum*-induced embryonic death was believed to be the cause. Other malformations were linked to *Veratrum* ingestion including skeletal malformations such as carpal and tarsal shortening and tracheal stenosis occur in lambs when ingestion occurred during GD 28-33 (Keeler and Stuart, 1987).

While cyclopamine is believed to be the putative teratogen in *Veratrum* other naturally occurring steroidal alkaloids such as jervine and veratramine are also present in varying concentrations in the plant. Keeler et al., 1993, determined the structural requirements for these steroidal alkaloids to impart teratogenic activity. These structure activity studies determined that cyclopamine and jervine possessed the required structural requirements for teratogenic activity while veratramine did not. Furthermore, it was shown that teratogenic activity was enhanced by the synthetic derivative cyclopamine-4-en-3-one (Brown and Keeler, 1978).

The mechanism of cyclopamine-induced birth defects has been shown to result from the inhibition of the Sonic Hedgehog (Shh) signal transduction pathway (Cooper et a., 1998; Incardona et al., 1998). The Hedgehog signaling pathway plays a key role in cellular differentiation especially in neurological tissue including the eyes (Lum and Beachy, 2004). The alkaloids jervine, cyclopamine and cyclopamine-4-en-3-one inhibit Sonic Hedgehog signaling and produce cyclopia and holoprosencephaly in gastrulation-stage embryos (Gaffield et al. 2000). Cyclopamine inhibits Shh at 0.12 μM and completely blocks the induction of floor plate and motor neurons at 12 μM and which is believed to be the mechanism of *Veratrum*-induced "monkey faced" lamb disease.

The importance of *in vitro* fertilization techniques which includes *in vitro* oocyte maturation, *in vitro* oocyte fertilization and *in vitro* embryo culture using bovine oocytes from a local abattoir to screen for cytotoxicity of certain natural toxins has been previously demonstrated (Panter et al., 2004; Wang et al., 1999,

2004, 2005). Specifically, biological activity of reproductive toxins from locoweeds, ponderosa pine needles and *Solanum* spp. has been evaluated using these methods.

The objective of this research was to determine the effects of selected teratogens and reproductive toxins from *Veratrum californicum* on oocyte maturation and pre-implantation embryo development using *in vitro* fertilization (IVF) techniques. We believe these IVF techniques provide an economical, rapid through put and effective method to screen natural toxins, especially suspected reproductive toxins for cytotoxicity.

## MATERIALS AND METHODS

***Production of bovine embryos in vitro.*** Ovaries were collected from a local abattoir. Oocytes were aspirated from small antral follicles (3 to 8 mm in diameter) as described by Hawk and Wall (1994). Cumulus oocyte complexes (COCs) with evenly granulated ooplasm and surrounded by several layers (at least 3 layers) of compact cumulus cells were selected for use according to the oocyte grading system of Hawk and Wall (1994). Oocytes were washed three times with HEPES-TALP solution (Parrish et al., 1988) and once with maturation medium. *In vitro* maturation (IVM) of oocytes followed the procedure of Sirard et al. (1988) and Bavister et al. (1992) with minor modifications. The maturation medium consisted of M-199 plus 10% (vol/vol) fetal bovine serum (FBS, A-1111, HyClone Laboratories, Inc., Logan, UT, USA), 25 mM HEPES, 2 mM glutamine, 0.25 mM sodium pyruvate, 0.5 µg/ml ovine FSH (F-4520, Sigma Chemical Company, St. Louis, MO, USA), 5.0 µg/ml ovine LH (L-5269, Sigma), and 1.0 µg/ml estradiol (E-2258, Sigma). Polystyrene plastic 4-well culture petri dishes (Nunclon, Nunc Inc., Naperville, IL, USA) were used for IVM culture. Each well contained 500 µL IVM medium covered with paraffin oil (6358, Mallinckrodt Inc., Port, KY, USA). Approximately 40 to 65 oocytes were transferred to the IVM medium per well and cultured in a humidified 5% $CO_2$ atmosphere at 39°C for 24 h.

