Oral, Osmotic Minipump, and Intramuscular Administration to Sheep of the *Veratrum* Alkaloid Cyclopamine (42970)

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Abstract. **Logistic and biologic aspects of three separate means of administration of cyclopamine for experimental induction of terata or embryonic death in sheep were examined. Oral capsule administration of crystalline cyclopamine is logistically simple and biologically effective, but costly in terms of amount of compound required. Embryos were affected in five of seven ewes dosed cyclopamine orally at higher levels (four nonpregnant and one with cyclopia). Intramuscular administration of cyclopamine dissolved in ethanol was logistically simple but without biologic effect at levels tested. Three of three treated ewes had normal offspring. Osmotic minipump administration of powdered cyclopamine suspended in propylene glycol was logistically unsatisfactory with serious delivery complications. Osmotic minipump administration of cyclopamine dissolved in ethanol was logistically very satisfactory, and one ewe among three treated animals was nonpregnant at term. There were no nonpregnant ewes nor deformed offspring in 17 controls.** [P.S.E.B.M. 1989, Vol 192]

Experimental induction of terata or embryonic death in sheep by oral administration of the various *Veratrum* steroidal alkaloid teratogens including cyclopamine (1, 2) is costly because of the amount of teratogen required. Although craniofacial defects or embryonic death can be induced on the 14th day of administration, oral doses on that day totaling nearly 2.0–3.0 g/animal are generally required (1, 2). The compound cyclopamine is available in reasonable amounts only by isolation from *Veratrum californicum*. This requires collection of the plant in remote high mountain areas, processing and extraction of the plant material, and purification of the extract to prepare the compound (3–5). It is a major logistic problem to secure adequate amounts of cyclopamine and this has hampered efforts to determine whether the compound is responsible for the other expressions of malformations in sheep (6–8). Only by intrauterine injection has the dosage requirement in sheep been reduced (9), but the common expression of cyclopia was not among the terata induced. Inducing embryonic effects in sheep while using about 1/10 or less the cyclopamine presently required would solve the logistic time and cost problem.

We report here a preliminary assessment of the practicality of using osmotic minipumps or im administration rather than oral administration to induce terata or embryonic death. We assessed the logistic constraints of nonoral administration and the effectiveness of low dose levels compared with the traditional oral route.

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Materials and Methods

Collecting and Processing of Plant Material. Root/rhizome portion of *V. californicum* was collected and prepared for extraction as previously described (3). Several methods have been used in the past to isolate cyclopamine (3–5). The method described herein was a further modification facilitated by the fortuitous direct crystallization of cyclopamine on the walls of cold containers in which the benzene extract was stored for several months as described below. A 358-kg quantity of the dried ground root/rhizome material was moistened with 5% NH_4OH (about 190 liters) and extracted with benzene at 20°C by percolation (about 750 total liters). This extract was stored at 5°C or below for several months and copious crystals that had formed on the inner walls of the containers were recovered in warm ethanol (about 60 liters). After evaporation of the ethanol and washing in cold benzene and acetone

(about 2 liters each), a 100-g portion of the 600 g of crude crystalline alkaloid mixture therefrom was utilized to yield cyclopamine of suitable purity for the present experiment. The 100-g portion was washed in a few milliliters of hot acetone, recovered on a fritted disk funnel, recrystallized three times from an ethanol:acetone:water system by dissolving the crude crystals in a minimum of hot ethanol (100–200 ml), on a steam bath, slowly adding 3 liters of acetone and allowing slow cooling after the addition of about 30 ml of H_2O. The resulting crystals were then recrystallized twice out of acetone in small batches because of the low acetone solubility and pooled for the final double recrystallization from hot ethanol:acetone:water performed as above. Yield was slightly under 20 g. The crystals had the expected (10) direct inlet mass spectrometric fragmentation pattern and infrared (IR) spectrum of good quality (about 95% pure) cyclopamine (mass spectrometric-411M^+[5], 396[26], 145[7], 126[31], 125[94], 124[91], 114[34], 110[100]; IR-3400, 3250, 2900, 1450, 1118, 1067, 1060, 1042, 984, 927, 809).

Workers interested in isolation of cyclopamine should be aware that isolation is complicated by the presence of several dozen related steroids and steroidal alkaloids, with similar chemical properties, each of which varies in total and relative concentration from one plant collection to the next. Because cyclopamine is difficult to separate from components of this variable mixture, one must rely, during purification, on repeated assays for cyclopamine to ensure that purification is actually being achieved. Simple IR analysis (KBr pellet) of the crystallized sample from organic solvents containing at least a trace of water provides the necessary rapid assay needed. Specifically, each time the material is recovered, an IR analysis is performed with particular attention paid to the height and sharpness of the 927, 984, and 1118 cm^{-1} ring oxygen peaks of cyclopamine as well as the 1725 and 1126 cm^{-1} carbonyl peaks of muldamine and 963 cm^{-1} unassigned peak of veratramine (the two principle contaminant compounds) (5). Repeated recrystallization should continue at the final step until an IR pattern like that of Figure 1 is obtained.

