Isocupressic Acid Blocks Progesterone Production from Bovine Luteal Cells


*Department of Animal Science, †Department of Physiology and ‡Department of Chemistry, National Taiwan University
§Department of Pharmacy, Taipei Medical College
Taipei, Taiwan, Republic of China

Abstract: The needles of ponderosa pine (Pinus ponderosa Laws.) were reported to induce abortions when fed to late-term pregnant beef cows in North America. An in vivo study of pregnant cows suggested that isocupressic acid (IA) was the main abortifacient isolated from needles and bark of the pine. However, the mechanism of abortifacient activity of IA is not clear yet. In a pregnant cow, the corpus luteum of the ovary helps the maintenance of pregnancy by its progesterone production. This study involved the IA extracted from the root of the Taiwan cypress (Juniperus formosana) and used a frozen-thawed bovine luteal cell culture system to investigate the action of IA on progesterone production.

Thawed bovine luteal cells (1 × 10^5 cells/ml/well) in M199 medium were cultured in 24-well culture plates at 37°C in a 5% CO₂ incubator. Ten ml of tested drugs, IA at 1 to 1000 ng/ml and/or ovine luteinizing hormone (oLH) at 1 to 100 ng/µl or 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) with 0.1–10 mM, were added into each well. After 4 hours of incubation, the media were harvested and assayed for progesterone by an enzyme immunoassay. Progesterone production from cells was the indicator used to evaluate the action of IA. All tested doses of IA significantly inhibited progesterone production in both basal and oLH stimulating conditions. Also those dosages inhibited cyclic adenosine-3',5' monophosphate (cAMP) stimulation, suggesting a post-cAMP mechanism is involved in the IA action. We concluded that IA can induce pregnant cows to abort partly through blocking luteal function and may be identified as a new abortifacient chemical.

Keywords: Ponderosa Pine; Isocupressic Acid; Bovine Luteal Cells; Progesterone; Enzyme Immunoassay.

Correspondence to: Prof. Jen-Hsou Lin, Department of Animal Science, National Taiwan University, 1 Rooslev Rd., Sec. 4, Taipei 106, Taiwan, R.O. C. Tel: (+886) 2-2363-0231 (ext. 3103), Fax: (+886) 2-2733-7095, E-mail: jhlin@ccms.ntu.edu.tw

533
Introduction

Ponderosa pine, often called yellow pine and black jack pine, is a large coniferous tree. It has characteristic yellowish-green needles (18–28 cm long) in bundles of three, and has yellow and black cones. Ponderosa pine is extremely important for the lumber industry and can be found throughout the western United States, southern British Columbia, and northern Mexico, growing at elevations of 7000–9000 feet. Many cattle ranges in those states have dense ponderosa pine forests, in which cattle have access to the trees.

Since the early 1900s, there has been concern that the ingestion of ponderosa pine needles by pregnant cattle caused them to abort their calves (Bruce, 1927). Abortions may occur from two to 14 days after ingesting pine needles. Cows in late gestation have a high incidence of abortion when exposed to pine needles (Short et al., 1992). Controlled feeding trials demonstrated the abortifacient effects of the pine needles experimentally (MacDonald, 1952). Many other feeding trials also confirmed this (James et al., 1977 and 1989; Gardner et al., 1996).

Since the early 1960s, many attempts were made to identify the abortifacient toxin in the needle (Allen and Kitts, 1961; Cook and Kitts, 1964), researches eventually identified isocupressic acid (IA) as the abortifacient compound shown in Fig. 1 (Gardner et al., 1994, 1997 and 1999). The pine needle toxin or its metabolites is thought to disrupt uterine vascular flow resulting in decreased placental perfusion. However, IA does not appear to act directly at the site of the uterine arterial muscle (Short et al., 1996) and its specific abortifacient mechanism remains unknown at this point.

Figure 1. Structure of IA, an abortifacient compound isolated from ponderosa pine (Pinus ponderosa).

Normal function of corpus luteum is as a main controller that maintains animal pregnancy. There is no research that focuses on the relationship between IA and corpus luteum. In this study, we investigate the action of IA in an in vitro system of frozen-thawed bovine luteal cell culture system (Yuan et al., 1994).
Materials and Methods

Materials

Professor Y.H. Kuo, Department of Chemistry, National Taiwan University, supplied the isocupressic acid (Fig. 1), extracted from the roots of Taiwan cypress (*Juniperus formosana*) (Shiu, 1998). The culture media were purchased from Pharmacia Co. Ltd. The National Hormone and Pituitary Program, USA kindly provided the ovine luteinizing hormone (NIDDK-oLH-I-3, Bethesda, MD), and the staff in our laboratory made the enzyme immunoassay (EIA) kits for the progesterone assay (Wu *et al.*, 1997). Heng-Chun Station, Taiwan Livestock Research Institute, supplied the bovine luteal tissues. Most of the chemical compounds, such as bovine serum albumin, collagenase, deoxyribonuclease I, etc. were purchased from Sigma Chemical Co.

