AN ABSTRACT OF THE THESIS OF

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Title: Teratogenicity of Coniine, a Nicotinic Alkaloid from *Conium maculatum* (Poison Hemlock)

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Coniine, an alkaloid from *Conium maculatum,* is a known teratogen in many domestic species with maternal ingestion resulting in arthrogryposis (twisted limbs) of the offspring. Arthrogryposis is also a common teratogenic outcome in humans but the mechanisms remain largely unknown. Therefore, the overall objectives of this thesis were 1) to develop an experimental model of coniine-induced arthrogryposis using a laboratory animal and 2) to use the experimental laboratory animal model to design and perform mechanistic studies to better understand mechanisms of arthrogryposis. Coniine-induced teratogenicity was evaluated in Sprague-Dawley rats, New Zealand white rabbits, and New Hampshire X White leghorn chicks. Fetal weights were significantly lower in coniine-exposed rat and rabbit fetuses but the only statistically significant treatment-related visceral or skeletal malformation was a reduction of cranial ossification of rabbit fetuses. Coniine-exposed rabbit litters were affected by arthrogryposis more than controls (2/6 vs. 0/9)

with the lesion characterized as hyperflexion of the antebrachial-carpal joint with supination. Chicks treated with coniine showed a dose-dependent teratogenic response of excessive flexion or extension of one or more toes. Coniine caused malformations in the chick similar to those caused by nicotine and there was a statistically significant ($P \leq 0.01$) decrease in movement in coniine and nicotine sulfate treated chicks as determined by ultrasound. Control chicks were in motion an average of 33.67% of the time while coniine (1.5%) treated chicks were only moving 8.95% of a 5 minute interval and no movement was observed for nicotine sulfate (5%) treated chicks. Neither the peripheral nicotinic receptor antagonist d-tubocurarine chloride nor the central nicotinic receptor antagonist trimethaphan camsylate blocked the teratogenesis of 1.5% coniine in chicks. The $IC_{50}$ for coniine binding to nicotinic receptors in the rat was 314μM while that for the chick was 70μM.

The chick embryo provides a reliable and simple experimental animal model of coniine-induced arthrogryposis. The data in this thesis support the hypothesis that the mechanism of coniine-induced teratogenesis is blockade of the nicotinic receptor. Differences in the receptor affinity for coniine between susceptible and nonsusceptible species may explain, in part, cross-species variation in teratogenicity of coniine.

# Teratogenicity of Coniine, a Nicotinic Alkaloid from *Conium maculatum* (Poison Hemlock)

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## CONTRIBUTIONS BY AUTHORS

Dr. Tony Frank was involved in the design, analysis, and writing of each manuscript. Dr. Barbara Watrous performed the ultrasound recording and Dr. Andrea Bohn assisted in data collection for that study. The receptor binding assays were performed in the laboratory of Dr. Robert C. Speth who also assisted in data interpretation.

# TERATOGENICITY OF CONIINE, A NICOTINIC ALKALOID FROM *Conium maculatum* (POISON HEMLOCK)

## INTRODUCTION

Limb development, including joint formation, is a complex and poorly understood process. Bones and joints are self differentiating structures that have critical periods of development which are susceptible to damage (Gardner 1971). Mechanistically defined models of abnormal joint formation could be very useful in understanding normal joint formation. Although arthrogryposis ("twisted limbs") is a common teratogenic outcome in both livestock and humans, the mechanisms of arthrogryposis in the majority of these human and animal conditions remain largely unknown although viral- and plant-induced arthrogryposis have been documented.

In 1988 it was estimated that poisonous plants cost the livestock industry more than $250 million annually (Nielsen 1988). This includes death loss as well as decreased reproductive efficiency. Maternal ingestion of *Conium maculatum* (poison hemlock, CM) causes limb malformations in cattle (Keeler and Balls 1978), pigs (Panter, et al. 1985a), goats (Panter, et al. 1990), and sheep (Panter, et al. 1988a). Coniine, a major piperidine alkaloid of CM,produces arthrogryposis in cattle (Keeler 1974), but hamsters (Panter 1983) and rats are not susceptible and rabbits only weakly susceptible (Forsyth and Frank 1993) to coniine-induced teratogenesis. The mechanism of CM-induced malformations in

livestock is unknown, as is the reason for the variation in species susceptibility to coniine, although a great deal is known concerning the nicotinic effects of coniine in terms of acute intoxication.

Therefore, the overall objectives of this thesis are: 1) to develop an experimental model of arthrogryposis using a laboratory animal, and 2) to use the experimental laboratory animal model to design and perform mechanistic studies aimed at better understanding mechanisms of arthrogryposis. Coniine was evaluated in several laboratory animal species using routine teratology studies and due to the severe teratogenic response of the chick to nicotine, coniine was also evaluated for teratogenicity in the chick. Coniine caused malformations and lethality in the chick in a dose-dependent manner providing a reliable and reproducible model with which to study coniine-induced arthrogryposis. This model was subsequently used to determine histopathological changes induced by coniine, to test mechanistic hypotheses by comparison with chemicals that have similar and dissimilar mechanisms of action to coniine, and to study interspecies differences to coniine susceptibility.

# REVIEW OF LITERATURE

## Limb and Joint Formation

Limbs are self differentiating structures which do not begin to form until the primordia of most organs have formed and the body wall musculature is well differentiated. In humans, the fore limb buds appear on day 25 and hind limb buds on day 28 of gestation (Szabo 1989). Other animals vary greatly in the appearance of limb buds during gestation: rats d10 and d11 (approximately 48% of gestation), rabbits d10 and d10 (32% of gestation), and cattle d24 and d26 (approximately 9% of gestation) for fore and hind limb buds, respectively (Szabo 1989). In the chick, wing and leg buds appear at stage 17 (about 60 hours or 12% of incubation, Hamburger and Hamilton 1951).

Limb primordia arise close to the somites from a condensation of lateral plate mesoderm and its overlying ectoderm (Carlson 1988). In the *Drosophila* embryo, the segment polarity gene *wingless* is involved in determining the positions along the antero-posterior axis of the embryo at which limb primordia will develop (Cohen 1990). Similar genes are probably present in mammals. As limb primordia grow off the body wall, ectoderm covering the tip of the bud thickens to form the apical ectodermal ridge (AER, Carlson 1988). The AER acts to stimulate outgrowth of the bud and to influence condensation of the mesenchyme at the center of the limb bud by a mechanism called centripetal packing

(Wilson 1986). The outer cell layer of the resulting core forms fibrous connective tissue while the inner mesenchymal cell layer forms cartilage. At the distal aspect of the limb bud, aggregates of cells are formed which are called digital rays (Carlson 1988). These will become digits as the tissue between them is eliminated via apoptosis (Hurle and Gañan 1986).

Certain homeobox genes are transcribed within the limb bud coincident with precartilage differentiation. Precartilage condensation in the chick limb is divided along the proximodistal axis by the Hox-1 genes but cells undergoing chondrogenic differentiation cease expression of these genes (Yokouchi, et al. 1991; Rogina, et al. 1992). The branching process of precartilaginous condensations coincides with expression of Hox-4 genes (Yokouchi, et al. 1991) which also act to maintain positional signalling along the antero-posterior axis of the limb (Nohno, et al. 1991). As mesenchymal cells differentiate into chondroblasts, the crucial stage of chondrogenic condensation coincides with a marked increase in cytoplasmic type II collagen mRNA. The newly differentiated chondroblasts now produce type II collagen and cease producing type I collagen through transcriptional regulation (Kosher, et al. 1986).

Spacial patterning of limbs is also influenced by gradients of morphogens such as retinoic acid and its analogues (Thaller and Eichele 1990). One retinoic acid analog, 13-*cis*-retinoic acid, has been shown to act as a

morphogen at low doses by enhancing cartilage production or a teratogen at high doses by depressing or eliminating cartilage development (Kwasigroch and Bullen 1991) in mouse limb buds. The effects of retinoic acid are mediated by gradients of cellular retinoic acid binding proteins (CRABP) and retinoic acid receptors (RAR). In the mouse, RARα and γ transcripts are uniformly distributed along the limb bud on day 10 post-coitus, however CRABP transcripts are differentially expressed along a proximo-distal gradient. Later in development, RARγ becomes specific to differentiating cartilage and skin cells and RARβ concentrates in the interdigital mesenchyme (Dollé, et al. 1989). Studies with limb regeneration in the adult newt (Giguère, et al. 1989) indicate that the morphogenic gradient is established through differential activation of pre-existing RAR's rather than differential gene expression.

At the joint region, type I collagen predominates and there is a decrease in keratan-sulfate-containing proteoglycans (Craig, et al. 1987). Cavitation begins after the form of the joint is established and the joint region represents an area of localized weakness. Shear forces generated by the musculature cause the formation of a hinge and the eventual separation of the cartilaginous elements (Craig, et al. 1987). Movements of chick embryos correspond with joint formation. Complete cavitation occurs on days 10-11 for the hip and knee, days 12-13 for the ankle, and days 14-15 for the metatarsophalangeal and interphalangeal

joints (Llusa-Perez, et al. 1988). The cavity increases in absolute size while maintaining relative size (Gardner 1971) and by the end of the embryonic period, the form of the bones and joints resembles that of the adult (Gardner 1971). In humans, joint development begins at about 5-6 weeks and by 8 weeks there is movement of the limbs. Limb motion is essential for normal joint development (Smith 1988, Llusa-Perez, et al. 1988).

The final stage of limb development, besides growth, is ossification of the skeleton. In humans, skeletal changes associated with abnormal joint formation caused by neuromuscular disease include bone thinning leading to fractures and calluses but not major structural defects in bone (Rodriguez, et al. 1988). Bone is produced from the cartilaginous precursor by a mechanism known as endochondrial ossification. Mineralization is initiated by production of extracellular matrix vesicles which arise from either pinching off of the cytoplasmic membrane or as apoptotic bodies of dying chondrocytes (Anderson 1989). Matrix vesicles serve as the initial site of calcification and contain high concentrations of calcium binding phospholipids and alkaline phosphatase. Within the matrix vesicle membrane, calcium and phosphate combine to form crystals of hydroxyapatite (HA) which protrude through the vesicle membrane. Exposure of HA to the extravesicular environment leads to rapid proliferation of extracellular HA (Anderson 1989). Osteoblasts enter the developing bone with

the invasion of blood vessels (Carrington and Reddi 1991) and use these seed crystals of HA to lay dawn osteoid. The process of ossification is accompanied by degradation of cartilage and production of type I collagen (Carrington and Reddi 1991) and is stimulated by proteins such as bone morphogenic protein (Carrington, et al. 1991a; Lyons, et al. 1990).

## Arthrogryposis

Limb malformations, including arthrogryposis ("twisted limbs"; persistent flexure or contracture of a joint), are among the most common malformations in humans and animals. One in 200 human infants is born with dislocated hips, one in 500 has clubfoot, and one in 3000 is born with multiple contractures (Hall 1989). The spontaneous occurrence of arthrogryposis per 1000 live births in rats, rabbits, and cows is 0.03, 5.8, and 0.2, respectively (Szabo 1989).

Causes of arthrogryposis in humans and animals are varied. Decreased intrauterine movement by human fetuses has been associated with arthrogryposis and may be induced by neuropathic agents, myopathic agents, abnormal connective tissue or joints, or decreased space in which the fetus can move (Hall 1989). Causes of arthrogryposis in livestock include plants such as poison hemlock, locoweed, tobacco, and lupine, and viruses such as bluetongue, Akbane, bovine viral diarrhea, and equine encephalitis (Noden 1985). Arthrogryposis is also suspected to be hereditary in

Charolais (Nawrot, et al. 1980) and Hereford (Shupe, et al. 1967) cattle.

Experimentally, arthrogryposis has also been shown to be associated with decreased fetal movement. In a classic study, Drachman and Coulombre (1962), induced arthrogryposis in chick embryos by infusion of *d*-tubocurarine. They demonstrated that temporary paralysis of the chick in ova results in limb malformations. Tendons from the limbs of these chicks were taut and resisted joint movement. Dissection of all the periarticular and intraarticular ligaments was required before joint movement returned. Rat fetuses paralyzed by transuterine injections of curare also developed arthrogryposis (Moessinger 1983) but the limbs were not analyzed histopathologically to determine soft tissue involvement. Lack of fetal movement may also be part of the pathogenesis of arthrogryposis induced by CM in livestock. Panter, et al. (1990) observed fetal movements by ultrasound in pregnant goats gavaged with CM seed or plant. Both treatments resulted in significant reduction of fetal movements and kids were born with arthrogryposis (fixed carpal joints and rigidity of hock, elbow, and stifle joints). Fetal movement in pregnant ewes gavaged with CM was also shown by ultrasound to be greatly reduced. However, limb malformations of lambs were transient and resolved by 8 weeks post partum (Panter, et al. 1988b). The exact mechanism of decreased fetal movement from maternal ingestion of CM and relation to arthrogryposis is unknown

but may be due to interaction of coniine with the nicotinic receptor. Neither kids nor lambs were examined for neurological or soft tissue defects and the relationship between the nicotinic receptor and joint malformation is unknown.

