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# Absence of neurotoxicity with medicinal grade terbutaline in the rat model

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ABSTRACT

To evaluate neurological effects of terbutaline, rats were injected with saline, terbutaline (Sigma or American Pharmaceutical Partners (APP$^{TM}$)) at 0.5 mg/kg-d or 10 mg/kg-d between postnatal days (PND) 2–5 or 11–14. Brains collected 24 h after last injection were used to determine corpus-callosum thickness, Purkinje cell and neuronal number in the cerebellum. Ambulation, distance traveled, resting time and time on rotarod were analyzed. Terbutaline (both doses/grades at PND 11–14) decreased corpus-callosum thickness. Ambulation time was significantly decreased in the 10 mg/kg-d (Sigma) and 0.5 mg/kg-d of terbutaline (APP$^{TM}$) (PND 2–5) juvenile-rats and 10 mg/kg-d-Sigma adult-rats, 0.5 mg/kg-d APP$^{TM}$ (PND 11–14) adult-rats. Resting time was increased in both doses of APP$^{TM}$ (PND 2–5) in juvenile-rats, 10 mg/kg-d Sigma adult-rats. 10 mg/kg-d-Sigma (PND 2–5) decreased distance traveled in adult-rats. 0.5 mg/kg-d-Sigma (PND 2–5 and PND 11–14) decreased the time spent on rotarod (30 RPM) in adult-rats. Sigma terbutaline Sigma had 2× as much free base compared to APP$^{TM}$. In conclusion, APP$^{TM}$ terbutaline did not have a deleterious effect on the developing rat brain.



## 1. Introduction

Preterm birth remains the single most common and costly obstetric complication in the United States, accounting for the majority of neonatal mortality and morbidity. In addition, preterm delivery is linked to the majority of childhood neurobehavioral problems [1]. According to recent statistics, preterm birth has risen from 8.2% in the 1980s to just over 10% in the 1990s and over 12% in 2003 [2]. As a large portion of preterm births are found in women who develop preterm labor, it is likely that such patients are treated with tocolytic drugs in an effort to prolong the gestation and permit corticosteroid administration [3].

In addition to the desired $\beta_2$ effects of blocking uterine contractions and bronchiole relaxation, stimulation of the $\beta_1$ adrenergic receptors accounts for maternal β-agonist side effects. These include tachycardia, anxiety, nervousness, insomnia and, in some cases, angina and pulmonary edema. The vast majority of serious maternal adverse effects have been reported using intravenous β-agonist dosing for acute preterm labor treatment. Infants delivering just after high dose intravenous β-agonist tocolytic therapy for preterm labor have demonstrated short term renal, pulmonary, and cardiovascular side effects [4]. No consistent, adverse, long-term effects in children exposed to intravenous β-agonists have been noted when stratified for the effects of prematurity and long term neurologic studies following low dose β agonists have not demonstrated any adverse consequences in children [5–7].

A series of studies by the Slotkin laboratory using terbutaline, obtained from Sigma Chemical Company, have found that perinatal administration of terbutaline causes profound changes in behavior, neurochemistry and morphology in the developing rat as well as changes in brain neurochemistry and activation of fetal β-adrenoreceptors on developing neurons and glia [8,9]. Therefore the objective of this study was to determine if a pharmaceutical grade of terbutaline was also capable of changing basic neurobehavior among rats, we examined the effects of different preparations of terbutaline using the same administration schedule and doses of terbutaline as the Slotkin laboratory [8,9]. We used the combination of histology and neurobehavioral testing to determine if this was also the case for American Pharmaceutical Partners$^{TM}$ terbutaline, a tocolytic agent in clinical practice.

## 2. Materials and methods

The Institutional Animal Care and Use Committee approved the animal procedures in this study at the University of Mississippi Medical Center. Fifty-three timed pregnant, Sprague–Dawley rats were obtained (day 15 of gestation) from Harlan Laboratories. Pregnant rats were placed in separate cages under a 12/12-h light/dark cycle and allowed to acclimate and deliver without interference. Water and food were available *ad libitum*.