A Frozen-Thawed Bovine Luteal Cell System to Screen Luteotropic Activity in Drugs

Due to its simplicity and reliability, the frozen-thawed bovine luteal cell system, established since 1994, is a useful system for studying the steroidogenesis of chemicals and herbal extracts (Yuan *et al.*, 1994; Wu *et al.*, 2000). Once a good batch of frozen cells is established, data from each set of experiments are quite similar between cryopreservatives. Dispersed luteal cells were obtained enzymatically from corpora lutea enucleated aseptically *per vaginam* from Holstein or beef cows between days 9–11 of the estrous cycle (estrus = day 0) (Hickey and Hansel, 1987). Cryotubes, each containing $1 \times 10^7$ cells/ml and freezing media (10% DMSO; Gibco 320-1101PF), were frozen in liquid nitrogen for storage. Before the test, the frozen cells in the cryotube were rapidly thawed in a water bath at 37°C. The thawed cells were washed twice and resuspended in medium 199. One ml of media was put in each well of 24-well culture plates (Coaster) containing $1 \times 10^5$ viable cells/ml. Test drugs, i.e. ovine luteinizing hormone (oLH) extracts were added in volumes of 10 µl. The plates were incubated at 37°C and 5% CO₂ for 4 hours. During the incubation period, and depending on the experimental design, 50 µl of media were collected periodically and frozen at −20°C until needed for progesterone assay. Each drug test was done in triplicate and each experiment was performed at least three times. The data shown were either selected from the best set of results with corresponding response tendency, or from the average of three experiments.

Enzyme Immunoassay for Progesterone

A valid competitive enzyme immunoassay for progesterone was established in our laboratory using monoclonal antibody G7 (Wu *et al.*, 1997). Briefly, 50 µl of diluted test medium + 150 µl of progesterone-horseradish peroxidase were added to 96 wells of a microtiter plate (Costar 3590) coated with G7 monoclonal antibody. After incubation at room temperature for 15 minutes and washing with phosphate buffered saline (PBS) twice, the color was developed in 200 µl of 2.2 mM o-phenylenediamine in 0.003% H₂O₂ at room temperature for 30 minutes. The reaction was stopped by 50 µl of 8 N sulphuric acid.
Absorbance rate at 490 nm was compared with a progesterone standard curve. The variation coefficients within and between assays were approximately 7 and 10% respectively, and the sensitivity was 0.3 pg / well.

Statistical Analysis

Data obtained from pharmacological experiments were expressed as mean ± SD and were analyzed by ANOVA and Duncan’s multiple range test and t-test for comparison of two means. A value of $p < 0.05$ was considered a statistically significant difference.

Results

The objective of this experiment is to study the action of IA on bovine luteal tissue leading to its abortive mechanism in pregnant cows. This in vitro experiment was conducted on the bovine luteal cells, which were used for assaying the progesterone production by incubation of IA alone or in combination with oLH or cAMP. The dose-dependent production of progesterone by the luteal cells in response to the stimulation by oLH and 8-Br-cAMP is shown in Fig. 2; thus indicating the reliability of the sensitive bioassay used. The viability

![Graph showing effect of oLH and 8-Br-cAMP on progesterone secretion](image)

Figure 2. Effect of oLH and 8-Br-cAMP on progesterone secretion of bovine luteal cells in vitro. Each point represents the mean ± SD ($n = 3$). *$p < 0.05$, **$p < 0.01$, compared to control group.
Figure 3. Effect of IA on basal secretion of progesterone in bovine luteal cells *in vitro*. Each point represents the mean ± SD (n = 3). *p < 0.05, **p < 0.01, compared to control group.

Figure 4. Effect of IA on stimulated secretion of progesterone by oLH (upper panel) and cAMP (lower panel) in bovine luteal cells *in vitro*. Data are expressed in units of inhibition index (progesterone conc. in treatment/ progesterone conc. in control) and obtained from three different experiments with triplicate in each test. *p < 0.05, compared to control group.
of the cells in the culture for experimental purpose was tested by staining with trypan blue and there was no cell death when incubated with IA between 1–1000 ng/ml for 4 hours, suggesting that the reduced progesterone production by IA did not result from the death of luteal cells. The level of progesterone production by the experimental bovine luteal cells resulting from the incubation of IA alone or in combination with oLH or cAMP is shown in Figs. 3 and 4. The results indicate progressive reduction in progesterone production by the bovine luteal cell culture in vitro trials when IA was added at the dose rate of 10 to 1000 ng/ml to the luteal cells (Fig. 3). The addition of oLH at the dose of 10 ng/ml to the various doses of IA between 100 to 1000 ng/ml was not able to stimulate progesterone secretion; 8-Br-cAMP at 1mM along with different doses of IA also could not increase progesterone production (Fig. 4). This data implies that IA inhibits progesterone production by blocking oLH and cAMP actions at the cellular level of luteal cells.

