Anesthesiology
76:999–1003, 1992

Developmental Toxicity of Nondepolarizing Muscle Relaxants in Cultured Rat Embryos

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Evidence of developmental toxicity of clinically used nondepolarizing muscle relaxants was sought in rat embryos grown in culture. Embryos were explanted at 8 AM on day 9 of gestation (presomite stage, plug day = day 0), and were cultured in rotating bottles with medium containing various concentrations of *d*-tubocurarine, pancuronium, atracurium, and vecuronium. At 10 AM on day 11 of gestation (forelimb bud stage), culture was terminated and embryos were examined for general morphology. Treatment with tested agents resulted in dose-dependent developmental toxicity; namely, growth retardation seen as decreased crown–rump length, decreased number of somite pairs, and morphologic abnormalities. However, the concentrations that caused toxicity were at least 30-fold greater than serum concentrations clinically achieved in the mother. We conclude that these muscle relaxants have a low potential for causing developmental toxicity during organogenesis. (Key words: Animals: rat. Culture: whole embryo culture. Neuromuscular relaxants: atracurium; *d*-tubocurarine; pancuronium; vecuronium. Pregnancy: toxicity.)

MUSCLE RELAXANTS are difficult to test for reproductive toxicity in standard, *in vivo* animal models because of the respiratory depression that they cause in the mother. It is very difficult to compensate for this effect with artificial ventilation because of technical difficulties and the large number of animals required for most toxicity studies. This problem can be avoided by using *in vitro* models that are not influenced by respiratory muscle relaxation. One such model is the whole-embryo culture system in which direct effects of test agents on embryonic development can be examined during organogenesis, when the embryo is most susceptible to xenobiotics. In the present study, we used this model to examine developmental toxicity of the commonly used nondepolarizing muscle relaxants, *d*-tubocurarine, atracurium, pancuronium and vecuronium.

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Received from the Department of Anesthesia, Stanford University School of Medicine, Stanford, California and Anesthesiology Service, Palo Alto Veterans Affairs Medical Center, Palo Alto, California. Accepted for publication February 9, 1992. Supported by the Veterans Administration and the Anesthesia/Pharmacology Research Foundation.

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Materials and Methods

Male and female Sprague-Dawley rats were purchased from the breeder (Hilltop Laboratory Animals, Scottdale, PA), and were provided food and water *ad libitum* and artificial lighting between 6 AM and 6 PM. Timed-pregnant rats were obtained by breeding the rats for 2 h between 8 AM and 10 AM.[1] The presence of a copulatory plug was sought immediately after mating, and the day it was found was defined as day 0 of gestation. Care of the rats was in accordance with institutional guidelines, and the experimental protocol was approved by the institutional animal care committee before the study was begun.

The procedures for explantation and culture of embryos were originally established by New.[2] Briefly, at 8 AM on day 9 of gestation (presomite stage), the uterus was excised from a halothane-anesthetized rat and individual implantation sites were harvested into a sterile Petri dish containing Hank's balanced salt solution. The decidua was then dissected from the egg cylinder using a dissecting microscope. Reichert's membrane (parietal yolk sac) was removed from the egg cylinder starting from the opposite side to the embryonic disc after the ectoplacental cone and roof of the ectoplacental cavity had been excised.[3]

Three to five embryos were placed in a 60-ml glass culture bottle that contained 1.5 ml per embryo of culture medium consisting of 30% pregnant rat serum, 50% male rat serum, and 20% Hank's balanced salt solution. Serum was obtained from blood that had been centrifuged immediately after collection and heat-inactivated for 30 min at 56° C. The nondepolarizing muscle relaxants *d*-tubocurarine chloride (Eli Lilly and Co., Indianapolis, IN), atracurium besylate (Burroughs Wellcome, Co., Research Triangle Park, NC), pancuronium bromide (Organon, Inc., West Orange, NJ), and vecuronium bromide (Organon, Inc., West Orange, NJ) were added individually to the culture medium as less than 3% of the total volume to achieve the concentrations shown in table 1. All of these commercial preparations except for *d*-tubocurarine chloride contain 0.9–1.0% benzyl alcohol. Bottles were

TABLE 1. Outcome for Embryos Cultured from the Presomite Stage (8 AM on Day 9 of Gestation) to the Forelimb Bud Stage (10 AM on Day 11 of Gestation) in the Presence of Nondepolarizing Muscle Relaxants