## *Conium maculatum*

CM was introduced to North America from Europe and Asia and has now spread throughout most of the continent (Knight 1987). The plant can be found along roadsides, waterways, and edges of cultivated fields. Botanical features allow ready identification of CM (Knight 1987, Kingsbury 1964). It is a 3-9 foot tall biennial herb with many branches, a hollow stem, and a whitish tap root. Stems have irregular purple spots, especially near the base and leaves are fernlike, compound, and triangular. Small white flowers occur in umbrella-like clusters and produce pale brown seeds. When crushed, stems, leaves, and roots emit a pungent, mousey odor.

Domestic livestock as well as humans are susceptible to CM intoxication. Poisonings by CM have been reported for rabbits (Short and Edwards 1989), tule elk (Jessup, et al. 1986), turkeys (Frank and Reed 1987), dairy cattle (Galey, et al. 1992), horses, pigs, and goats (Panter, et al. 1988c). Human fatalities have occurred when CM is mistaken for edible plants such as wild carrot, parsley, or anise (Knight 1987). Hemlock was used as a formal state poison in

ancient Greece and Italy and an extract of CM was used to kill Socrates who denounced established gods (Kingsbury 1964; Kennedy and Grivetti 1980). Coturnism, or poisoning by toxic quail, has been blamed on hemlock seeds which were fed on by European migratory quail but a definitive link has not been established (Kennedy and Grivetti 1980; Frank and Reed 1990). However, coniine (an alkaloid from CM) was found in the urine of 8 people after ingestion of small birds (skylarks, chaffinches, or robins) which had eaten hemlock buds prior to being killed. These patients also had rhabdomyolysis and acute tubular necrosis (Rizzi, et al. 1989).

Five piperidine alkaloids isolated from CM have been associated with acute intoxication: coniine, gamma-coniciene, N-methylconiine, conhydrine, and pseudoconhydrine (Keeler 1977). Coniine and gamma-coniciene, the major toxic and teratogenic alkaloids, gradually increase and become predominant in seeds as the plant matures (Kingsbury 1964; Knight 1987).

## Coniine

Coniine, a piperidine alkaloid, (Fig. 1) is derived from acetate (Leete 1963). The compound is a colorless, alkaline,liquid at room temperature with a density of 0.85 g/ml and a boiling point of 166°C. Coniine has a distinct mouse-like odor and was the first alkaloid isolated from CM.

The effects of coniine are similar to nicotine both centrally and peripherally. Toxic action of coniine is biphasic with first stimulation then depression of the autonomic ganglia. Coniine also causes end-plate depolarization unrelated to nicotinic receptor blockade (Bowman and Sanghvi 1963). Symptoms of acute poisoning include nervousness, fasiculations of skeletal muscles, clonic and tonic contractions of separate limbs and convulsions followed by weakened and slowed heart rate, coma, and death (Bowman and Sanghvi 1963; Kingsbury 1964; Knight 1987). Coniine has been shown to block the patellar reflex and crossed extensor and flexor reflex in cats indicating action at the spinal cord (Bowman and Sanghvi 1963). The alkaloid readily penetrates cell membranes implicating extra- and intracellular sites of action (Bowman and Sanghvi 1963).

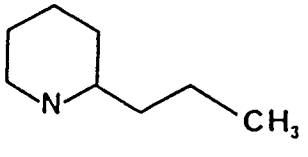


Fig. 1. Coniine

Nicotinic receptors are distributed both centrally and peripherally and are distinguished structurally and pharmacologically (Maelicke 1992). The receptor exists as a pentameric complex ($\alpha_2\beta\delta\gamma$ or $\alpha_2\beta\delta\varepsilon$) in which the subunits vary during development and between tissues creating a multiplicity of subtypes. For example, in embryonic or denervated muscle, nicotinic receptors contain a $\gamma$ subunit while receptors from adult innervated muscle contain the $\varepsilon$ subunit (Gilman, et al. 1990). Subtypes of receptors occur depending upon the type of subunits present which account, in part, for ligand specificity.

Livestock vary markedly in their susceptibility to coniine. Cattle develop severe symptoms when dosed with 3.3 mg/kg body weight PO, mares develop mild signs of intoxication at 15.5 mg/kg body weight PO, while sheep tolerate doses of coniine up to 44 mg/kg body weight PO (Keeler, et al. 1980). Quail and chickens are affected at 50 mg/kg body weight PO but turkeys respond to 100 mg/kg body weight PO (Frank and Reed 1990) for intoxication to become evident. A single oral dose of CM seed of 1 g/kg body weight is lethal to swine (Cheeke 1985).

Besides acute nicotinic effects, coniine is also teratogenic. Although most arthrogryposis in cattle has been attributed to maternal ingestion of lupines, CM was implicated in an outbreak of limb malformations in Utah calves born to dams not exposed to lupines (Keeler 1974). Coniine was subsequently shown to produce arthrogryposis in

calves (Keeler 1974) after maternal gavage during days 55-75 of gestation at coniine levels (approximately 1.5 ml/day) that maintained slight signs of intoxication. Two of 3 calves were born with lateral rotation of front limbs, permanent front carpal flexure, and bowed rear limbs. Hereford cows gavaged with fresh CM material at various times during days 45-75 of gestation produced calves with arthrogrypotic lesions similar to those caused by coniine (Keeler and Balls 1978). These defects were not reproduced by maternal forced inhalation of coniine in which freshly chopped CM or cotton saturated with 3 cc of coniine was placed in the bottom of a strap-on feed bag and bags placed on the animals for 20 hours each day during days 45-75 of gestation (Keeler and Balls 1978). Edmonds, et al. (1972) reported intoxications and congenital malformations in a Missouri swine herd that had been allowed onto a pasture containing CM. Skeletal malformations, including arthrogryposis and scoliosis, were subsequently induced in newborn pigs from gilts fed fresh CM seed or plant on days 43-53 of gestation (Panter, et al. 1985a) at levels designed to maintain clinical signs of intoxication (approximately 126 g seed/day or 1,296 g plant/day). Cleft palate was also induced in newborn pigs from gilts fed seed or plant on days 30-45 of gestation (Panter, et al. 1985b). Pregnant goats gavaged with CM seed (130.3 g/day) or plant (387.3 g/day) produced kids with both arthrogryposis and cleft palate (Panter, et al. 1990). Fetal movements of kids were shown

by ultrasound to be significantly reduced up to 12 hours after maternal treatment of CM seed and up to 5 hours after maternal treatment of CM plant on days 45-60 of gestation. The authors showed a direct correlation between severity of malformation at birth and duration of reduced fetal movement during gestation. Reduction in fetal movement also occurred in pregnant ewes gavaged with 5-10 g CM/kg body weight on days 30-60 of gestation, however, all 7 lambs born with arthrogryposis appeared normal by 8 weeks post partum (Panter, et al. 1988b).

Analogs of coniine have been tested for their structure/activity relationship to teratogenicity. A double bond introduced between the nitrogen and C-1 does not alter activity. However, the degree of saturation and the length of the side chain affects the activity of the compound. The coniine analog with a fully unsaturated ring is not teratogenic and analogs with a side chain of less than three carbons do not have teratogenic activity (Cheeke and Shull 1985). Therefore, piperidine alkaloids with a fully saturated ring and a side chain $\alpha$ to the nitrogen and at least propyl in length have teratogenic activity.

## Summary

In order for an animal model to be useful for human terata, several requirements must be met: 1) similarity in teratogenic outcome, 2) susceptibility to teratogens of similar structure, 3) comparable metabolic processes, and 4)

similar mechanism(s) of action (Keeler 1988). The significance of this proposal is twofold. First, limb malformations are common in humans. Mechanisms in the majority of these human conditions remain largely unknown. Second, arthrogryposis, including CM-induced lesions, is a significant economic factor in the cattle industry. Development of this model system will add to our understanding of CM-induced teratogenesis in domestic species and mechanisms of arthrogryposis in nonhuman and human species.

# CONIINE DOSE RANGE FINDING STUDY IN RATS AND RABBITS

Purpose: The purpose of this study was to determine the maximum amount of coniine that could be given by gavage to rats and rabbits without causing death.

In much of the previous work involving coniine teratogenesis in domestic species (Keeler, et al. 1980, Keeler 1974, Panter, et al. 1985a, and Panter, et al. 1988a), animals were given an amount of coniine or CM necessary to cause marked clinical signs of intoxication and this dose was adjusted at each treatment to maintain maximum intoxication. Therefore, a dose of coniine needed to be found that caused clinical signs, but not death, in rats and rabbits. Using allometric equations, an arithmetically related dose was metabolically scaled (Schmidt-Nielsen 1984) for rats and rabbits from available estimates on exposures in cows (Keeler, et al. 1980).

Background: Allometry is the use of nonisometric scaling. Allometric equations are descriptive and used for relating a variable quantity to body size. The general form of the allometric equation is $y = a \cdot M_b^b$ where a is the proportionality coefficient (intercept at unity), $M_b$ is body mass, and $^b$ is the body-mass exponent (slope). For placental mammals, a = 70 and b = 0.75 when scaling metabolic rate in relation to body mass. For this experiment, the dose of coniine in cows was used to

calculate a dose of coniine for rats and rabbits based on the minimum energy cost (MEC), or basal metabolic rate, of each animal. For example, the MEC dose of coniine for the cow is the dose of coniine divided by the MEC of the cow. MEC dose is then multiplied by the rat or rabbit MEC to get a dose of coniine for that animal. Because allometric equations are generalizations of expected variables, the calculated dosages of coniine were used as a starting reference only. Adjustments were required to maintain coniine levels without resulting in death of the animal.

Results: The mathematically scaled doses of coniine for rats and rabbits were 23.7 mg/kg body weight and 10.6 mg/kg body weight, respectively, based on 3.3 mg/kg body weight in the cow (Keeler, et al. 1980). The results of the range finding tests are given in Tables 1 and 2 for rats and rabbits, respectively. Rats were treated for 3 days and rabbits for 5 days. These correspond to 60% and 33% of the fetal period for the rat and rabbit, respectively, although the susceptible period for cows is only 8% of the total fetal period. Mild signs of coniine intoxication for rats included head shaking, chewing motions, and lethargy while those for rabbits included chewing motions, drooping ears, and lethargy. Signs in either species were never of the magnitude reported in domestic livestock unless the rat or rabbit died. Based on preliminary studies in the rat, rabbits were treated 3 times daily in order to maintain

higher levels of coniine in the body throughout the day instead of spiking every 12 hours or less. On the basis of this study, doses of 25 mg coniine/kg body weight and 40 mg coniine/kg body weight were chosen for rats and rabbits, respectively.