Experimental Animal Care. Western white-faced crossbred ewes weighing 53 ± 6 kg were bred to Suffolk rams and randomly assigned to experimental groups. Breeding day was considered Day 0 of gestation and accurately timed by observing time of coitus. Ewes were provided alfalfa hay, trace mineralized salt, and water *ad libitum* throughout gestation.

Dosages and Methods of Administration of Cyclopamine. Experimental preparations were administered orally by gelatin capsule, by (im) injection, or by implanted osmotic minipump (Alzet Corp.). A total of 10 ewes received capsules orally at doses of 1.2, 1.8, or 3.0 g/animal at 8:00 AM on Day 14 of gestation or as a divided dose for both 3.0 g/animals at 8:00 AM and at 3:00 PM. Three ewes received cumulative doses of 100, 300, or 600 mg/animal of cyclopamine dissolved in ethanol by im injection. Doses were administered at 8:00 AM and 3:00 PM on each of Days 13 and 14 of gestation using one-fourth of the cumulative dose each time. Individual dose volume did not exceed 3 ml and was injected at six multiple sites using ½ ml at each site to minimize tissue trauma and maximize absorption of cyclopamine. Ethanol was used as carrier because cyclopamine is virtually insoluble in anything having conventional physiologic characteristics. Osmotic minipumps (model 2M136S) with 2.260-ml volume and 46 liter/hr mean pumping rate were used so that delivery

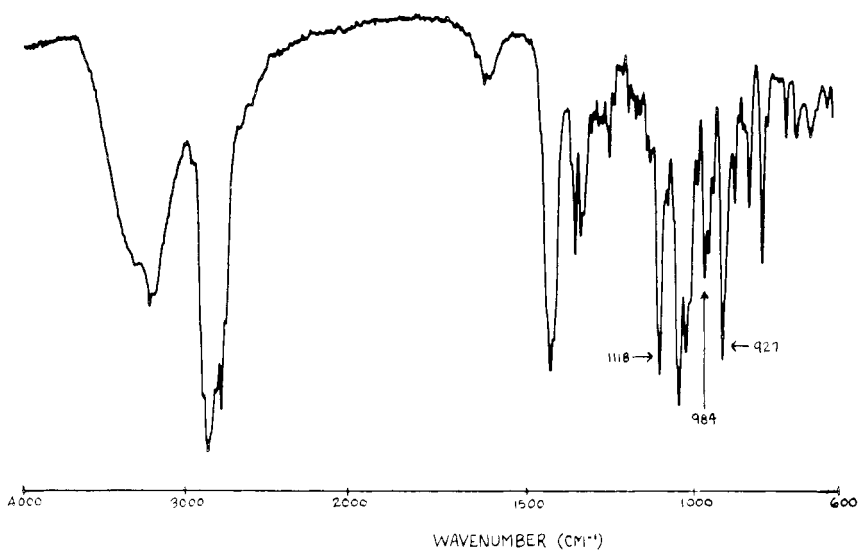

Figure 1. The IR spectrum (KBr pellet) characteristic of excellent quality cyclopamine, crystallized from solvents containing a trace of water, indicating ring oxygen peaks at 927, 984, and 1118 cm^{-1} of cyclopamine useful in following the purification.

of the total 2.260 ml would take place in about 2 days. They were loaded aseptically with 100 mg of cyclopamine either suspended in 2.2 ml of propylene glycol for three animals or dissolved in 2.2 ml of ethanol for three animals. These unusual vehicles for minipump use were selected because they allowed sustained suspendibility or solubility, respectively, of the cyclopamine. Minipumps were then surgically implanted in experimental animals at 3:00 PM on Day 13 of gestation. Minipumps were implanted subcutaneously in the neck on the upper left side in a deep pocket formed below the incision, and the incision was closed with staples. The manner of placement ensured that delivery of cyclopamine would be to the lower reaches of the pocket and not to the outside. Control animals received empty capsules (five animals), im injection of ethanol carrier only (six animals), or surgically implanted minipumps holding carrier only (three with propylene glycol and three with ethanol). Minipumps were removed and the incision reclosed 1 week after implantation.