Cryopreserved bovine semen was used for *in vitro* fertilization (IVF). Live sperm were separated with Percoll (P-4937, Sigma) gradients (45% and 90% on the upper and lower layers, respectively) and centrifuged at 500 x *g* for 30 min. Motile spermatozoa were added to the fertilization medium (Fert-TALP, Parrish et al., 1988) to provide a final concentration of 1.5 x $10^6$ per ml. Capacitation of spermatozoa occurred in Fert-TALP containing 10 µg heparin/ml and 0.6% fatty acid free bovine serum albumin. IVM matured oocytes were added to Fert-TALP containing spermatozoa and cultured in plastic 4-well petri dishes under paraffin oil in a humidified 5% $CO_2$ atmosphere at 39°C for 17 h. Each well contained 500 µL Fert-TALP and approximately 40 to 65 oocytes.

Cumulus and corona cells were removed from ova by mixing in HEPES-TALP supplemented with 0.3% (w/v) bovine serum albumin for 3 min. The presumptive

zygotes were then cultured in plastic 4-well Petri dishes under paraffin oil at 39ºC in a humidified 5% $CO_2$ atmosphere. A modified CR2 medium (Wang et al., 1997) comprising 108.3 mM NaCl, 2.9 mM KCl, 24.9 mM $NaHCO_3$, 2.5 mM hemicalcium lactate, 0.5 mM sodium pyruvate, BME amino acids (B-6766, Sigma), MEM nonessential amino acids (M-7145, Sigma), 0.5 mM glycine, 0.5 mM alanine, 1.0 mM glutamine, 1.0 mM glucose, and antibiotics was used to culture embryos. Each well contained 500 µL CR2 medium with approximately 40 to 60 oocytes. During culture, medium was changed every other day.

***Preparation of Veratrum Alkaloids:*** The steroidal alkaloids were extracted from *Veratrum californicum* roots, isolated and purified according to Gaffield and Keeler (1996). Briefly, dried ground *Veratrum* roots were extracted using organic solvents and separated into active fractions. Each alkaloid was isolated and purified by repeated chromatographic separations on silica gel and following re-crystallization, purity was determined by chromatographic (TLC) and spectroscopic (GC-MS) analysis. The cyclopamine-4-en-3-one was prepared by Oppenauer oxidation of cyclopamine as previously described (identified as 11-dexojervine-4-en-3-one, Brown and Keeler, 1978 and references therein).

***In vitro experiments.*** Two experiments were designed to test the effects of steroidal alkaloids on bovine oocyte maturation and embryo development. Experiment 1 was aimed to evaluate the effect of exposure of bovine oocytes to alkaloids during maturation stage only. Experiment 2 was to exam the influence of the steroidal alkaloids on the pre-implantation embryo development of *in vitro* generated bovine zygotes.

In Experiment 1, oocytes (n=2665) aspirated from the same collection of abattoir ovaries were used for IVM culture and subjected different treatments. There were 5 experimental treatments: Control (TRT 1), in which no alkaloid was added to the IVM medium; TRT 2, 12 µM of jervine; TRT 3, 12 µM of veratramine; TRT 4, 12 µM of cyclopamine; and TRT 5, 12 µM of cyclopamine-4-en-3-one were applied to the IVM culture medium, respectively. The *in vitro* matured oocytes then went through *in vitro* fertilization (IVF) followed by *in vitro* embryo culture (IVC) procedures under same conditions, i.e., no steroidal alkaloids mentioned above were added into either IVF or IVC media. Oocyte cleavage rates were determined 48 h after the exposure of matured oocytes to spermatozoa. The pre-implantation embryo development was further evaluated, i.e., morula production at day 6, blastocysts production at day 8, and expanded blastocysts and/or hatched blastocysts at day 10 of IVC (IVF = day 0).