Concentration ($\mu g \cdot ml^{-1}$)	Number of Embryos Studied	Number of Malformed Embryos (%)	Crown–Rump Length (mm, mean ± SD)	Number of Somite Pairs (mean ± SD)	Number of Embryos with Situs Inversus (%)
Control	107	0 (0.0)	3.45 ± 0.19	24.8 ± 2.4	9 (8.4)
d-Tubocurarine					
3	20	0 (0.0)	3.43 ± 0.21	24.3 ± 1.3	0 (0.0)
30	34	0 (0.0)	3.35 ± 0.20*	24.4 ± 1.3	3 (8.8)
60	25	1 (4.0)	3.19 ± 0.17*	23.4 ± 1.0*	2 (8.0)
90	25	9 (36.0)*	2.82 ± 0.19*	22.1 ± 1.9*	0 (0.0)
120	25	17 (68.0)*	2.77 ± 0.19*	22.0 ± 2.1*	3 (12.0)
150	25	25 (100.0)*	2.59 ± 0.18*	19.8 ± 2.2*	0 (0.0)
Atracurium					
25	30	0 (0.0)	3.49 ± 0.15	24.9 ± 1.0	0 (0.0)
50	28	0 (0.0)	3.35 ± 0.16*	24.7 ± 0.8	5 (17.9)
75	31	8 (25.8)*	2.94 ± 0.25*	23.0 ± 1.7*	13 (41.9)*
100	35	16 (45.7)*	2.78 ± 0.20*	22.4 ± 1.2*	16 (45.7)*
125	29	25 (86.2)*	2.47 ± 0.32*	20.7 ± 2.1*	12 (41.4)*
Pancuronium					
1	19	0 (0.0)	3.40 ± 0.11	24.8 ± 1.0	2 (10.5)
10	34	0 (0.0)	3.23 ± 0.20*	24.9 ± 1.4	2 (5.9)
20	25	0 (0.0)	3.08 ± 0.12*	23.4 ± 0.8*	2 (8.0)
30	25	13 (52.0)*	2.48 ± 0.27*	20.4 ± 2.9*	2 (8.0)
40	24	22 (91.7)*	2.11 ± 0.36*	16.1 ± 3.5*	2 (8.3)
Vecuronium					
50	26	0 (0.0)	3.42 ± 0.14	24.8 ± 0.7	2 (7.7)
200	23	0 (0.0)	3.02 ± 0.20*	24.1 ± 0.7	0 (0.0)
250	25	0 (0.0)	2.84 ± 0.16*	22.5 ± 1.2*	5 (20.0)
275	25	0 (0.0)	2.81 ± 0.14*	22.6 ± 1.1*	2 (8.0)
300	23	23 (100.0)*	2.61 ± 0.21*	21.7 ± 2.0*	1 (4.3)

* $P < 0.05$ *versus* control.

flushed for 1 min with a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 and then were capped with rubber stopcocks and rotated at 20 rpm in a 37–38° C incubator. Bottles were reflushed with a gas mixture of 20% O_2, 5% CO_2, and 75% N_2 at 12 AM on day 10 and with a gas mixture of 95% O_2 and 5% CO_2 at 7 AM on day 11.

At 10 AM on day 11 of gestation, when embryos were at the forelimb bud stage, culture was terminated, and the embryos were examined for crown–rump length, number of somite pairs, and gross morphology. In addition, the location of the chorioallantoic placenta relative to the embryo (normally right sided), the side of the embryo to which the tail (lower body) flexed (normally right sided), and the curvature of the bulboventricular loop (normally C-shaped, and so called the D-loop) were determined. Embryos with alteration of sidedness of one or more of these structures were designated as having situs inversus.

Nonparametric data from treated and untreated groups were analyzed using a contingency table; chi-square analysis was used as an *a posteriori* test when there were differences. Parametric data were analyzed by one-way analysis of variance; Fisher's protected least significant difference test was used as an *a posteriori* test when differences were found. A P value less than 0.05 was considered significant.