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### 2.1. Drug administration

Upon delivery, the number of pups for each litter was recorded and remained unadjusted, and the pups were maintained with dams until the beginning of the experimental period. The litters were assigned to one of the five regimens: (1) saline, (2) 0.5 mg/kg-d terbutaline in the hemisulfate form (Sigma Chemical Company, St. Louis, MO) terbutaline, (3) 10 mg/kg-d of terbutaline obtained from Sigma [10], (4) 0.5 mg/kg-d, pharmaceutical grade (American Pharmaceutical Partners, Inc. (APP$^{TM}$), Schaumburg, IL) terbutaline and (5) 10 mg/kg-d pharmaceutical terbutaline. Terbutaline doses used were based on previous studies by the Slotkin laboratory indicating developmental neurotoxicity and β-adrenoreceptor stimulation [8,9,11]. Saline was used as a vehicle to obtain the different concentrations of terbutaline. Previous animal studies by Slotkin's group also utilized saline as a vehicle [8,9].

All animals were administered saline or terbutaline between PND 2–5 or PND 11–14 [9,10]. Single subcutaneous doses were administered daily to all groups during the appropriate dosing period using a 25-gauge needle inserted into the subcutaneous tissue of the lower hind legs. Equivalent volumes of saline were given at both time periods to control animals and all injections were given at the same time each day. All animals remained with their dams until PND 30 at which time they were weaned and placed into new cages immediately following behavioral testing at PND 30. As both neonatal handling and prenatal stress have been shown to increase locomotion in rats, especially Sprague–Dawley rats, we elected to wean rats after their first exposure to the locomotor activity chamber [12,13].

### 2.2. Histological examination

Histological examination of brains was performed in accordance (brain regions, and parameters studied) to previous studies by the Slotkin laboratory [8]. Twenty-four hours after the last terbutaline injection, PND 6 or PND 15, 2–3 pups per litter were sacrificed and their brains were harvested. There was equal sex distribution between the treatment groups at the time of sacrifice. After decapitation brains were extracted from the skull, immediately weighed followed by immediate placement into 10% formalin buffered solution. Paraffin embedded sections were prepared, cut to 8 μm-thickness, mounted and stained with hematoxylin and eosin (H&E). For each animal, two slides containing 3 adjacent brain sections were examined. H&E sections were submitted for pathological review and examined microscopically for alterations in histopathology including thickness of the corpus callosum, number of Purkinje cells in the cerebellum and the thickness of the external granular cerebellar layer. The pathologist (JWA) was blind to the treatment group.

### 2.3. Motor and balance assessment

Perinatal terbutaline treatment in rats was reported to increase hyperactivity in the open field and decrease the number of Purkinje cells in the cerebellum [8,9]. Animals with decreases in Purkinje cell density or function often exhibit deficits in motor coordination and balance [14,15]. In order to determine if either terbutaline obtained from Sigma or pharmaceutical grade terbutaline could lead to impairment in these behaviors the remaining pups were subjected to basic neurobehavioral testing (motor coordination, balance, locomotion and exploratory behavior) in order to determine long-lasting or delayed effects of perinatal drug injections on motor or neurobehavioral development [16,17]. On the day of behavioral testing, rats were brought to a sound-attenuated testing room and allowed to acclimate for 1 h. Animals from each experimental group underwent behavioral testing at two different ages, PND 30–35 (juvenile) and PND 50–55 (adulthood). All testing was done at the same time of day for each time period.

To measure locomotor activity, rats were placed individually into a monitoring system (Opto-Varimex-Minor System, Columbus Instruments, Columbus, OH) under low lights for 30 min to test locomotion. A computer system recorded horizontal and vertical activity as determined by the breaking of infrared detectors and sensors. Data were analyzed for distance traveled, resting time and ambulatory time.

To measure motor coordination and balance, rats were placed on a rotating cylinder (Economy Rotamex, Columbus Instruments, Columbus, OH) at a slow rotational speed (15 RPM) for 3 min. At the end of 3 min the speed was increased to 30 RPM for an additional 3 min. The first 30 s in each time period was used as an acclimation period, and any falls were not scored. The time spent on the rota-rod was the main outcome.

### 2.4. Terbutaline composition by mass spectrometry

Mass spectrometry was used to ascertain if a difference exists in either of the molecular ion spectra of the two terbutaline preparations (Sigma vs APP$^{TM}$) or their fragmentation patterns. Terbutaline obtained from Sigma was dissolved in water at a concentration of 1 μg/mL and medicinal grade terbutaline diluted to 1 μg/mL, were extracted with 1 mL of deionized water:methanol:methylene chloride (1:1:1) by mixing for 10 min at room temperature. After centrifugation at 1400 × *g* for 5 min, the organic phase was transferred to recovery vials and evaporated to dryness under a stream of nitrogen gas. The residue was re-dissolved in 1 mL of methanol:deionized water (1:1) with 1% acetic acid. This step was undertaken to purify the free base from the salt to improve ionization efficiency. All solvents used were analytical grade and obtained from Fisher Scientific.