Assessment of Protocol and Biologic Effect. Ewes were observed for signs of toxicosis, trauma at injection sites, or infection at minipump implantation sites. Offspring were examined for gross evidence of malformations. Ewes that were nonpregnant at time of expected delivery were noted and compared statistically with controls by Yates corrected chi-square techniques (11) to determine whether there was likelihood that nonpregnant ewes represented early embryonic death induced by the cyclopamine.

Results and Discussion

Assessment of Protocol. Oral administration by capsule of crystalline cyclopamine resulted in complete delivery to the gastrointestinal tract without loss. Intramuscular administration of cyclopamine dissolved in ethanol resulted in complete delivery with no gross evidence of tissue encapsulation of dose or trauma. Osmotic minipump administration of powdered cyclopamine suspended in propylene glycol resulted in incomplete delivery of compound to the tissue pocket. Plugging of the minipump delivery tubes (even though cyclopamine particulate size was smaller than delivery tube diameter) resulted in only partial delivery in each case. About one-third of the cyclopamine was actually delivered to the tissue pockets as judged by weighing the amount of cyclopamine remaining in the minipump after removal from the tissue pockets. Not all that was delivered had been absorbed by the tissue since considerable residue was found in the pockets and on the outside of the minipumps upon removal. The residue proved to be cyclopamine by IR analysis. Osmotic minipump administration of cyclopamine dissolved in ethanol resulted in complete delivery of the dissolved cyclopamine to the tissue pockets. Minipumps were empty upon removal from the pockets. The cyclopamine delivered was more completely absorbed by the tissue than was the case with powdered cyclopamine suspended in propylene glycol, since only a small amount of cyclopamine residue was found on the minipumps and in the tissue pockets upon minipump removal. This method of administration was logistically satisfactory.

Assessment of Biologic Effects. There was no clinical evidence of maternal toxicity from administration of cyclopamine except with ewes 4734 and 4737 on the high oral dose. These two had modest excess salivation as was expected at that dose from good purity cyclopamine.

There was evidence of embryotoxicity in several instances. Of 10 ewes dosed cyclopamine by capsule, one, at a 3-g dose, had a lamb with anophthalmic cyclopia, and four others were nonpregnant at term (one on a 3-g and three on 1.8-g doses) and presumably represent early embryonic deaths induced by cyclopamine. A total of 5 of 7 ewes dosed at 1.8 g/animal/day or above had embryonic effects ($P < 0.05$ compared with 5 normal capsule controls, or $P < 0.005$ when compared to all 17 controls). Effects were dose dependent. None of three were affected at the dose of 1.2 g, three of five were affected at the dose of 1.8 g, and two of two were affected at the 3.0-g cumulative dose.

There were no embryonic deaths or deformed offspring from ewes that were administered by im injection cyclopamine dissolved in ethanol. Three of three treated (at 100 mg/animal or above) and three of three controls had normal offspring.

There were no embryonic deaths or deformed offspring from ewes that were administered by minipump 100 mg of cyclopamine suspended in propylene glycol. Three of three treated and three of three controls had normal offspring.

There was one nonpregnant ewe at term among three ewes administered by minipump 100 mg of cyclopamine dissolved in ethanol. That incidence was not statistically significant, however, compared with the three normal minipump ethanol controls.

Delivery by minipump of cyclopamine dissolved in ethanol may hold some promise in reducing cyclopamine dosage requirements compared with the oral route if the single embryonic death in that group (1 of 3 = 33%) may be ascribed to induction by cyclopamine rather than failure to conceive (despite lack of statistical significance). The insolubility problem with cyclopamine was effectively circumvented by using ethanol as vehicle. Furthermore, tissue absorption of cyclopamine dissolved in ethanol appeared to have been much more complete compared with particulate cyclopamine suspended in propylene glycol. The minipump manufacturer does not ordinarily recommend ethanol as carrier, but that recommendation is based on minipumps designed for much longer periods of delivery than 2 days. Our recovered minipumps with ethanol vehicle did not appear to have malfunctioned in any way during their

2-day exposure to ethanol, and cyclopamine delivery to the pocket was complete. Certainly, a more uniform level of cyclopamine in maternal circulation would be expected from minipump delivery than by discontinuous oral or im doses. Further experiments are warranted using minipump-delivered cyclopamine dissolved in 100% ethanol at somewhat higher dose levels than 100 mg/animal. Intravenous administration of cyclopamine dissolved in 100% ethanol should also be tested, although we deem this route to be less useful from a practical point of view for large numbers of animals because of the need for more continuous attention during delivery over a long enough time to cover the appropriate embryonic susceptibility period.

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