Table 1: Results of coniine range finding in rats.

| Dose (mg/kg) | S.I.D. | B.I.D. | T.I.D. |
|---|---|---|---|
| 150 | killed 1 of 1 after 1 dose | not done | not done |
| 100 | killed 1 of 1 after 3 doses | not done | not done |
| 75 | killed 2 of 2 after 2 doses | not done | not done |
| 50 | no deaths, signs in 1 of 2 after 3 doses | killed 1 of 2 after 4 doses, signs in 2 of 2 after 6 doses | killed 2 of 2 after 3 and 4 doses, respectively |
| 40 | not done | signs in 2 of 2 after 6 doses | not done |
| 25 | no deaths, no signs in 2 of 2 after 3 doses | no signs in 2 of 2 after 6 doses | signs in 2 of 2 after 9 doses |

Table 2: Results of coniine range finding in rabbits.

| Dose (mg/kg) | T.I.D. |
|---|---|
| 120 | killed 1 of 1 after 3 doses |
| 80 | killed 1 of 1 after 3 doses |
| 60 | killed 1 of 1 after 8 doses |
| 40 | signs in 2 of 2 after 3 doses |
| 30 | no signs in 1 of 1 after 15 doses |
| 20 | no signs in 1 of 1 after 15 doses |
| 10 | mild signs in 2 of 2 after 8 doses |

# EVALUATION OF DEVELOPMENTAL TOXICITY OF CONIINE TO RATS AND RABBITS

Carol S. Forsyth and Anthony A. Frank

Abstract

*Conium maculatum* (poison hemlock, CM) is teratogenic in several domestic species, presumably due to its piperidine alkaloids, including coniine. Coniine has been verified to be teratogenic in cattle. Coniine/CM teratogenicity culminates in production of arthrogryposis. The purpose of this study was to evaluate coniine-induced teratogenicity in two laboratory animal species, Sprague-Dawley rats and New Zealand white rabbits. Pregnant rats were given coniine (25 mg/kg body weight) by oral gavage at 8 hour intervals on gestation days 16-18. Pregnant rabbits were given coniine (40 mg/kg body weight) by oral gavage at 8 hour intervals on gestation days 20-24. Rats were killed on day 19 and rabbits on day 29. Fetuses were immediately removed, weighed, and examined for external abnormalities. Alternate fetuses were either stained for skeletal examinations with alizarin red-S or fixed in Bouin's solution for visceral examination. Symptoms of maternal intoxication due to coniine administration were observed in both the rat and rabbit, and higher doses were uniformly lethal. Rabbits treated with coniine appeared to lose more weight and eat less than controls but there was no statistically significant difference between groups. Fetal

weights were significantly lower in coniine-exposed rat and rabbit fetuses indicating fetotoxicity. The only statistically significant treatment-related visceral or skeletal malformation was a reduction of cranial ossification of rabbit fetuses, probably related to maternal toxicity. Coniine-exposed rabbit litters tended to be affected by arthrogryposis (no bony deformities noted on skeletal exam) more than controls (2/6 vs 0/9). The lesion was characterized as hyperflexion of the antebrachial-carpal joint with supination. While this was not statistically significant, this incidence rate was significantly ($P < 0.01$) increased when compared to historical controls. This study indicates coniine is not teratogenic to rats and weakly teratogenic to rabbits. The reason for the lack of sensitivity to teratogenicity in these laboratory animal species as compared to domestic species is currently unknown.

## Introduction

Limb malformations, including arthrogryposis ("twisted limbs"), are among the most common malformations in humans and domestic species. One in 200 infants is born with dislocated hips, one in 500 has club foot, and one in 3000 is born with multiple contractures or arthrogryposis (Hall, 1989). Arthrogryposis is also a common teratogenic outcome in livestock and a significant economic factor in the cattle industry. Arthrogryposis is suspected to be

hereditary in Charolais (Nawrot, et al. 1980) and Hereford (Shupe, et al. 1967) cattle. Non-heritable causes of arthrogryposis in livestock include toxic plants as well as viruses such as bluetongue, Akabane, bovine viral diarrhea, and equine encephalitis (Noden and de Lahunta 1985). With respect to plant causes of arthrogryposis, Panter, et al. (1990) found goats and cattle are susceptible to *Conium*, *Nicotiana*, and *Lupinus formosus*. Although *Lupinus* sp. (lupine) ingestion is believed to be the most common toxic plant etiology of arthrogryposis in livestock ("crooked calf disease"), maternal ingestion of *Conium maculatum* (poison hemlock, CM) also causes limb malformations in cattle (Keeler and Balls 1978), pigs (Panter, et al. 1985a), goats (Panter, et al. 1990), and sheep (Panter, et al. 1988a). Coniine, a major piperidine alkaloid of CM, has been shown to produce arthrogryposis in cattle (Keeler, et al. 1980). Mechanisms of arthrogryposis in the majority of human and animal conditions remain largely unknown.

Much of the difficulty in establishing mechanisms of arthrogryposis lies in the poor understanding of normal limb formation. Bones and joints are self differentiating structures that have critical periods of development during which they are susceptible to damage. By the end of the embryonic period, the form of the bones and joints resembles that of the adult (Gardner 1971). In humans, joint development begins at about 5-6 weeks of gestation and by 8 weeks there is movement of the limbs which is

essential for joint development (Smith 1988). Decreased intrauterine movement by human fetuses has been associated with arthrogryposis and is suggested to be caused by either neuropathic agents, myopathic agents, abnormal connective tissue or joints, or decreased space in which the fetus can move (Hall 1989). The detailed mechanisms of coniine-induced malformations in livestock are unknown but may also be related to decreased fetal movement (Panter, et al. 1990).

The purpose of this study was to evaluate the potential of coniine-induced arthrogryposis in two laboratory animal species, Sprague-Dawley rats and New Zealand white rabbits, to provide a model system for the study of mechanisms of arthrogryposis in human and nonhuman species. Development of this model system will add to our understanding of CM-induced teratogenesis in domestic species and mechanisms of arthrogryposis in general.

## Materials and Methods

### *Chemicals*

Coniine and Bouin's solution were purchased from Sigma Chemical Co. (St. Louis, MO).

### *Animals*

Pregnant, nulliparous Sprague-Dawley rats were obtained from Simonsen (Gilroy, CA). Day zero of gestation was defined as the day sperm were present in the vaginal smear. Rats were housed individually in wire-bottom cages

with water and commercial rodent chow available *ad libitum*. Pregnant first-litter New Zealand white rabbits were obtained from Western Oregon Rabbit Co. (Philomath, OR). Day one of gestation was defined as 24 hours *post coitus*. Does were housed individually in wire-bottom cages with water and commercial rabbit chow available *ad libitum*. Maternal body weights and food consumption were measured on gestation day (GD) 10-19 for rats and GD 18-29 for rabbits.

*Treatment Protocol*

Pregnant rats were given 25 mg coniine (in corn oil)/kg body weight by oral gavage at 8 h intervals on GD 16-18. Control rats received 1 ml corn oil per 0.2 kg body weight. Rabbits were given 40 mg coniine (in corn oil)/kg body weight by oral gavage at 8 h intervals on GD 20-24. Controls were given 0.5 ml corn oil alone per 1 kg body weight. Dose and time of administration were metabolically and chronologically scaled (Schmidt-Nielsen 1984) from domestic species data in the literature (Keeler, et al. 1980) in an effort to maximize the probability of a teratogenic outcome. Pilot studies (data not included) indicated these doses were the maximum nonlethal dosages tolerated by the dams. Mild symptoms of coniine intoxication, such as head shaking and tremors, were observed at these doses, especially in the rat. The period of late gestation was chosen for both the rat and rabbit based on the susceptible period for CM-induced arthrogryposis in the cow (Keeler, et al. 1980). Treatment

of rats with coniine throughout organogenesis (days 6-15, preliminary studies; data not included) did not result in induction of arthrogryposis.

*Fetal Evaluation*

Rats were killed on GD 19 by barbiturate anesthesia followed by exsanguination. Rabbits were killed on GD 29 by injection of approximately 2 ml Buthanasia-D (Schering-Plough) into the marginal ear vein. All fetuses were immediately removed from the uterus and surrounding membranes, weighed, and examined for external abnormalities. Alternate fetuses were placed in either 95% ethanol or Bouin's solution.

Ethanol-fixed fetuses were stained with alizarin red-S as described by Taylor (1986) except that the integument was removed from the rabbit fetuses only. Rat and rabbit fetuses were eviscerated and fat patches removed. Fetuses were cleared in potassium hydroxide then transferred to glycerin for storage and examination.

All fetuses fixed in Bouin's solution were examined for visceral abnormalities using the Staples (1974) technique.

*Statistical Analyses*

Maternal body weight gain and food consumption were evaluated by one-way analysis of variance (Siegel 1956). Differences between fetal weights were determined by nested analysis of variance (Healy 1972). The incidence of any given fetal malformation between litters was analyzed by

the Fisher Exact Probability Test (Siegel 1956). The level of significance for all tests was $P \leq 0.05$.

## Results

Signs of maternal intoxication due to coniine administration were observed in both the rat and rabbit. In the rat, head shaking and chewing motions, occasionally accompanied by mild tremors, were observed. Lethargy was the only sign observed in the rabbit. Signs subsided in both species within 30 minutes of treatment. Higher doses of coniine produced severe tremors in both species similar to those reported for cattle (Keeler, et al. 1980), but only when preceding death of the animal. Even at the doses given in this study, two rabbits died during treatment: one after 8 doses, the other after 11 doses. Data from these animals were excluded from statistical evaluation.

In addition to clinical signs of intoxication, maternal toxicity in teratology studies is also determined by reduction in food consumption and/or body weight of the dam (Khera 1984). Although rabbits treated with coniine appeared to lose more weight and eat less than control rabbits, these differences were not statistically significant (Table 3). The high degree of variation in the data is due to a few animals that stopped eating during treatment. Rat maternal weight gain and feed consumption were not statistically significantly different between

treated and control animals and those trends observed in rabbits were less apparent (Table 3).

There were no statistically significant differences in the numbers of viable fetuses between control and coniine-exposed rat or rabbit litters. No dead fetuses were found in any of the control or coniine-exposed rat litters. However, 2 of 9 control and 3 of 8 treated rabbit litters contained dead fetuses although the number per litter was small (controls: 2 of 11 and 1 of 11; treated: 2 of 11, 1 of 8, and 1 of 10). All dead fetuses were excluded from further analyses.

While fetal weights were significantly lower in coniine-exposed rat and rabbit fetuses (Table 3), skeletal and visceral morphology were only minimally affected by coniine exposure (Tables 4 and 5). In skeletal examinations, the only statistically significant treatment related malformation was a reduction in cranial ossification of rabbit fetuses. No skeletal anomalies in rats or visceral abnormalities in either species were found to be statistically significant or to show any apparent trends.

The only external malformation observed was arthrogryposis in rabbit fetuses. This lesion was observed in 2 of 8 coniine-exposed litters with intralitter incidence rates of 1 of 9 and 2 of 9, respectively. The lesion was hyperflexion of the antebrachial-carpal joint with excessive supination (Fig. 2). The lesion was only modestly reducible at birth and returned to the original

position following manipulation. In two cases, the alteration was unilateral (involving the left forelimb in the first case and the right forelimb in the second case), while the third case was bilateral. While the incidence of arthrogryposis in coniine-exposed rabbit litters was not statistically significant when compared to study controls (0 of 9 litters affected), it is statistically significant when compared to historical controls (see discussion).

## Discussion

Coniine was fetotoxic, probably associated with maternal toxicity, in rats and rabbits as evidenced by a decrease in fetal weights (Black and Marks 1986; Khera 1984) in both species. At the doses administered in rabbits, coniine caused clinical signs of intoxication. Two deaths occurred at this dose and only marginally higher doses were uniformly lethal in both species. The slight reduction in maternal weight gain of rats and greater weight loss and lower daily food consumption for rabbits, although not statistically significant, may further represent maternal toxicity. Maternal toxicity has also been associated with delays in fetal ossification (Black and Marks 1986) and this could explain decreased cranial ossification observed in coniine-treated rabbits. Embryotoxicity, as measured by reduced litter size (data not included) or increased fetal death, was not observed.

In order to classify coniine as a teratogen in the species tested, a statistically significant increase in a treatment-related, permanent structural alteration not associated with maternal toxicity should be demonstrated (Black and Marks 1986). Coniine was clearly not teratogenic in rats as administered in the present study. The only potential evidence of coniine teratogenicity in rabbits was arthrogryposis in 2 of 8 litters. Arthrogryposis without skeletal malformations detectable by alizarin red-S is not unusual. In humans, skeletal changes associated with arthrogryposis caused by neuromuscular disease include bone thinning leading to fractures and calluses (Rodriguez, et al. 1988) but not major structural defects in the bone. While the incidence of arthrogryposis was not a statistically significant effect when compared to study controls, the present data are statistically significant ($P < .01$) when historical control incidence data for rabbits are used (5.8/1000 live births; Szabo 1989). However, unlike numerous domestic animal species, coniine did not induce a high incidence of arthrogryposis in rabbit litters and few animals within rabbit litters were affected (1 of 9 and 2 of 9). In short, coniine (as administered to rabbits in the present study) is, at worst, a weak teratogen at doses which induced marked maternal toxicity.

The apparent lack of sensitivity to the teratogenic effects of coniine in laboratory animals is interesting given that coniine/CM is a potent teratogen in many

domestic species. Coniine is only weakly teratogenic in rabbits and not teratogenic in rats or mice (preliminary observations in our laboratory). CM seed containing 51% coniine was not found to be teratogenic in Golden hamsters (Panter 1983). Coniine has been shown to cause arthrogryposis in cattle (Keeler, et al. 1980) and ingestion of CM resulted in arthrogryposis in cattle (Keeler and Balls 1978), goats (Panter, et al. 1990), sheep (Panter, et al. 1988a), and pigs (Panter, et al. 1985a) as well as cleft palate in pigs (Panter, et al. 1985b) and goats (Panter, et al. 1990).