Discussion

The total economic losses resulting from livestock poisonings have been estimated to be as high as US$20 million for the direct losses from pine needle abortions (Miner et al., 1987; Lacey et al., 1988). The indirect losses from increased management costs, supplemental feeding, lost forage, veterinary care, increased postpartum interval, and smaller weaning weights should also be considered in the overall effect these plants have on the economic losses for the livestock and supporting industries.

There are no known methods to prevent abortions after pregnant cattle consume pine needles. Currently, the best method to prevent abortions is to deny cows access to pine needles during the late periods of pregnancy (third trimester) by either removing pregnant cows from the pines or eliminating the pine trees by burning or clear cutting. If we know the mechanism of abortion of the pine needles, we may develop some other preventive and therapeutic methods for the intoxication.

James et al. (1989) suggested that many factors may be associated with and/or predispose cattle to abort after ingestion of pine needles: the stage of gestation, amount of needles ingested, environmental stress, nutritional status of the cows, and current management practices. Abortions may occur from two to 14 days after ingesting pine needles (Short et al., 1992). Somehow, IA was identified as the major abortifacient pine toxin (Gardner et al., 1994). IA also exists in other plant species like in Cypress but at a low concentration level (Shiu, 1998), with no reported cases of abortion in cows when compared to ponderosa pine trees. However, pine needles mainly from Pinus tabulaeformis Carr. are largely used in China as feed additives for increasing meat and egg production and are not reported to induce any abortive action in cattle (Hsieh et al., 1996). The varying results may be attributed to many factors, ranging from palatability of the plant species, its IA concentration and other factors, which would be an interesting topic for further investigation.

It is, however, speculated that pine needles from Ponderosa trees affect the fetal/placental unit, reducing blood flow to the fetus and subsequently initiating the parturition mechanism (Ford et al., 1992 and 1997). Christensen et al. (1992 and 1993) showed that pine needles reduced uterine blood flow by up to 56%, and this is believed to initiate the fetal parturition
mechanism during pregnancy. Caruncular arterial blood flow is regulated by short-term (phasic) and long-term (tonic) contractile mechanisms controlled by a balance of adrenergic receptors, potential-sensitive Ca++ channels, and various hormone-sensitive receptors (Ford et al., 1997). Recent reports suggest the role of progesterone in maintaining pregnancy is by supporting the blood flow and maintaining vascular structure of uterine tissues (Reynolds et al., 1992). The consequence of reduced progesterone results in the apoptosis of corpus luteum derived endothelial cells (Friedman et al., 2000). This will affect the blood circulation ultimately leading to abortion.

There are still no reports concerning basic research of IA on bovine luteal tissue. The current study focused on the role of IA in its inhibitory action on bovine luteal cells for progesterone secretion. This study proposed that IA may inhibit the luteal function and also established a frozen-thawed bovine luteal cell system (Yuan et al., 1994; Wu et al., 2000) for the investigation (Fig. 2). The results suggested that IA may block luteal functions in pregnant cows and subsequently induce abortion. They also imply that the abortifacient actions may involve a post-cAMP mechanism (Fig. 4), similar to that of cantharidin which is a defensive chemical isolated from Chinese blister flies or Spanish flies, and is capable of inhibiting testosterone production in the rat Leydig cell system (Lin et al., 1995). Both compounds are known to have abortifacient effects. However, through irritant stimulation of the bladder and urethra, cantharidin also possesses aphrodisiac actions in males. Whether this same effect exists in IA requires further studies though both compounds may be considered as new drugs for inducing abortions in the future. In addition, acetyl and succinyl compounds, two naturally occurring ester derivatives of IA, were shown to be abortifacient (Gardner et al., 1996). Therefore, further research is needed on derivatives of IA.

In summary, it can be said that IA results in the decrease in progesterone secretion, which affects the angiogenesis of the female reproductive system. The decrease in blood flow and apoptosis of corpus luteum-derived endothelial cells of the uterine tissue results in abortion. However, a more detailed and in-depth study is needed to obtain strong supporting evidence for this hypothesis of IA-induced abortive mechanism in pregnant cows.

**Acknowledgments**

We thank The National Science Council, Republic of China for their financial support (NSC 89-2313-B-002-129 and 90-2313-B-002-301). Phil Rogers MRCVS, Dublin, Ireland is duly acknowledged for his help in the final preparation of this manuscript.

**References**


Bruce, E.A. Astragalus serotinus and Other Stock-Poisoning Plants of British Columbia. Dominion of Canada, Department of Agriculture Bulletin No. 88, 1927, P. 44.


Shiu, L.L. Studies on the chemical components of heartwood of Juniperus chinesis and leaves and roots of Juniperus formosana. PhD thesis of the National Taiwan University, Taipei, Taiwan, ROC, 1998.