Results

All embryos in the control group developed normally except for a small background incidence (8.4%) of situs inversus (table 1 and fig. 1A). Treatment with all tested agents resulted in dose-dependent developmental toxicity seen as growth retardation (decreased crown–rump length and number of somite pairs; fig. 1B) and morphologic abnormalities (severe head malformations sometimes accompanied by failure of axial rotation; fig. 1C). The growth retardation occurred at somewhat lower concentrations than did the morphologic abnormalities (table 1). This pattern of toxicity, including the types of morphologic abnormalities observed, is seen with high doses of many drugs and is considered to be nonspecific. Atracurium deviated slightly from this pattern in that it caused a high incidence of situs inversus at concentrations of 75 $\mu g \cdot ml^{-1}$ and greater (table 1), in addition to the abnormalities mentioned above.

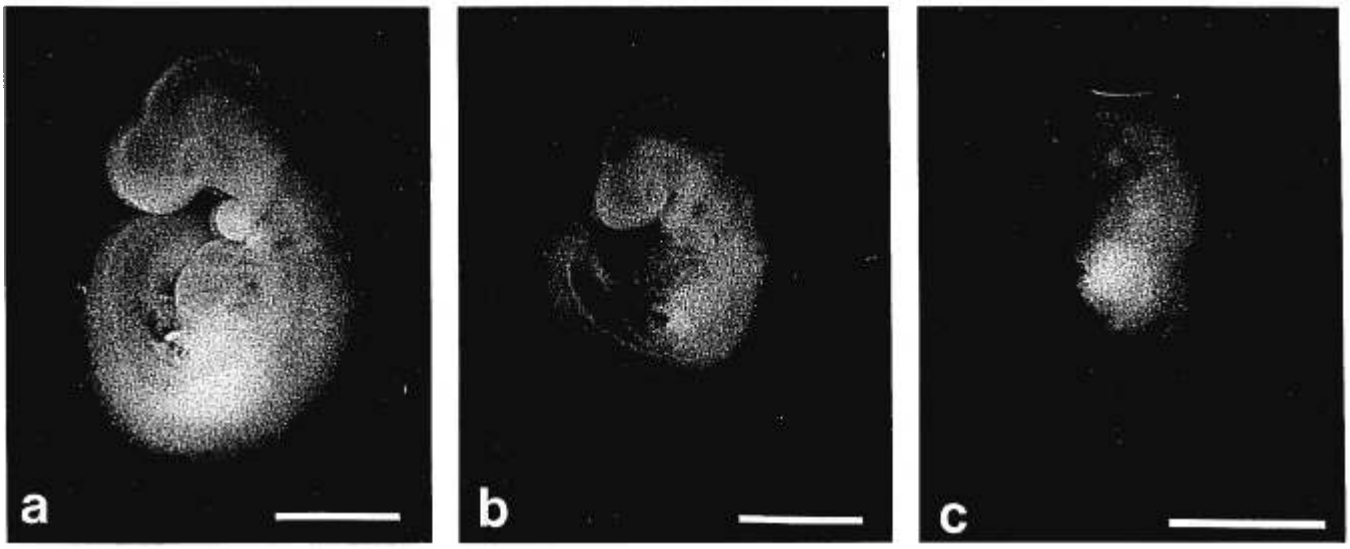

FIG. 1. Embryos at the forelimb bud stage after 49 h of culture from the presomite stage (bar = 1 mm). *A:* Normal embryo with 25 somite pairs. *B:* Embryo treated with 50 $\mu g \cdot ml^{-1}$ atracurium showing growth retardation, *i.e.*, smaller crown–rump length and fewer somite pairs, but no significant morphologic abnormalities. *C:* Embryo treated with 100 $\mu g \cdot ml^{-1}$ atracurium showing severe growth retardation and morphologic abnormalities. Axial rotation has not occurred, and the head is small and severely malformed. These morphologic abnormalities were seen with all tested muscle relaxants at high concentrations.

Discussion

Results from the present study indicate that the commonly used nondepolarizing muscle relaxants *d*-tubocurarine, atracurium, pancuronium, and vecuronium produce developmental toxicity in rat embryos cultured during organogenesis. However, the lowest concentrations of relaxants that caused any adverse effects were at least 30-fold greater than serum concentrations achieved in humans under normal clinical circumstances. The steady state-plasma concentrations that result in 50% paralysis for each muscle relaxant are as follows: 0.2–0.6 $\mu g \cdot ml^{-1}$ for *d*-tubocurarine[4–6] and atracurium,[7,8] 0.1–0.3 $\mu g \cdot ml^{-1}$ for pancuronium,[9–13] and approximately 0.1 $\mu g \cdot ml^{-1}$ for vecuronium.[10] Serum concentrations of muscle relaxants in the fetus are only about 10–20% of those in the mother.[14–17] Thus, our results indicate a wide margin of safety for these muscle relaxants on embryonic development during organogenesis.