In contrast to its species-specific teratogenicity, CM is uniformly acutely toxic to a wide range of species via the nicotinic effects of piperidine alkaloids. Five piperidine alkaloids isolated from CM have been associated with intoxication: coniine, gamma-coniciene, N-methylconiine, conhydrine, and pseudoconhydrine (Keeler and Balls 1978). Coniine and gamma-coniciene, the major toxic and teratogenic alkaloids, gradually increase and become predominant in seeds as the plant matures (Fairbairn and Challen 1959). Acute toxic action of coniine is biphasic with stimulation followed by depression of autonomic ganglia, neuromuscular junctions, and central nicotinic synapses. Symptoms of acute poisoning include nervousness, fasiculations of skeletal muscles, clonic and tonic contractions of separate limbs, and convulsions followed by

weakened and slowed heart rate, coma, and death (Bowman and Sanghvi 1963).

The proposed mechanism of teratogenicity for coniine is via nicotinic receptor blockade resulting in decreased motion of the fetus (Panter, et al. 1990) although there is no direct proof for this supposition. If correct, it seems likely given the cross-species conservation of nicotinic receptors, that coniine would be teratogenic in any species given an exposure resulting in significant access of chemical to the fetus. We utilized the highest possible non-lethal dose of coniine in rats and rabbits and were unable to produce the marked teratogenic effects observed in domestic species. Potential reasons for this differential effect include differences in metabolism and distribution of the compound or a difference in nicotinic receptors. The validity of these explanations is unclear and remains to be tested.

In summary, coniine is not teratogenic to rats and weakly teratogenic to rabbits. Decreased cranial ossification and reduced fetal weights were observed due to fetal toxicity probably as a result of maternal toxicity. Sprague-Dawley rats and New Zealand white rabbits are not appropriate laboratory animals for a model of coniine-induced arthrogryposis. The reason for the resistance to teratogenicity in these laboratory animal species is unknown at this time.

Acknowledgements

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Table 3: Maternal weight gain, food consumption, and fetal weights[1].

| | average daily weight gain | | average daily food consumption | | fetal weight | |
|---|---|---|---|---|---|---|
| | control | coniine | control | coniine | control | coniine |
| Rat[2] | 5.02 ± 3.07 | 4.65 ± 1.87 | 20.00 ± 2.49 | 20.44 ± 1.12 | 3.03 ± 0.74 | 2.76 ± 0.49* |
| Rabbit[3] | -23.33 ± 20.00 | -35.00 ± 23.90 | 74.12 ± 40.33 | 45.28 ± 48.76 | 36.56 ± 9.72 | 32.49 ± 13.30* |

[1] Data in grams; mean ± S.D.
[2] Rat control litters n = 8; treated litters n = 10.
[3] Rabbit control litters n = 9; treated litters n = 8.
* Mean of treated group significantly different than control, $P \leq 0.05$.

Table 4: External anomalies in offspring exposed to coniine in utero*.

| | Rat[1] | | Rabbit[2] | |
|---|---|---|---|---|
| | control | coniine | control | coniine |
| Flexed limbs | 0/8 | 0/10 | 0/9 | 2/6 |
| Scoliosis | 0/8 | 0/10 | 0/9 | 1/7 |
| Umbilica hernia | 1/7 | 0/10 | 0/9 | 1/7 |
| Edema | 0/8 | 1/ 9 | 0/9 | 0/8 |
| Cleft palate | 0/8 | 0/10 | 0/9 | 1/7 |

* Data expressed as affected litters/normal litters. The incidence of edema within the affected rat litter was 1/4. The incidence of any anomaly within an affected rabbit litter never exceeded 2/4.

[1] Rat control litter size = 8.1 ± 5.2; treated litter size = 9.6 ± 5.1 (mean ± S.D.).

[2] Rabbit control litter size = 8.4 ± 4.1; treated litter size = 6.6 ± 3.7 (mean ± S.D.).

Table 5: Skeletal anomalies in offspring exposed to coniine in utero[1].

| | Rat | | Rabbit | |
|---|---|---|---|---|
| | control | coniine | control | coniine |
| Rib anomalies[2] | 0/8 | 0/10 | 6/3 | 6/2 |
| Reduced ossification | | | | |
| sternum | 4/4 | 9/ 1 | 1/8 | 1/7 |
| cranium | 4/4 | 2/ 8 | 1/8 | 5/3* |
| phalanges | 0/8 | 0/10 | 0/9 | 1/7 |

[1] Data expressed as affected litters/normal litters.
[2] Includes missing, extra, and wavy ribs.
* Statistically different from control. $P \leq 0.05$.


Fig. 2. Alizarin-red S stained forelimbs from day 29 rabbit fetuses. The fetus from which the limb on the right was taken was exposed to 40 mg coniine/kg maternal body weight at 8 hour intervals on gestation days 20-24. The fetus from which the limb on the left was taken was identically treated with corn oil vehicle. Note the hyperflexion of the antebrachial-carpal joint with excessive supination in the exposed fetus.

# EFFECT OF CONIINE ON THE DEVELOPING CHICK EMBRYO

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## Abstract

Coniine, an alkaloid from *Conium maculatum* (poison hemlock), has been shown to be teratogenic in livestock. The major teratogenic outcome is arthrogryposis, presumably due to nicotinic receptor blockade. However, coniine has failed to produce arthrogryposis in rats or mice and is only weakly teratogenic in rabbits. The purpose of this study was to evaluate and compare the effects of coniine and nicotine in the developing chick. Concentrations of coniine and nicotine sulfate were 0.015%, 0.03%, 0.075%, 0.15%, 0.75%, 1.5%, 3%, and 6% and 1%, 5%, and 10%, respectively. Both compounds caused malformations and lethality in a dose-dependent manner. All concentrations of nicotine sulfate caused some lethality but a no observed effect level for coniine lethality was 0.75%. The malformations caused by both coniine and nicotine sulfate were excessive flexion or extension of one or more toes. No histopathological alterations or differences in bone formation were seen in the limbs or toes of any chicks from any group, however, extensive cranial hemorrhage occurred in all nicotine sulfate treated chicks. There was a statistically significant ($P \leq 0.01$) decrease in movement in coniine and nicotine sulfate treated chicks as

determined by ultrasound. Control chicks were in motion an average of 33.67% of the time while coniine treated chicks were only moving 8.95% of a 5 minute interval and no movement was observed for nicotine sulfate treated chicks. In summary, the chick embryo provides a reliable and simple experimental animal model of coniine induced arthrogryposis. Data from this model support a mechanism involving nicotinic receptor blockade with subsequent decreased fetal movement.

## Introduction

Coniine is a major piperidine alkaloid of *Conium maculatum* (poison hemlock, CM). In utero exposure to coniine has been shown to produce arthrogryposis in cattle (Keeler, et al. 1980) and ingestion of CM during gestation also causes limb malformations in cattle (Keeler and Balls 1978), pigs (Panter, et al. 1985a), goats (Panter, et al. 1990), and sheep (Panter, et al. 1988a). The proposed mechanism of teratogenicity for coniine is via nicotinic receptor blockade resulting in decreased motion of the fetus (Panter, et al. 1990). Given the broad cross-species teratogenicity of coniine in domestic animals and the cross-species conservation of nicotinic receptors, it is interesting that hamsters (Panter 1983), mice, and rats are resistant to the teratogenic effects of coniine and rabbits are only weakly susceptible (Forsyth and Frank 1993). The reason for teratogenic species sensitivity remains unknown at present.

Because nicotine produces a more consistent and severe teratogenic response in the chick embryo (Landauer 1960) than the rat embryo (Slotkin, et al. 1987), we hypothesized that the chick embryo could provide an experimental animal model of coniine-induced arthrogryposis. Such a model would be useful to specifically study the mechanisms of species sensitivity of coniine teratogenicity as well as providing a general model for mechanistic investigations into arthrogryposis. The purpose of the present study was to evaluate and compare coniine- and nicotine-induced malformations in the chick.

## Materials and Methods

### *Chemicals*

Coniine (97% purity by gas chromatography) and nicotine sulfate (40% solution) were purchased from Sigma Chemical Corp. (St. Louis, MO). Coniine was diluted in corn oil and nicotine sulfate in 0.9% NaCl.

### *Experimental Design*

Study 1 was designed to characterize the effects of coniine and nicotine on chick embryos. Dosing schedule and chemical concentration ranges were determined from similar studies in the literature (Hosseini and Hogg 1991a; Drachman and Coulombre 1962). Concentrations of coniine used were 0.015%, 0.030%, 0.075%, 0.150%, 0.75%, 1.50%, 3.00%, and 6.00% and concentrations of nicotine sulfate used were 1.0%, 5.0%, and 10.0% (v/v). Fertile chicken

eggs from a New Hampshire X White leghorn cross were incubated in a Jamesway forced-draught incubator at 38°C for the entire experimental period. Treatment solution application was modified from the method described by Hosseini and Hogg (1991a). On days 6, 8, and 10 of incubation, a small hole was punched in the egg above the air cell and 50 µl of treatment solution was injected into the air space for absorption through the chorioallantoic membrane. The hole was then sealed with glue. These time periods correspond to Stages 29, 34, and 36, respectively (Hamburger and Hamilton 1952). Chicks were removed from the shell on day 17 of incubation and examined grossly for external malformations. Only live chicks were included in the malformation analysis.

Study 2 was an ultrasonic analysis of embryonic movement and histopathologic analysis of coniine, nicotine, and vehicle treated chicks. Each treatment group contained 25 eggs. Based on the results of study 1, 1.5% coniine and 5% nicotine sulfate were chosen due to a consistently high rate of malformations. Corn oil was used as the control for this study because a few malformations were observed in corn oil treated chicks from study 1 (no effect was seen in 0.9% NaCl treated chicks; see results).

*Ultrasonic and Histopathological Analyses*

Gray-scale real time sonography (Ausonics Microimager 1000, Universal Medical, Bedford Hills, NY) was used to monitor chick embryo movements 1 hour after the third

treatment (d10). Preliminary studies indicated this was the time of maximum paralysis of coniine treated chicks (data not included). For access to the embryo, a hole was cut in the shell above the air cell and the space filled with 0.9% NaCl. The mechanical sector transducer was 7.5MHz with a 100mm scan depth. Embryo localization was confirmed by identification of a fetal heart beat. Real time images were stored on a videotape. The percentage of time chicks were in motion was determined for a 5 minute interval without knowledge of treatment group. Chicks in motion equal to or less than 10% of the 5 minute interval were defined as severely inhibited. After ultrasonic evaluation, chicks were fixed in 10% neutral buffered formalin and legs, toes, and brains processed routinely for light microscopic evaluation. Histopathological interpretation was performed without knowledge of treatment groups. Only data from live chicks were included in the analysis.

### *Statistics*

Differences in chick motion were evaluated by one-way analysis of variance with the level of significance $P \leq 0.01$.

## Results

Coniine and nicotine sulfate caused malformations and lethality in chick embryos in a dose-dependent manner (Table 6). Lethality occurred at all concentrations of nicotine sulfate tested with 38%, 67%, and 100% of chicks dying at 1%, 5%, or 10% nicotine sulfate, respectively. A

no effect level of coniine on chick lethality was found at 0.75%. Above this dose, a sharp dose-response was observed with 15%, 45%, and 100% of chicks dying at 1.5%, 3.0%, and 6% coniine, respectively. Corn oil alone (diluent for coniine) appeared to cause mild reduction in body size as compared with 0.9% NaCl and decreased body size was commonly observed in both treatment groups although body weights and measurements were not obtained, prohibiting statistical comparison.

Coniine and nicotine sulfate limb lesions were similar: excessive flexion or extension of one or more toes (Fig. 3). Malformations of the toes were reducible but returned to their original position following manual manipulation. None of the toes appeared specifically to be affected more than the others. Malformations were observed at all doses of coniine and nicotine sulfate tested (Table 6). At 1% and 5% nicotine sulfate, 20% and 100% of chicks were affected, respectively. Corn oil alone resulted in a 20% incidence of malformations. Coniine caused malformations in 25%, 29%, 53%, 43%, 43%, 70%, and 100% of chicks at 0.015%, 0.03%, 0.075%, 0.15%, 0.75%, 1.50%, and 3% coniine, respectively. Nicotine treated chicks were generally more severely affected than coniine treated chicks. The most severely affected nicotine treated chicks displayed some of the morphological symptoms of the crooked-neck dwarf lethal mutation: neck rigid, vertebra palpable, and hypotrophy of leg musculature; however, rigid legs and shortened upper

beak were not observed (Landauer 1960; Romanoff 1972). No histopathological alterations or differences in bone formation were seen in the limbs or toes of chicks from any group in study 2.