In Experiment 2, oocytes (n=2114) aspirated from the same collection of abattoir ovaries were subjected to the *in vitro* maturation and in *vitro* fertilization procedures to generate zygotes. Steroidal alkaloids were added to the IVC media in which the *in vitro* generated (presumptive) zygotes were cultured for embryonic development. There were 5 IVC experimental treatments: Control, in which no

alkaloid was added (TRT 1); 12 µM of jervine (TRT 2), 12 µM of veratramine (TRT 3), 12 µM of cyclopamine (TRT 4), or 12 µM of cyclopamine-4-en-3-one (TRT 5) were applied to IVC media, respectively. Cleavage rates were determined 48 h after the exposure of zygotes to these chemicals. The pre-implantation embryo development was evaluated at days 6, 8, and 10 of IVC as described in Experiment 1 (IVF = day 0).

***Statistical analysis.*** A complete randomized block experimental design was used to establish the effects of these toxins on *in vitro* culture of IVM/IVF derived bovine embryos. Percentage data were angularly transformed and analyzed by the use of a general linear model (GLM) ANOVA. The Fisher's Least Significant Difference (LSD) at the 5% significant level ($P < 0.05$) was used to test the differences between treatment means. The NCSS 97 (Number Cruncher Statistical System) computer software package (Hintze, 1997) was used for all statistical calculations.

## RESULTS

***In vitro maturation experiment.*** The results from *in vitro* maturation experiment (Table 1, Experiment 1) showed the effects of steroidal alkaloids on oocytes

**Table 1.** The effects of *Veratrum* alkaloids with jervine ring on *in vitro* bovine oocyte maturation (Experiment 1).

| Steroidal alkaloid concentration in medium (12 µM) | Number of Oocytes n | Cleavage Rate at 48 h[f] n (%)[g] | Morulae[h] at day 6[i] n (%)[j] | Blastocysts at day 8 n (%) | Expanded Hatched Blastocysts at day10 n (%) |
|---|---|---|---|---|---|
| Control | 449 | 389(87.1)[a] | 225(57.4)[a] | 112(28.3)[a] | 95(24.1)[a] |
| Jervine | 448 | 370(82.7)[a] | 178(48.5)[a] | 79(21.4)[a] | 96(25.6)[a] |
| Veratramine | 463 | 381(82.6)[a] | 193(50.6)[a] | 83(21.7)[a] | 66(17.4)[a] |
| Cyclopamine | 457 | 303(65.6)[b] | 92(27.9)[b] | 24(6.9)[b] | 26(7.4)[b] |
| Cyclopamine-4-en-3-one | 448 | 229(49.3)[c] | 43(17.1)[c] | 10(3.2)[b] | 6(1.8)[c] |

Within a column, values with different letters (a-d) are significantly different ($P < 0.05$)

[f] 0 h = the time when the in vitro matured oocytes were added to Fert-TALP containing spermatozoa.

[g] The percentage data were angularly transformed and analyzed by general linear model (GLM) ANOVA. The percentage of each treatment in this table represents 10 replications.

[h] Morulae rates were based on nine replications for Experiment 1 and eight replications for Experiment 2.

[i] Day 0 = IVF

[j] Percentage development of morulae/blastocysts/expanded blastocysts was calculated with respect to cleaved oocytes.

maturation and subsequent embryonic development. The cleavage rates were 87.1%, 82.7%, 82.6 %, 65.6%, and 49.3% for TRT 1, TRT 2, TRT 3, TRT 4, and TRT 5 respectively. The percentage of morulae at day 6 of IVC was 57.4%, 48.5%, 50.6%, 27.9% and 17.1%; the percentage of blastocysts at day 8 of IVC was 28.3%, 21.4%, 21.7%, 6.9%, and 3.2%; and the percentage of expanded and hatched blastocysts at day 10 was 24.1%, 25.6%, 17.4%, 7.4%, and 1.8% for TRT 1-5 respectively (Table 1, Experiment 1).

As shown in development of pre-implantation embryos derived from oocytes matured in media containing either cyclopamine or cyclopamine-4-en-3-one was inhibited compared to control, jervine or veratramine ($P < 0.05$). Cleavage rates were lower and numbers of embryos developing to morulae, blastocyst and expanded and hatching blastocyst stages were also significantly reduced ($P < 0.05$). The production of morulae and hatched blastocysts was further reduced for cyclopamine-4-en-3-one compared to cyclopamine ($P < 0.01$).