Samples were analyzed in an Electrospray Ionization – Triple Quadrupole mass spectrometer (API 365 – Applied Biosystems). Analytes were introduced by direct infusion into the mass spectrometer at a flow rate of 10 μL/min. Nitrogen was used as the nebulizer gas at 40-psi pressure. Data were collected by positive ionization in MS mode and the origin of the fragment ions confirmed by MS/MS analysis. The source was optimized and the orifice and ring voltages were set at 51 and 210 V.

### 2.5. Statistical analysis

One-way analysis of variance (ANOVA) or Student's *t*-test with Bonferroni's correction was used to determine statistical significance for the thickness of the corpus callosum and external cerebellar granular cell layers, as well as the number of Purkinje cells within each treatment group. Locomotor and rota-rod data were analyzed using a mixed model which allowed us to incorporate within-rat correlations and also to adjust for dams per treatment group and litters using variance components models. An ANOVA incorporating random effects for dams and litters with drug treatment (per injection regimen) serving as the repeated measure was used to analyze behavioral data. Tukey–Kramer multiple comparisons tests were used for post hoc analysis. Data are represented as mean ± standard error mean. $p$ values <0.05 were considered significant.

## 3. Results

In agreement with previous reports [8,9] neither the 0.5 mg/kg-d or 10 mg/kg-d terbutaline (Sigma and APP$^{TM}$) regimen had a significant effect on brain ($p = 0.853$) or pup weight ($p = 0.712$). In addition, none of the pups died following injections and body weights were similar among groups regardless of treatment type (data not shown).

### 3.1. Terbutaline alters corpus callosum thickness based on age at exposure

In the PND 2–5 injection group, terbutaline (neither Sigma or APP$^{TM}$ at either dose) did not significantly increase corpus callosum thickness compared to animals in the saline group ($p = 0.626$, Table 1) when analyzed at PND 6. Terbutaline (neither Sigma nor APP$^{TM}$ at either dose) injection between PND 2–5 did not significantly decrease Purkinje cell density ($p = 0.386$, Table 1) or significantly decrease neurons in the cerebellar external granular cell layer ($p = 0.133$, Table 1) when analyzed at PND 6.

In the PND 11–14 injected group, terbutaline injection, both Sigma and APP$^{TM}$ at 0.5 mg/kg-d and 10 mg/kg-d, significantly decreased corpus callosum thickness compared to animals in the saline group ($p = 0.003$, Table 1, Fig. 1) when analyzed at PND 15. Terbutaline injection (neither Sigma nor APP$^{TM}$ at either dose) between PND 11–14 did not significantly decrease Purkinje cell density ($p = 0.445$, Table 1) or significantly decrease neurons in the cerebellar external granular cell layer ($p = 0.161$, Table 1) when analyzed at PND 15.

### 3.2. The effect of terbutaline on motor skills

#### 3.2.1. Ambulation time and behavior

As there were no statistical differences in ambulation time between male and female rodents at either the juvenile (PND 2–5 regimen, $p = 0.097$; PND 11–14 regimen, $p = 0.315$) or adult time (PND 2–5 regimen, $p = 0.751$; PND 11–14 regimen, $p = 0.917$) period among either the 0.5 mg/kg-d or 10 mg/kg-d dose, males and females are reported together (data not shown).Juvenile animals in the PND 2–5 regimen that received 0.5 mg/kg-d of terbutaline from APP$^{TM}$ or the 10 mg/kg-d dose of terbutaline obtained from Sigma had significantly decreased ambulation times ($p < 0.05$), in comparison to the saline group (Fig. 2A). There were no differences in ambulation time seen in juvenile animals in the PND 11–14 regimen regardless of terbutaline treatment ($p = 0.089$, Fig. 2B).

Adult animals in the PND 2–5 regimen injected with 10 mg/kg-d of terbutaline obtained from Sigma had significantly decreased ambulation times ($p < 0.05$) when compared to the saline and