Extensive cranial hemorrhage occurred in all nicotine sulfate treated chicks. Microscopically, this hemorrhage surrounded the rhombencephalon with the most severe cases having hemorrhage within the fourth ventricle. Mild hemorrhage was occasionally observed in coniine treated or control animals.

There was a statistically significant ($P \leq 0.01$) decrease in movement in coniine and nicotine sulfate treated chicks (Table 7) as determined by ultrasound. Control chicks were in motion an average of 33.67% of the time while coniine treated chicks were only observed to move 8.95% of the 5 minute interval. While coniine dramatically decreased the amount of movement observed, nicotine sulfate completely paralyzed all chicks (no movement was observed). For coniine treated chicks, the incidence of severe motion inhibition (75%) closely approximated the incidence of malformations at this concentration: 70% malformations at 1.5% coniine (Table 7).

## Discussion

Because of coniine's acute nicotinic effects, the mechanism of coniine teratogenicity is proposed as a nicotinic receptor blockade resulting in decreased motion

of the fetus (Panter, et al. 1990), a general mechanism associated with arthrogryposis in humans (Hall 1989). In this study, coniine caused malformations in chick embryos similar to those caused by nicotine sulfate and both compounds produced defects in a dose-responsive manner. Lack of complete paralysis in coniine treated chicks as compared to nicotine sulfate treated chicks correlates with the less severe arthrogrypotic effects of coniine. The close similarity of degree of effect on fetal movement and incidence of malformation supports the hypothesis that coniine causes arthrogryposis via a reduction of fetal movement, most likely due to nicotinic receptor blockade. If coniine produces arthrogryposis by decreased movement secondary to nicotinic receptor blockade, the mechanism of marked teratogenic species specificity remains unknown. Possibilities include differences in nicotinic receptor kinetics and/or biotransformation pathways. Studies are underway to evaluate these possibilities.

The physical basis of coniine- and nicotine-induced malformations observed in this study is unknown. Decreased intrauterine movement caused by either neuropathic agents, myopathic agents, abnormal connective tissue or joints, or decreased space in which the fetus can move has been associated with arthrogryposis (Hall 1989). The limb malformations observed in coniine and nicotine treated chicks were not associated with histological alterations on d10 and bone shape appeared normal (gross observation) on

d17. Hosseini and Hogg (1991b) also reported no change in bone formation at d10 for paralysed chick embryos. Therefore, the malformations observed here may be due to tendon contracture, although the mechanism by which fetal movement would affect tendon structure remains unknown.

Coniine and nicotine both produced lethality in a dose-responsive manner. Cranial hemorrhage seen in nicotine treated chicks may be related to lethality as cotreatment with d-tubocurarine chloride reduced the severity of both hemorrhage and lethality but limb malformations were not reduced (data not included). It should be noted that the mechanism by which nicotine would cause such a hemorrhage is unknown and marked increases in cranial hemorrhage were not observed with the higher concentrations of coniine that resulted in increased mortality. In short, the relationship of the cranial hemorrhage and mortality remains unclear.

In summary, the chick embryo provides a reliable and simple experimental animal model of coniine induced arthrogryposis. Data from this model support a mechanism involving nicotinic receptor blockade with subsequent decreased fetal movement. This model will be useful in determining why some species are resistant to coniine-induced arthrogryposis, information which could provide a better understanding of arthrogrypotic mechanisms in general.

Table 6: Dose response of lethality and malformation rates for coniine and nicotine sulfate treated chicks[1].

| Chemical | Live Chicks | % Live | Malformed Chicks[2] | % Malformed |
|---|---|---|---|---|
| Corn oil | 25 of 20 | 100 | 5 of 20 | 20 |
| % Coniine | | | | |
| 0.015 | 8 of 8 | 100 | 2 of 8 | 25 |
| 0.030 | 7 of 7 | 100 | 2 of 7 | 29 |
| 0.075 | 7 of 7 | 100 | 4 of 7 | 53 |
| 0.150 | 7 of 7 | 100 | 3 of 7 | 43 |
| 0.750 | 7 of 7 | 100 | 3 of 7 | 43 |
| 1.500 | 47 of 53 | 89 | 33 of 47 | 70 |
| 3.000 | 6 of 9 | 67 | 6 of 6 | 100 |
| 6.000 | 0 of 9 | 0 | --- | --- |
| 0.9% NaCl | 9 of 9 | 100 | 0 of 9 | 0 |
| % Nicotine | | | | |
| 1.0 | 5 of 8 | 63 | 1 of 5 | 20 |
| 5.0 | 9 of 27 | 33 | 9 of 9 | 100 |
| 10.0 | 0 of 8 | 0 | --- | --- |

[1] chicks treated with 50 µl of coniine or nicotine sulfate solution on days 6, 8, and 10; see Methods for details.
[2] only live chicks included.

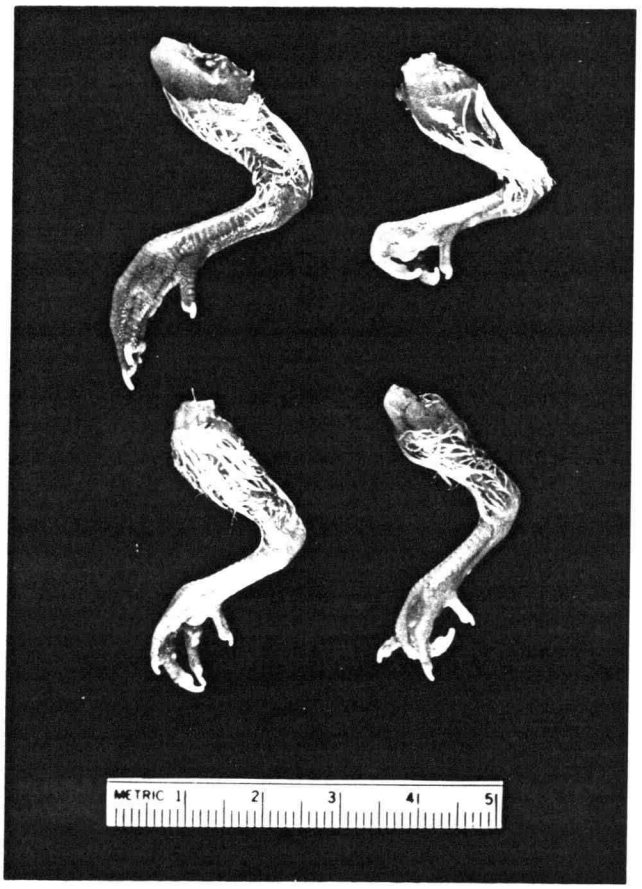


Fig. 3: Limb malformations on d17 of coniine and nicotine sulfate treated chicks. Chicks were treated with 50 µl of either 0.9% NaCl (upper left), 5% nicotine sulfate (upper right), corn oil (lower left), or 1.5% coniine (lower right) on days 6, 8, and 10 of incubation.

Table 7. Malformation rate and depressed movement of d10 chick embryos 1 hour after treatment with either coniine or nicotine sulfate[1].

| Treatment | Time in motion (%)[2] | % Chicks Malformed | % Chicks with Depressed Movement[3] |
|---|---|---|---|
| Control | 33.67 ± 5.81 | 20 | 0 |
| 1.5% Coniine | 8.95 ± 7.84* | 70 | 75 |
| 5% Nicotine | 0.00 ± 0.00** | 100 | 100 |

[1] chicks treated on days 6, 8, and 10; corn oil used for control; see text for details.
[2] per cent of 5 minute interval; mean ± S.D.; n = 25 per group.
[3] in motion ≤ 10% of a 5 minute interval.
* significantly different from control group, $P < 0.01$.
** significantly different from control and coniine groups, $P < 0.01$.

# CONIINE BINDING TO NICOTINIC RECEPTORS IN THE RAT AND CHICK

Carol S. Forsyth, Anthony A. Frank, and Robert C. Speth

Abstract

Coniine, an alkaloid from *Conium maculatum* (poison hemlock), is a known teratogen in many domestic species with maternal ingestion resulting in arthrogryposis of the offspring. We have previously shown that rats are not susceptible and rabbits only weakly susceptible to coniine-induced arthrogryposis. However, the chick embryo does provide a reproducible laboratory animal model of coniine-induced teratogenesis. The reason for this cross-species variation is unknown. Because the proposed mechanism of coniine teratogenesis is interaction with nicotinic receptors, the purpose of this study was to evaluate coniine binding to nicotinic receptors in susceptible and nonsusceptible species. Using the chick model, neither the peripheral nicotinic receptor antagonist d-tubocurarine chloride nor the central nicotinic receptor antagonist trimethaphan camsylate blocked the teratogenesis or lethality of 1.5% coniine (50 μl/egg). Trimethaphan camsylate enhanced coniine-induced lethality in a dose dependent manner. Neither nicotinic receptor blocker prevented nicotine sulfate-induced malformations but d-tubocurarine chloride did block lethality in a dose-dependent manner. Coniine binding to nicotinic receptors isolated from rat

diaphragm and chick leg muscle was also assessed. The $IC_{50}$ for coniine binding in the rat was 314μM while that for the chick was 70μM. Overall, coniine displacement of [$^{125}$I]-α-bungarotoxin was low for both the rat and the chick compared to nicotine and [$^{125}$I]-α-bungarotoxin binding alone was low in rat fetal thigh tissue. Differences in receptor affinity and/or quantity between the rat and chick may explain, in part, the differences in susceptibility of coniine-induced teratogenesis between these two species.

## Introduction

Coniine, an alkaloid from *Conium maculatum* (poison hemlock, CM), is a documented teratogen in many domestic species (Panter, et al. 1988c). The acute toxic action of coniine is biphasic with first stimulation then depression of the autonomic ganglia (Bowman and Sanghvi 1963). Similarly depression, or blockade, of nicotinic receptors resulting in reduced fetal movement is the proposed mechanism of teratogenic action of coniine (Panter, et al. 1990). Evidence consistent with this hypothesis is supported by the reduction in fetal movements and similar malformations observed in CM treated goat kids (Panter, et al. 1990) and in coniine and nicotine sulfate treated chicks (Forsyth, et al. 199x).

While a teratogenic mechanism involving blockade of nicotinic receptors would seem likely to result in cross-species teratogenicity, coniine is teratogenic in several

domestic species (Panter, et al. 1988c) and the chick (Forsyth, et al. 199x) but hamsters (Panter 1983) and rats (Forsyth and Frank 1993) are unaffected and rabbits are only minimally susceptible (Forsyth and Frank 1993). The reason for this cross species variation in susceptibility to coniine-induced teratogenesis is unknown but may be due to differences in receptor affinity/number or biotransformation.

Nicotinic receptors are distributed both centrally and peripherally and are distinguished structurally and pharmacologically (Maelicke 1992). Using the chick model of coniine-induced teratogenesis, the first part of this study attempted to determine whether coniine-induced malformations and lethality are mediated through central or peripheral nicotinic receptors. The central nicotinic receptor blocker trimethaphan camsylate and the peripheral nicotinic receptor blocker d-tubocurarine chloride (Gilman, et al. 1990) were evaluated for their ability to block the malformations and/or lethality caused by coniine and nicotine sulfate. The competition binding curves of coniine and nicotine sulfate for nicotinic receptors isolated from rat diaphragm and chick leg muscle were compared to assess binding affinity and receptor quantity.

## Materials and Methods

### *Chemicals*

Coniine, nicotine sulfate, and α-bungarotoxin were purchased from Sigma Chemical Corp (St. Louis, MO).

Trimethaphan camsylate was purchased from Roche Laboratories (Nutley, NJ) and d-tubocurarine chloride from ICN Biochemicals (Irvine, CA). [$^{125}$I]-α-Bungarotoxin was purchased from New England Nuclear (Boston, MA).

*Experimental Design*

*Nicotinic Receptor Blockers.* Study 1 determined the effects of central or peripheral nicotinic blockers on coniine-induced malformations and lethality in the chick. Fertile chick eggs from a New Hampshire X White leghorn cross were incubated in a Jamesway forced-draught incubator at 38°C. Treatment solutions were applied as described previously (Forsyth, et al. 199x; Hosseini and Hogg 1991a). Briefly, a small hole was punched in the shell above the air cell on days 6, 8, and 10 of incubation and 50 µl of a nicotinic receptor blocker followed by 50 µl 1.5% coniine or 5% nicotine sulfate solution were injected into the air space. The hole in the shell was then sealed with glue. Based on preliminary observations of 25% lethality or less, concentrations of d-tubocurarine chloride used were 2% and 3% and of trimethaphan camsylate 0.6%, 1.2%, and 1.8% (v/v in 0.9% NaCl). Numbers of chicks evaluated at each concentration are given in Figures 4 and 5. On day 17 of incubation chicks were removed from the shell and examined for gross external malformations; only live chicks were included in the malformation analysis. On d10 some chicks were remeved from the shell and fixed in 10% neutral buffered formalin and brains processed routinely for light

microscopic evaluatuion. Histopathological interpretation was performed without knowledge of treatment groups.