***In vitro culture experiment.*** The cleavage rates from *in vitro* culture experiment in which the steroidal alkaloids were added to the embryo culture media were 85.6%, 83.9%, 69.1%, 85.5% and 71.2% for TRT 1-5 respectively (Table 2, Experiment 2). The percentage of morulae at day 6 of IVC was 57.3%, 54.3 %,

**Table 2.** The effects of *Veratrum* alkaloids with jervine ring on *in vitro* bovine oocyte maturation (Experiment 2).

| Steroidal alkaloid concentration in medium (12 μM) | Number of Oocytes n | Cleavage Rate at 48 h[f] n (%)[g] | Morulae[h] at day 6[i] n (%)[j] | Blastocysts at day 8 n (%) | Expanded Hatched Blastocysts at day10 n (%) |
|---|---|---|---|---|---|
| Control | 426 | 363(85.6)[a] | 209(57.3)[a] | 84(22.8)[a] | 73(20.0)[a] |
| Jervine | 420 | 352(83.9)[a] | 189(54.3)[a] | 65(18.7)[b] | 45(12.8)[b] |
| Veratramine | 424 | 292(69.1)[b] | 143(48.3)[a] | 47(15.5)[c] | 26(9.1)[c] |
| Cyclopamine | 432 | 369(85.5)[a] | 177(48.0)[a] | 66(17.8)[b] | 19(5.3)[d] |
| Cyclopamine-4-en-3-one | 412 | 293(71.2)[b] | 42(12.9)[b] | 12(3.6)[d] | 15(4.7)[d] |

Within a column, values with different letters (a-d) are significantly different ($P < 0.05$)

[f] 0 h = the time when the in vitro matured oocytes were added to Fert-TALP containing spermatozoa.

[g] The percentage data were angularly transformed and analyzed by general linear model (GLM) ANOVA. The percentage of each treatment in this table represents 10 replications.

[h] Morulae rates were based on nine replications for Experiment 1 and eight replications for Experiment 2.

[i] Day 0 = IVF

[j] Percentage development of morulae/blastocysts/expanded blastocysts was calculated with respect to cleaved oocytes.

48.3%, 48.0% and 12.9%; the percentage of blastocysts at day 8 of IVC was 22.8%, 18.7%, 15.5%, 17.8% and 3.6%; and the percentage of expanded and hatched blastocysts at day10 was 20.0%, 12.8%, 9.1%, 5.3% and 4.7% for TRT 1-5, respectively (Table 2, Experiment 2).

These results indicated that cleavage rates were lower for veratramine and cyclopamine-4-en-3-one, compared to controls, jervine or cyclopamine ($P < 0.05$). The percentage of pre-implantation embryos to reach the morulae stage of development was reduced for cyclopamine-4-en-3-one only ($P < 0.05$) but the reduction in percentage of blastocysts and expanded/hatched blastocysts was lower for all 4 steroidal alkaloids. Furthermore, the rank of inhibition for development to the blastocyst and expanded/hatched blastocyst stage was cyclopamine-4-en-3-one > cyclopamine > veratramine > jervine > control ($P < 0.05$).

## DISCUSSION

*Veratrum* alkaloids have long been known to cause early embryonic loss and severe birth defects in livestock (Binns et al., 1959; Binns et al., 1963; Keeler and Stuart,

