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Thrombocytosis in the Offspring of Female Mice Receiving DL-Methadone (40861)[1]

Y. BURSTEIN, R. W. GRADY, M. J. KREEK, A. R. RAUSEN, AND C. M. PETERSON

Rockefeller University, New York, New York 10021 and Beth Israel Medical Center, New York, New York 10010

Abstract. Subcutaneous methadone administration (2.5 mg/kg/day) to female mice beginning 2 weeks prior to conception and continuing through weaning resulted in a significant increase in the number of circulating platelets (1650×10^3 vs 1150×10^3/mm^3), an increase in marrow megakaryocytes per high power field (12.22 vs 9.59) and an increase in the percentage of young megakaryocytes (34.5 vs 28.4) in the offspring but not in the mothers. The effect was maximal 14 days after birth and diminished by 28 days. Injection of 5 mg/kg/day resulted in similar findings but the changes persisted for 35 days. These findings are consistent with those noted in the infants of human polydrug abusers and suggest that methadone plays a role in similar abnormalities in humans.

Methadone was first synthesized 40 years ago. It has several pharmacological properties which include analgesic activity, suppression of withdrawal symptoms in physically dependent addicts, sedation, respiratory depression, and depressant effects upon smooth muscle and the cardiovascular system (1). In addition, it is used for detoxification of pregnant women taking various addictive drugs (2–5). Numerous side effects have been observed in patients concomitant with methadone use (6–10). Decreased levels of estrogen, follicular stimulating hormone (FSH), and luteinizing hormone (LH) have been reported (11–13) while increased levels of thyroxine (T_4) and triiodothyronine (T_3) have also been observed (14). Patients on methadone maintenance exhibit increased serum globulin and albumin levels (10–14), and chronic methadone administration to rabbits resulted in sustained increases in albumin synthesis (15).

A variety of problems have been observed in infants born to mothers maintained on methadone. These include low birth weight, narcotic withdrawal symptoms, seizure activity, and sudden infant death (16, 17). The overall incidence of morbidity in infants of narcotic dependent mothers is higher than that of controls and include asphyxia, intrauterine growth retardation, prematurity, hyperbilirubinemia, sepsis, transient tachypnea, and hypocalcemia (18). Thrombocytosis and increased platelet hypersensitivity as measured by the platelet aggregate ratio were found in infants born to mothers using addictive agents (19). These thirty-three prospectively studied infants developed a significant thrombocytosis by the second week of life and platelet counts remained elevated for up to 16 weeks. These findings might contribute to the pathogenesis of the focal infarcts, subarachnoid, and germinal plate hemorrhages (20), seizure activity, and sudden infant death (16, 17) reported in newborn infants of mothers using addictive drugs. In the studies noted, the degree to which the mothers were using alcohol or other drugs was either not known or difficult to determine; thus it is not known whether the clinical problems observed were due to methadone, other agents, or combinations thereof.

In an attempt to determine the role methadone plays in causing the hematological abnormalities described, we designed a study utilizing female Balb/c mice to determine whether methadone administra-

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tion during pregnancy resulted in hematological changes in the offspring. The time course of abnormalities was noted as was the morbidity and mortality associated with these changes.

Methods. The Balb/c mice used in the study were obtained from Charles River Laboratories, Inc. (Wilmington, Mass.) or from the Texas Inbred Mice Company (Houston, Tex.). DL-Methadone·HCl was obtained from Mallincrodt Chemical Works (St. Louis, Mo.). A stock solution of the hydrochloride was prepared in physiological saline. Thirty-milliliter aliquots of this stock solution containing 0.25 mg of the free base/ml were placed in serum vials and stored at 4° until used.

Female mice were given daily injections of the hydrochloride corresponding to either 2.5 or 5.0 mg/kg of the free base for the duration of the study. The injections were given subcutaneously in the interscapular region. Two groups of controls were studied. One received an equivalent amount of normal saline and the second received no injections. On the 14th day of the study, a normal uninjected male was placed in a cage containing four to five females and allowed to remain there for 10 days. Those animals which became pregnant were placed in separate cages prior to delivery. The pups were counted and weighed within 48 hr of parturition.

Because of the number of pups and the time involved in determining platelet counts, platelet and white cell counts were performed on Days 1–3, 6–8, 13–15, 20–22, 27–29, and 34–36, and the counts averaged for each period. The number of platelets was determined by the procedure of Brecher and Cronkite (21), modified to utilize 5-μl capillary pipets (Becton-Dickenson, Rutherford, N.J.), ammonium oxalate diluent containing Sorensen's phosphate buffer, and thimerosal as an antibacterial agent. All blood samples were obtained from the tail after exposing the mice to infrared light for 2 to 3 min. The accuracy of this sampling technique was verified by direct comparison with the values determined on blood samples obtained via cardiac puncture.