Muscle relaxants are difficult to test for reproductive toxicity in the standard, *in vivo* animal models because of the respiratory depression that they cause. Nevertheless, some investigators have attempted to overcome the problem either by using artificial ventilation or by testing lower dosages than are used clinically. Suzuki *et al.*[18] administered 8 $\mu g \cdot kg^{-1}$ pancuronium intravenously to pregnant rabbits on days 8 and 16 of gestation and observed no teratogenic effects. Jacobs[19] administered 0.6 and 5 $mg \cdot kg^{-1}$ *d*-tubocurarine intramuscularly to pregnant mice on day 13.5 of gestation. Mice receiving 5 $mg \cdot kg^{-1}$ of *d*-tubocurarine had to be artificially ventilated because of apnea. The investigator aimed to study the effects of *d*-tubocurarine on palate formation but found no abnormalities.[19] Finally, Skarpa *et al.*[20] administered subcutaneously 0.15 $mg \cdot kg^{-1}$ atracurium daily or 0.10 $mg \cdot kg^{-1}$ twice daily (maximum tolerated doses) to pregnant rabbits on days 6 and 18 of gestation; they also found no teratogenic effects. These negative results are consistent with our own findings in the same concentration range.

Although there is no evidence that muscle relaxants produce adverse reproductive effects during organogenesis, there is some evidence that they do so later in gestation when they cannot be tested using the whole-embryo culture system. For example, prolonged disruption of muscle activity induced by various cholinergic agents,[21–25] including muscle relaxants,[24,26] causes axial deformities, peripheral skeletal malformations, and limb deformities in the chick. These effects are seldom seen in experimental mammals,[27–29] possibly because the placental barrier acts to reduce the concentrations of muscle relaxants presented to the fetus.[24] One would assume that, likewise, they would be seen in humans only under exceptional circumstances. In fact, we are aware of only one such case, an infant born with arthrogryposis (persistent flexure or contracture of a joint) to a mother who was treated with *d*-tubocurarine for tetanus for 19 days start-

ing about the 55th day of gestation.[30] The author speculated that drug-induced immobilization of the fetus by *d*-tubocurarine at the time of or shortly after the development of the joint cavities was the most probable cause. However, this is uncertain because the patient suffered several episodes of severe hypoxia, bronchopneumonia, and myocarditis and was treated with various other drugs including diazepam, chlorpromazine, digoxin, hydrochlorothiazide, and antibiotics.

A noteworthy finding in the present study in light of our other work on body asymmetry[31–33] was that atracurium caused a high incidence of situs inversus at concentrations equal to or greater than 75 $\mu g \cdot ml^{-1}$. Atracurium is known to undergo degradation at physiologic temperature and *p*H by a self-destroying mechanism (Hoffmann elimination) that produces laudanosine and a quaternary monoacrylate as metabolites.[34,35] Although we did not measure these metabolites, we would expect them to be produced by Hoffman elimination at the same rate *in vitro* as *in vivo* because physiologic temperature and *p*H were maintained throughout culture. Laudanosine increases the release of norepinephrine from the right atrium of the guinea pig.[36] We recently reported that phenylephrine, an α_1-adrenergic agonist, causes situs inversus in rat embryos cultured from the presomite stage.[31,32] Thus, a possible explanation for our results is that laudanosine produced in the culture medium or embryo caused situs inversus by releasing norepinephrine from embryonic tissues and stimulating α_1-adrenergic receptors. Again, it should be emphasized that effects were seen only at atracurium and presumably laudanosine concentrations well above those produced clinically.

In conclusion, we have demonstrated that the clinically used nondepolarizing muscle relaxants *d*-tubocurarine, pancuronium, atracurium, and vecuronium produce dose-dependent developmental toxicity on rat embryos cultured from the presomite stage to the forelimb bud stage. However, the concentrations that caused toxicity were much greater than those clinically achieved in humans, suggesting that these muscle relaxants have little potential for disrupting embryonic development during organogenesis under normal clinical conditions.

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