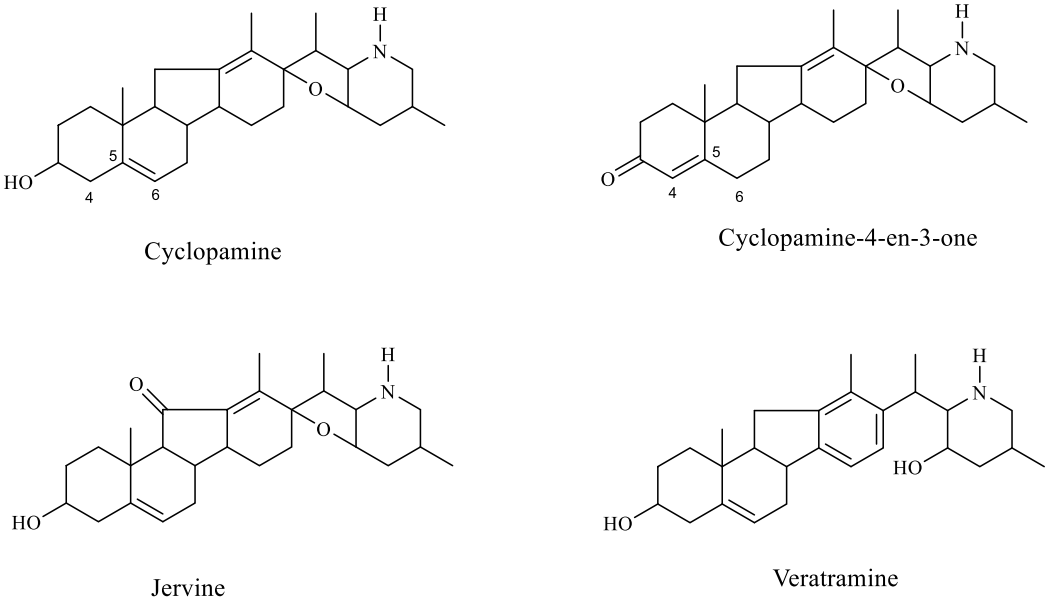


**Figure 1.** Steroidal alkaloids exposed to *in vitro* Oocytes/embryos. Cyclopamine and cyclopamine-4-en-3-one demonstrating the difference in functionality at the 5-6 and 4-5 unsaturation. Cyclopamine-4-en-3-one is 2x more potent in Hedgehog inhibition and is most toxic to oocytes and pre-implantation bovine embryos in the *in vitro* maturation (IVM) and *in vitro* culture (IVC) experiments.

1987). More recently, research has demonstrated that cyclopamine and other steroidal alkaloids from *Veratrum* and *Solanum* spp. possessing certain chemical functionalities may impart this abnormal growth and development through inhibition of the Hedgehog signaling pathway (Gaffield and Keeler, 1996; Gaffield et al., 2000). The cyclopamine-driven inhibition of hedgehog pathways has been exploited in cancer research and found to be active against pancreatic cancer and other cancers that express the Hedgehog pathway (Thayer et al., 2003). It has also been demonstrated that minor changes in chemical functionalities impart more potent effects, for example shifting the unsaturation from the 5-6 position as found in cyclopamine to the 4-5 position creating the unsaturated ketone as in cyclopamine-4-en-3-one changes Hedgehog inhibition almost 2-fold (Figure 1). Cyclopamine-4-en-3-one inhibited pre-implantation embryo development *in vitro* most significantly when maturing oocytes, as well as IVF embryos, were cultured in the presence of cyclopamine-4-en-3-one. This research suggests that the hedgehog signaling pathway is not only important in pre-implantation embryos but may have a role either directly or indirectly in the maturing oocyte.

The results presented in this study have been supported by research which has demonstrated that Sonic Hedgehog signaling is important for follicle development, oocyte maturation, and embryo development (Lee et al., 2017). In this regard, many studies now use cyclopamine as a research tool to demonstrate the role of hedgehog signaling in specific aspects of oocyte maturation and follicle development (Terauchi et al., 2020, Guo et al., 2022, Lee et al., 2018). However, some research suggests that the effects may be species specific (Liu et al., 2014).

## CONCLUSION

Exposure of bovine oocytes to cyclopamine and its synthetic analog cyclopamine-4-en-3-one during maturation inhibited cleavage rates and subsequent pre-implantation embryo development *in vitro*. Similarly, exposure of pre-implantation embryos immediately after IVF inhibited cleavage rates and subsequent embryonic development. This research indicates that ingestion of natural toxins such as these steroidal alkaloids can adversely affect oocyte maturation and subsequent pre-implantation embryo development. Furthermore, this research suggests a role of the Hedgehog signaling pathway in the maturation of oocytes as well as the normal development of the pre-implantation embryo. This IVF model provides an alternative to animal testing as a technique to screen potential reproductive and teratogenic toxins.

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