*Nicotinic Receptor Binding.* Study 2 was designed to measure coniine binding to nicotinic receptor preparations from muscle from chicks and rats. Chicks, incubated as described above, were euthanitized by placing at -20°C for several minutes on day 10 of incubation. Thigh muscle was removed and frozen at -70°C until receptor isolation. Rat diaphragm muscle was obtained from adult male Sprague-Dawley rats and used fresh. Rats were anesthetized with Equithesin (Jensen-Salsbury, Kansas City, MO) then killed by exsanguination. Diaphragm muscle end plate region was used to obtain a high concentration of nicotinic receptors. Attempts were made to determine the affinity of coniine for nicotinic receptors in rat fetal and adult thigh muscle. However, specific [$^{125}$I]-α-bungarotoxin binding in these tissues was too low to accurately measure coniine binding by this protocol (data not included; see discussion). Chick and rat tissues were homogenized for 10 seconds with a mechanical tissue homogenizer in 50mM Tris-HCl (pH 7.4) containing 100mM NaCl and 0.1mM phenylmethylsulfonylfluoride to inhibit proteolytic enzymes. Homogenates were filtered through 4 layers of gauze and centrifuged at 3000g for 10 minutes. The supernatant was centrifuged at 48,000g for 20 minutes. The resulting pellet was resuspended in the above buffer at approximately 0.1 g initial wet weight of tissue/ml. Tissue preparations (about 10 mg) were incubated

with 10nM [$^{125}$I]-α-bungarotoxin in the presence or absence of coniine or nicotine sulfate in 500 µl of 50mM Tris-HCl, 100mM NaCl (pH 7.4 buffer). After 2 hours at room temperature, the reaction was stopped by addition of 3 ml of room temperature 50mM sodium-potassium phosphate buffer, pH 7.4, and immediately filtered through a Schleicher and Schuell #32 glass filter which had been previously soaked with 1 mg/ml bovine serum albumin. Filters were rinsed 2 additional times with 50mM sodium-potassium phosphate buffer and the filter bound radioactivity determined by gamma scintillation counting. Specific [$^{125}$I]-α-bungarotoxin binding to nicotinic receptors was defined as binding that was displaced by 0.1mM d-tubocurarine chloride.

*Statistics*

The incidence of coniine- or nicotine sulfate-induced lethality or malformations in the presence or absence of a nicotinic receptor blocker was calculated using the chi-square analysis with the level of significance $P \leq 0.05$. $IC_{50}$ values of competing ligands for [$^{125}$I]-α-bungarotoxin binding were derived using Inplot (Graph Pad Software, San Diego, CA) based on the equation: $Y = 1-(I/(I+IC_{50}))$ where Y is the fraction of binding in the absence of competing ligand and I is the concentration of competing ligand. Average $IC_{50}$ values for 2 assays are reported.

## Results

Neither d-tubocurarine chloride nor trimethaphan camsylate blocked the teratogenicity or lethality of 1.5% coniine (Fig. 4). Trimethaphan camsylate actually enhanced coniine induced lethality in a dose responsive manner with statistically significant differences at 1.2% and 1.8%. Nicotine sulfate-induced malformations were unaffected by either d-tubocurarine chloride or trimethaphan camsylate (Fig. 5). Trimethaphan camsylate did not block the lethality of 5% nicotine sulfate. D-tubocurarine chloride did block lethality of 5% nicotine sulfate in a dose dependent manner with a statistically significant difference only at the highest d-tubocurarine chloride concentration tested (3%). Intracranial hemorrhage seen in nicotine sulfate treated chicks (Forsyth, et al. 199x) may be related to lethality as d-tubocurarine chloride also reduced the severity of this hemorrhage microscopically (data not included). Trimethaphan camsylate alone did not cause any malformations in chicks at any concentration tested up to 5% which resulted in 100% lethality. Twenty-five per cent lethality was observed at a concentration of 1.8% trimethaphan camsylate, the highest concentration used as a blocker with coniine (data not included). Malformations, similar to those observed with nicotine sulfate alone, occurred for 2% and 3% d-tubocurarine chloride at rates of 20% and 80%, respectively and 3% d-tubocurarine chloride alone resulted in 14% lethality (data not included).

Nicotinic receptors from both rat diaphragm and chick leg muscle bound coniine with lower affinity than nicotine sulfate (Figs. 6 and 7). The average $IC_{50}$ for coniine displacement of specific [$^{125}$I]-α-bungarotoxin binding to nicotinic receptors from chick muscle was 70μM and from rat diaphragm 314μM. This was considerably less than the $IC_{50}$'s for nicotine, 3.5μM and 25μM for chick muscle and rat diaphragm, respectively. Coniine displaced approximately 12,000 - 9000 cpm of [$^{125}$I]-α-bungarotoxin from rat diaphragm muscle over a concentration range of 1.26μM to 1.26mM while nicotine displaced 14,000 - 10,000 cpm over a concentration range of 0.04μM to 0.4mM (Fig. 6). Total [$^{125}$I]-α-bungarotoxin displacement from chick muscle was approximately 10,000 cpm to 7500 cpm for 1.26μM - 1.26mM coniine and 11,250 cpm to 8000 cpm for 0.04μM - 0.4mM nicotine (Fig. 7).

## Discussion

Using the chick model of coniine-induced teratogenesis, we were unable to block the effects of coniine and nicotine sulfate using central and peripheral nicotinic receptor antagonists. Because of the complicated nature of the interaction of nicotine sulfate and coniine with the nicotinic receptor (stimulation and blockade), the failure of the antagonists to prevent the teratogenic effects does not necessarily imply that the terata are not mediated by an effect at the nicotinic receptor, particularly given

existing data that support a role for the nicotinic receptor (Panter, et al. 1990; Forsyth, et al. 199x). If the teratogenic effects of coniine had been blocked by the antagonists, this would have provided direct evidence for the involvement of the nicotinic receptor. However, due to the biphasic response of the receptors to coniine with first stimulation then depression or blockade (Bowman and Sanghvi 1963), failure of the antagonists to block coniine effects is difficult to interpret. Also, the 100% rate of malformations induced by 5% nicotine sulfate alone may have masked any synergistic effects of the blockers. Teratogen concentrations were fixed such that a high malformation rate would occur allowing detection of even modest protection by the blockers.

Central and peripheral nicotinic receptor antagonists were also not definitive in determining the relationship of coniine binding to lethality. Nicotine sulfate induced lethality appears to be due to interaction of peripheral nicotinic receptors whereas coniine induced lethality appears to involve central nicotinic receptors (Figs. 4 and 5). The basis for this apparent difference is unknown. Further, why a peripheral nicotinic receptor antagonist would decrease intracranial hemorrhage is also unclear. In short, the relationship between coniine-induced lethality, intracranial hemorrhage, and the peripheral nicotinic receptor in the chick embryo is uncertain and deserves further study.

Differences in receptor binding of coniine may be one factor in the cross-species variation of susceptibility to coniine-induced teratogenesis. The $IC_{50}$ for coniine binding in the rat, a nonsusceptible species, was greater than 4 times that of the chick, a susceptible species. Thus, while coniine is presumably acting through muscular nicotinic receptors (Forsyth, et al. 199x), it appears to do so with a lower affinity than nicotine. If species susceptibility is related to nicotinic receptor affinity, nicotine should be more teratogenic than coniine in the chick and both nicotine and coniine should be stronger teratogens in the chick than the rat. We have previously shown that nicotine is a more potent teratogen in the chick than coniine (Forsyth, et al. 199x). Nicotine has also been shown to cause more pronounced structural defects in the chick (Landauer 1960; Forsyth, et al. 199x) than in the rat (Slotkin, et al. 1980; Joschko, et al. 1991). Coniine also causes pronounced defects in chicks (Forsyth, et al. 199x) but not rat embryos (Forsyth and Frank 1993).

Nicotinic receptors exist as a pentameric complex and the subunits vary during development and between tissues creating a multiplicity of receptor subtypes (Gilman, et al. 1990). Subtypes of receptors occur depending upon the type of subunits present which account, in part, for ligand specificity. The low amount of coniine binding as compared to nicotine sulfate binding that was observed for receptors from both the rat and chick (Figs. 6 and 7) may indicate a

second high affinity site for coniine binding but we have not further characterized this binding.

Differences in nicotinic receptor number/distribution could also play a role in interspecies differences in teratogenic susceptibility. Chick thigh muscle was used in the present study, but fetal rat thigh muscle analysis was not possible due to low binding of [$^{125}$I]-α-bungarotoxin. If peripheral nicotinic receptors are, in fact, decreased in the rat fetus as compared to chick, this would be expected to impart less coniine- and/or nicotine-induced paralysis in the rat fetuses resulting, in turn, in fewer terata. A combination of decreased receptor affinity, possible subtypes, and fewer receptors seems likely based on the data presented in this study.

In summary, both the peripheral nicotinic receptor antagonist d-tubocurarine chloride and the central nicotinic receptor antagonist trimethaphan camsylate failed to protect the chick embryo from coniine-induced teratogenesis. Differences in coniine binding to nicotinic receptors from rat diaphragm and chick leg muscle preparations may explain, in part, the cross-species variation in susceptibility to coniine-induced teratogenesis. Investigations are underway to determine whether differences in biotransformation of coniine between rats and chicks also exists.

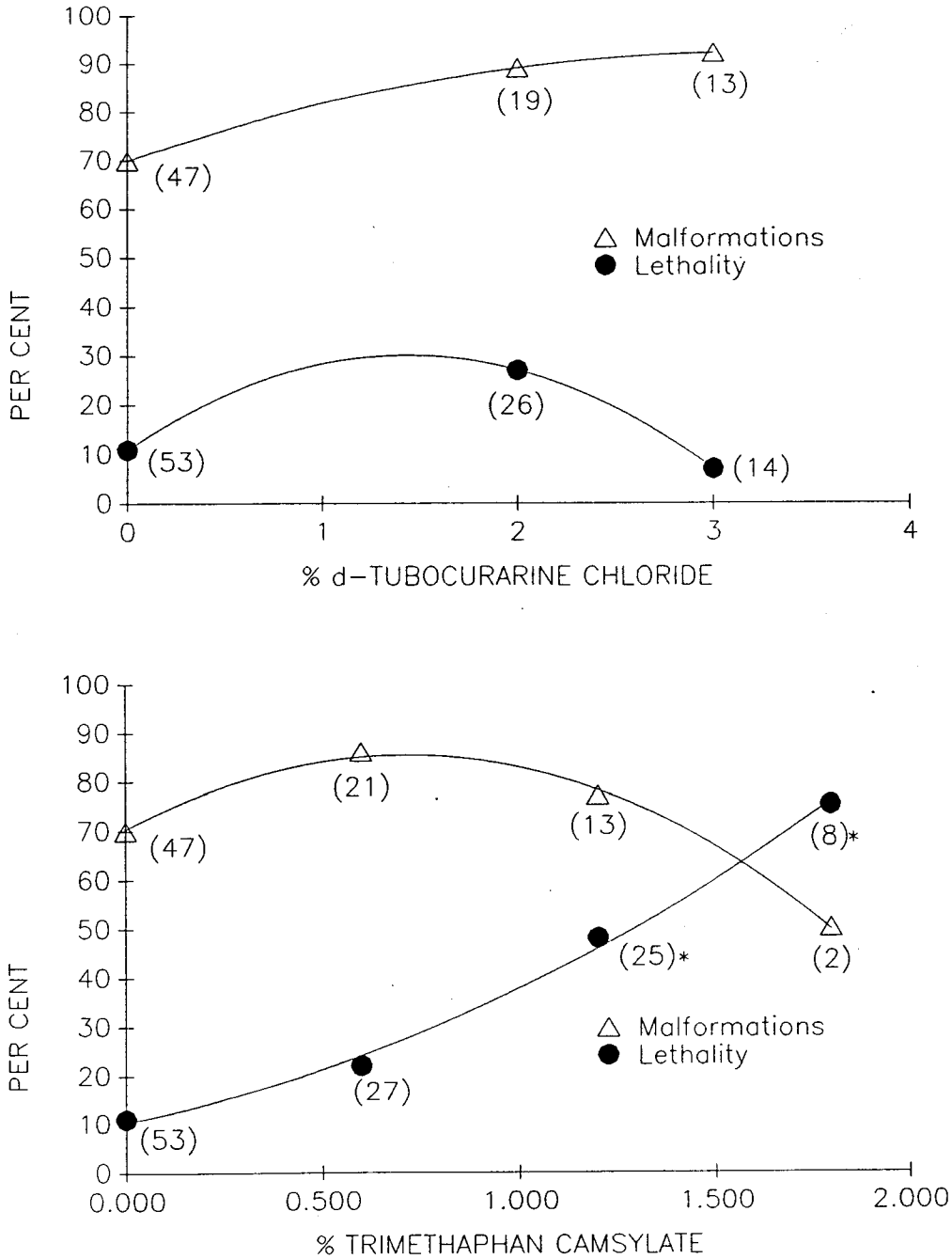


**Fig. 4:** Effect of d-turbocurarine chloride and trimethaphan camsylate on lethality and malformations caused by 1.5% coniine in the chick. Numbers in parentheses indicate number of chicks evaluated at each concentration of nicotinic blocker. Asterisks denote points on the curve that are statistically significant ($P \leq .05$) from 1.5% coniine alone.

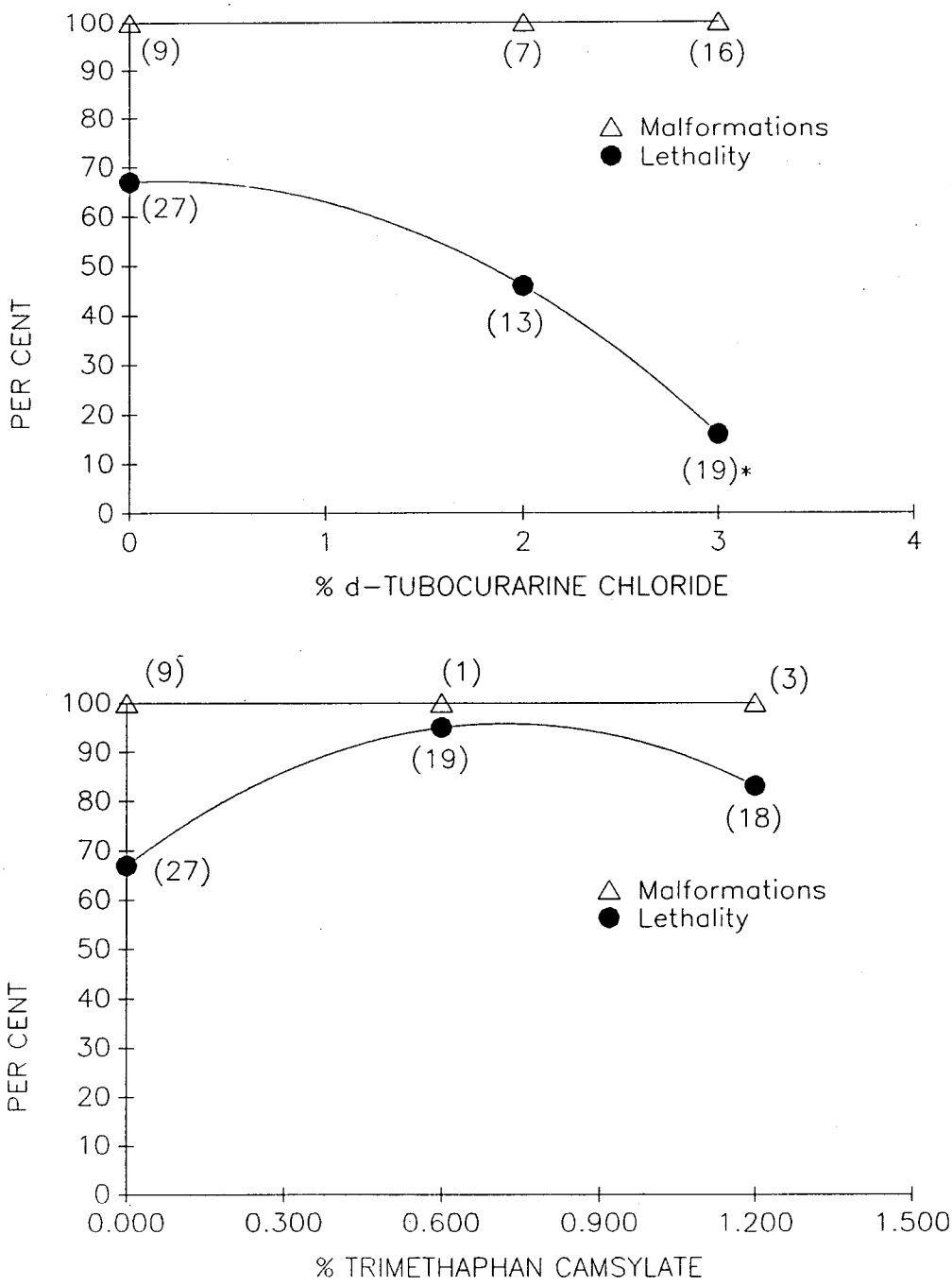


Fig. 5: Effect of d-turbocurarine chloride and trimethaphan camsylate on lethality and malformations caused by 5% nicotine sulfate in the chick. Numbers in parentheses indicate number of chicks evaluated at each concentration of nicotinic blocker. Asterisks denote points on the curve that are statistically significant ($P \leq .05$) from 5% nicotine sulfate alone.

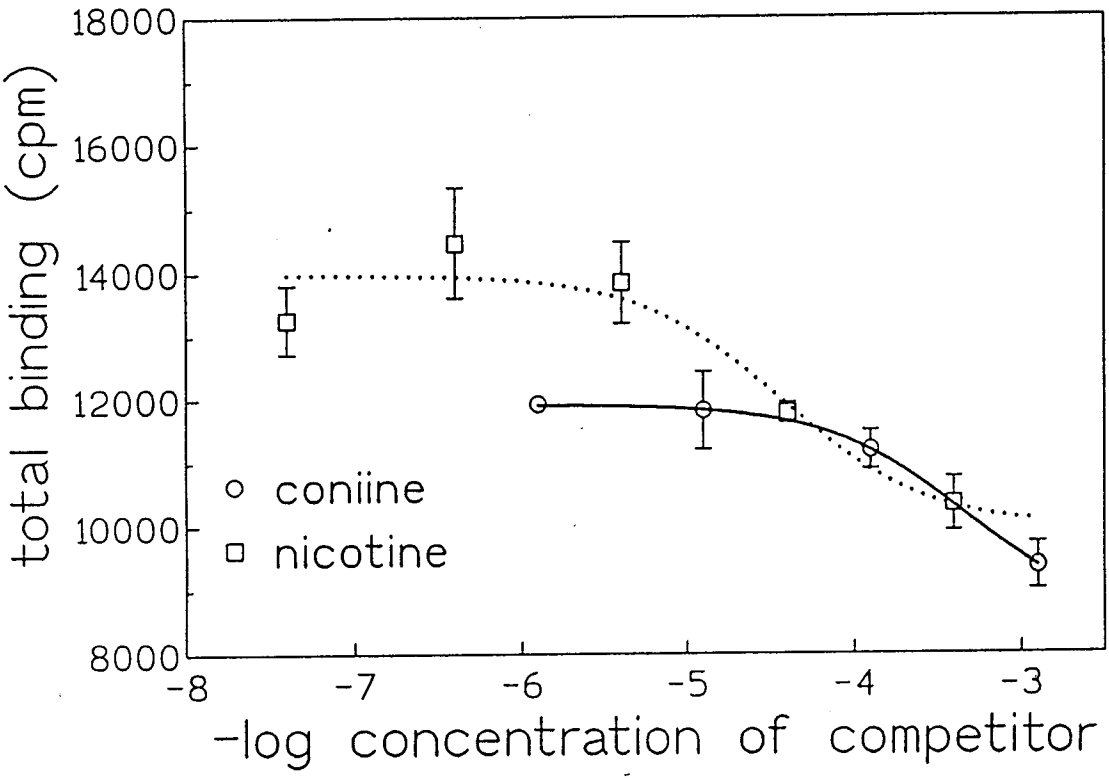


Fig. 6: Competition for [$^{125}$I]-α-bungarotoxin binding in rat diaphragm muscle.

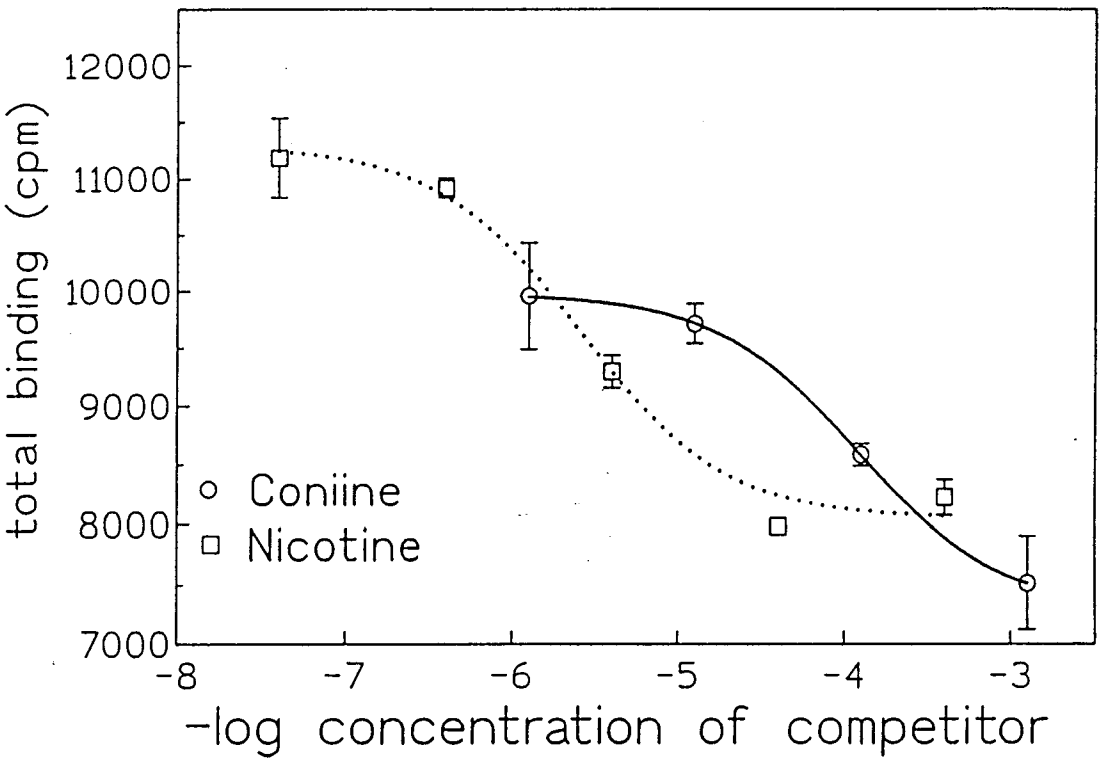


Fig. 7: Competition for [$^{125}$I]-α-bungarotoxin binding in chick muscle.

# CONCLUSIONS AND FUTURE DIRECTION

The Sprague-Dawley rat is not susceptible and the New Zealand white rabbit is only weakly susceptible to coniine-induced arthrogryposis. No coniine exposed rat litters (0/10) were malformed. However, 2 of 8 coniine exposed rabbit litters contained fetuses with arthrogryposis, although few fetuses within litters were affected (1 of 9 and 2 of 9). While this litter incidence rate in rabbits is not statistically significant within this study, the fetal incidence rate is significant when compared to the historical control incidence of arthrogryposis in rabbits of 5.8/1000 live births (Szabo 1989). Therefore, coniine is not teratogenic to rats and only weakly teratogenic to rabbits. The reason for the resistance to teratogenicity in these laboratory animal species is unknown, but may be due to differences in metabolism and/or receptor kinetics. Nicotinic receptor binding by coniine and nicotine was investigated in the rat (see below) but metabolic differences in coniine biotransformation remain unknown.

A statistically significant decrease in fetal weights of coniine-exposed rat and rabbit litters and a reduction of cranial ossification in coniine-exposed rabbit fetuses was observed. These differences were probably due to fetotoxicity as a result of maternal toxicity and not a direct effect of coniine on the fetuses. While maternal toxicity parameters of weight gain and feed consumption

were not affected by coniine treatment, acute signs of intoxication were observed in both species immediately after dosing.

Coniine and nicotine sulfate caused malformations and lethality in chick embryos in a dose-responsive manner. Limb malformations induced by both compounds were excessive flexion or extension of one or more toes. Therefore, the chick embryo provided a reliable and simple experimental animal model of coniine-induced arthrogryposis. Since the mechanism of coniine-induced teratogenicity is proposed as a nicotinic receptor blockade resulting in decreased fetal motion, this model was used to monitor fetal movements after coniine or nicotine treatment. At the concentration of coniine (1.5%) used for this study, the incidence of malformations (70%) closely approximated the incidence of severe motion inhibition (75%). Therefore, data from this model support a mechanism involving nicotinic receptor blockade with subsequent decreased fetal movement.

The physical basis of coniine- and nicotine-induced malformations observed in the chick embryo is unknown. Limb malformations were not associated with histological alterations on d10 and bone shape appeared normal by gross observation on d17. The lack of nervous, osseous, or articular changes in coniine or nicotine treated chicks is in accords with proximate tendinous/ligamentous contracture, although additional, more detailed microscopic studies will be required to confirm such alterations. Also, how

nicotinic receptor blockade and/or lack of fetal movement affects soft tissues is unknown.

Differences in receptor affinity and/or quantity between the rat and chick may explain, in part, the species specificity of coniine-induced teratogenesis. Nicotinic receptors from both the rat and chick bound coniine with lower affinity than nicotine. The average $IC_{50}$ for coniine displacement of specific [$^{125}$I]-α-bungarotoxin binding to nicotinic receptors from chick was 70μM and from rat 314μM. These were considerably greater than the $IC_{50}$'s for nicotine, 3.5μM and 25μM for chick and rat, respectively. Therefore, while coniine is presumably acting through muscular nicotinic receptors, it appears to do so with a lower affinity than nicotine. Differences in biotransformation of coniine between the rat and chick may also contribute to the differences in species susceptibility to coniine-induced teratogenesis, but remain to be investigated.

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# APPENDIX

# APPENDIX

## Dose Response Curves for Coniine, Nicotine Sulfate, d-Tubocurarine Chloride, and Trimethaphan Camsylate in the Chick

Purpose: The purpose of this experiment was to determine the dose/response relationship for lethality and malformation rate in chicks to coniine, nicotine sulfate, d-tubocurarine chloride, and trimethaphan camsylate. These results were preliminary to using the chick model of coniine-induced teratogenesis for studying the mechanism of action of coniine (Chapter 5). Coniine is a nicotinic alkaloid, therefore, nicotine was used as a positive control for comparison. The central nicotinic receptor blocker trimethaphan camsylate and the peripheral nicotinic receptor blocker d-tubocurarine chloride were evaluated in subsequent experiments for their ability to block the lethality and/or malformations caused by coniine and nicotine sulfate (Chapter 6).

Results: Coniine, nicotine sulfate, and d-tubocurarine chloride caused lethality and malformations in chick embryos in a dose dependent manner. No malformations were observed in chicks with trimethaphan camsylate but lethality occurred in a dose-dependent manner. A no effect level for coniine on chick lethality was observed at 0.75%. Above this dose, a sharp dose response was observed with 15%, 45%, and 100% of chicks dying at 1.5%, 3.0%, and 6.0%

coniine, respectively. Coniine caused malformations in 25%, 29%, 53%, 43%, 43%, 70%, and 100% of chicks at 0.015%, 0.03%, 0.075%, 0.15%, 0.75%, 1.50%, and 3.0% coniine, respectively (Fig. 8). Lethality occurred at all concentrations of nicotine sulfate tested with 38%, 67%, and 100% of chicks dying at 1%, 5%, and 10% nicotine sulfate, respectively. At 1% and 5% nicotine sulfate, 20% and 100% of chicks were malformed, respectively (Fig. 9). A no effect level of d-tubocurarine chloride on chick lethality was observed at 2% but 14%, 75%, 71%, and 100% of chicks died at 3%, 4%, 5%, and 10% d-tubocurarine chloride, respectively. Malformations were observed at all concentrations of d-tubocurarine chloride tested with 20%, 83%, and 100% of chicks affected at 2%, 3%, and 4%, respectively (Fig. 10). A no effect level for trimethaphan camsylate lethality was observed at 1.2% with 25%, 71%, and 100% of chicks dying at 1.8%, 2.4%, and 5% trimethaphan camsylate, respectively (Fig. 11).

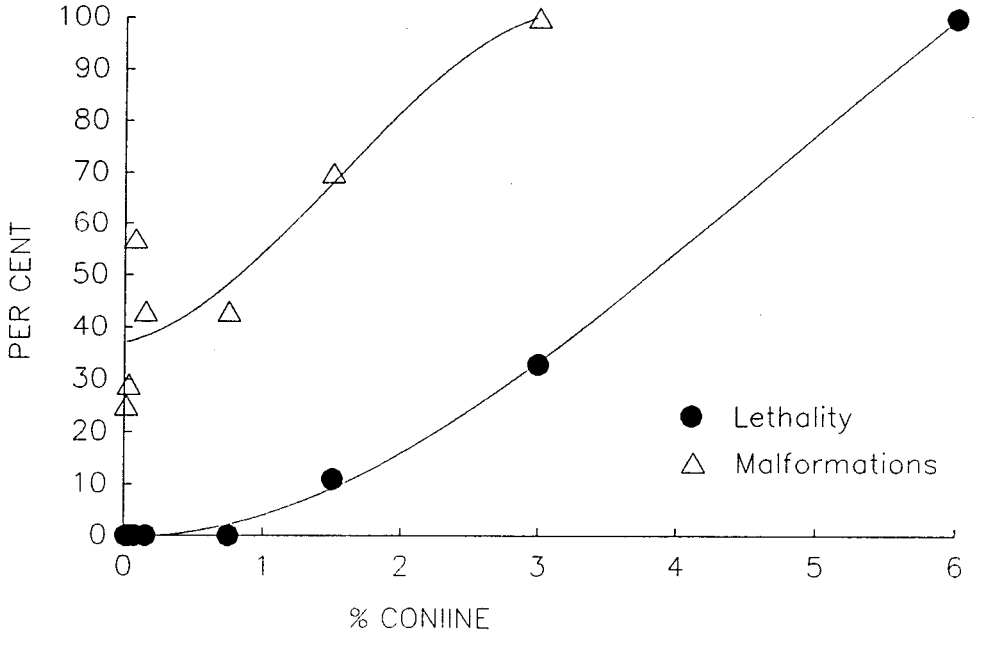


Fig. 8: Lethality and malformations induced in the chick on d17 after coniine treatment on days 6, 8, and 10.

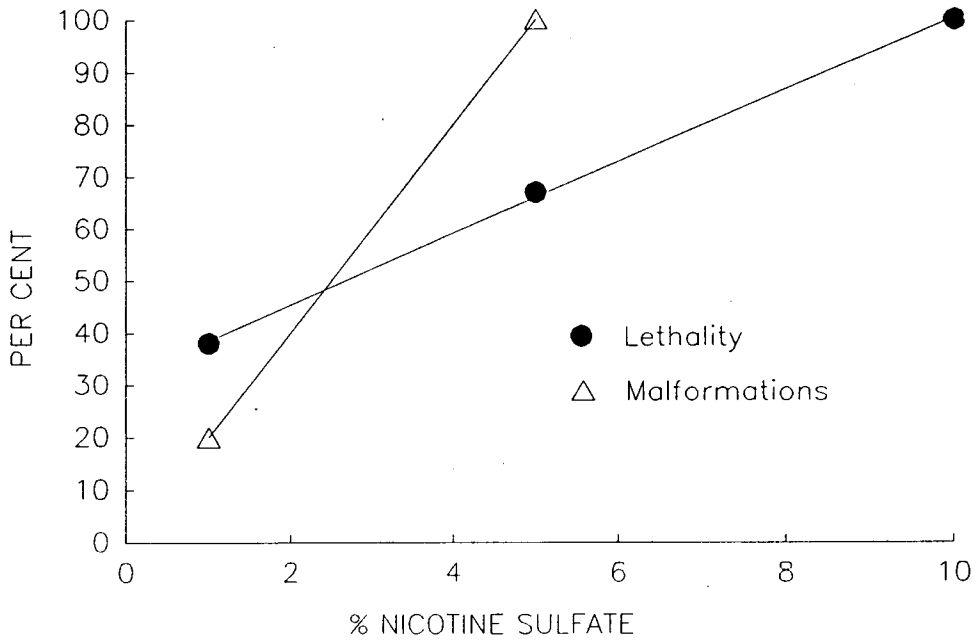


Fig. 9: Lethality and malformations induced in the chick on d17 after nicotine sulfate treatment on days 6, 8, and 10.

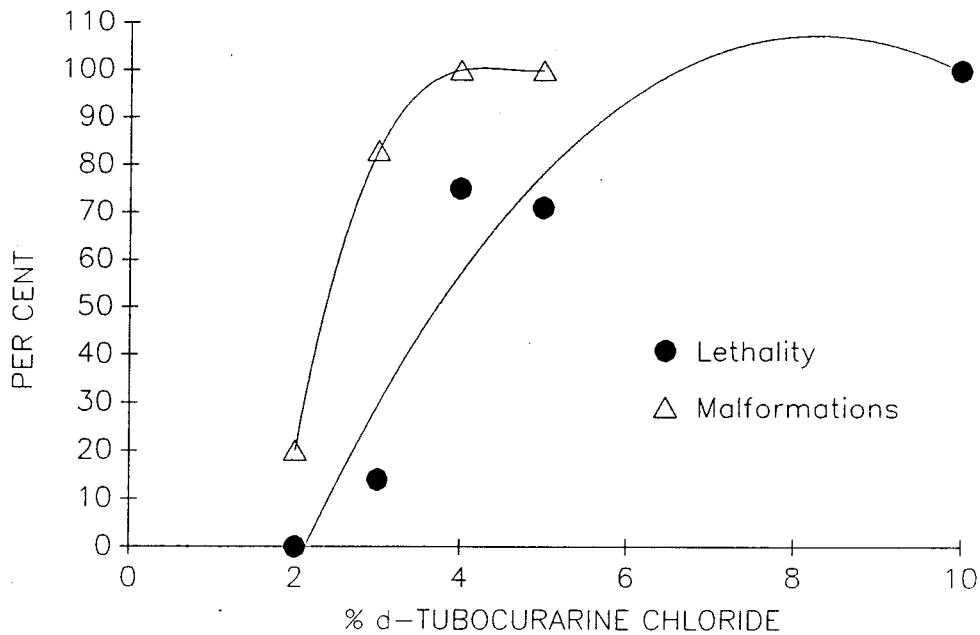


Fig. 10: Lethality and malformations induced in the chick on d17 after d-tubocurarine chloride treatment on days 6, 8, and 10.

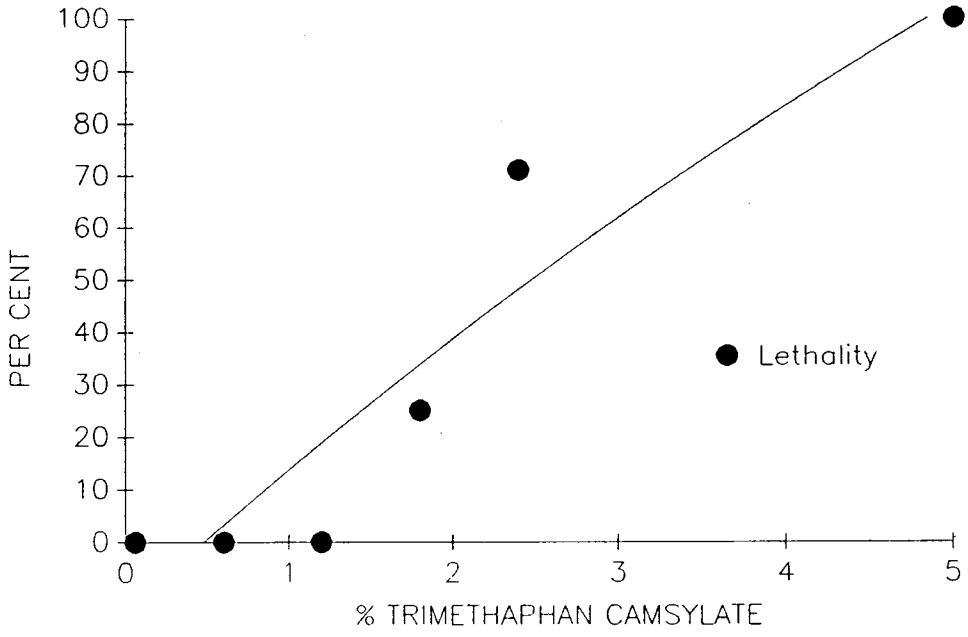


Fig. 11: Lethality induced in the chick on d17 after trimethaphan camsylate treatment on days 6, 8, and 10.