The number of megakaryocytes in bone marrow was determined in sections of femurs obtained on Days 14 and 28 of life. Whole femurs were fixed in 10% buffered formalin for 48 hr and decalcified in Omega decal solution for 8 hr. Sections were cut 4 to 5 μ thick and subsequently stained with hematoxylin and eosin. Three mice from the group receiving 2.5 mg/kg of methadone and three controls were chosen at random and studied. Marrow megakaryocyte counts were performed by two observers who were "blinded" as to the source of the sections. One section from each of the two femurs from each mouse was counted by both observers. Three high power fields (40×), one diaphysial and two epiphysial, were counted from each section. Both the total number of megakaryocytes and the number with less than five nuclear lobes were recorded. The latter were considered to be young cells (22). The mean number of megakaryocytes/hpf together with the percentage of young cells was calculated for each group of mice examined. Statistical analyses were performed by standard methods utilizing bivariant analysis of variance (23); and methadone levels were determined by gas–liquid chromatography (24, 25).

Results. Methadone was well tolerated by the mice; none of the mothers receiving either dose of methadone were obtunded nor demonstrably affected by drug administration. Maximum drug levels in plasma following a subcutaneous injection of 2.5 mg/kg of methadone (N = 3 mice) were found at 30 min with a value of 0.042 μg/ml ($\pm$0.004 SEM) with a half-life ($T_{1/2}$) of 112 min and values of 0.033 $\pm$ 0.008 at 60 min and 0.029 $\pm$ 0.003 at 90 min. Intrauterine demise was documented in two cases. The mothers having 10 and 11 pups, respectively, had received 2.5 mg/kg per day of methadone. Perinatal death occurred in two pups on Days 1 and 2, but no deaths occurred during periods of documented thrombocytosis.

Abnormal hematological findings were limited to the platelet system. No differences in white cell counts or hematocrits were noted between the various groups. At birth there was also no significant difference in platelet counts among the four

groups of pups studied, the means ranging from 510 to 620 × 10^3/mm^3. In all groups there was an increase in the platelet count with age. Adult values were reached by 14 days in both control groups. A significant increase in platelet counts over those of controls was observed in the pups of methadone-treated mothers. There was no difference between saline-injected and noninjected control animals. The difference in platelet counts was statistically significant by Days 13 to 15 (Fig. 1). Although there was no significant difference in the maximum platelet counts attained, the offspring of mothers receiving 5 mg/kg per day of methadone required longer to return to normal than those born to mothers receiving 2.5 mg/kg per day. Platelet counts performed on the mothers before, during, and after treatment with methadone showed no significant differences either within or between groups.

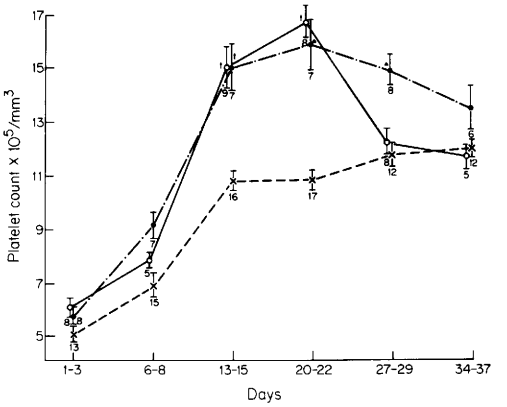

FIG. 1. Comparison of platelet counts in neonates born to normal and methadone-treated mice. Three groups of mice were studied, those born to mothers receiving subcutaneous injections of 2.5 (○) and 5.0 mg/kg per day (●) of methadone together with the offspring of controls (x). Bars indicate the SEM. Numbers below the bar indicate the number of animals tested at each time interval. Symbols above the bar indicate the levels of significance relative to the controls (↑ $P < 0.001$; ▲ $P < 0.005$).

Table I gives the megakaryocyte counts determined in bone marrow specimens obtained from mice whose mothers received methadone (2.5 mg/kg per day). The offspring of drug-treated mothers had a greater number of megakaryocytes/hpf than controls on Day 14 postpartum but this difference was obviated by Day 28 concomitant with the decrease in the circulating platelet count. Not only was the number of megakaryocytes increased, the number and percentage of younger cells (less than five nuclear lobes) were also increased in the pups of methadone-treated mothers (Table II). Again, the increase was no longer detectable by the 28th day of life.

Discussion. The offspring of methadone-treated female mice exhibited a significant increase in circulating platelet counts. This increase occurred concomitantly with an increase in the total number of megakaryocytes as well as an increase in the number of less than five-lobed (probably young) megakaryocytes in the bone marrow. The changes in the platelet counts which occurred in mice are similar to the changes observed in infants whose mothers were polydrug users during pregnancy (19). Still, differences do exist between the human and the animal studies. The platelet count of normal newborn mice increases with age and reaches adult values by 10 to 14 days, while human infants are born with

TABLE I. MEGAKARYOCYTES IN THE OFFSPRING OF METHADONE-TREATED MICE

Group	Days of life	No. of animals	Megakaryocytes/hpf[a]	P
Injected controls	14	3	9.59 ± 0.39[b]	<0.01
Methadone treated[c]	14	3	12.22 ± 0.61	
Injected controls	28	3	11.29 ± 0.94	NS[d]
Methadone treated	28	3	10.46 ± 0.69	

[a] Megakaryocytes per high power field.
[b] SEM.
[c] Methadone was administered to the mothers s.c. at a dose of 2.5 mg/kg per day.
[d] Not significant.

TABLE II. NUMBER AND PERCENTAGE OF YOUNG MEGAKARYOCYTES

Group	Days of Life	No. of Animals	No./hpf[a]	%/hpf[b]	P
Injected controls	14	3	2.72 ± 0.26[c]	28.4	<0.01
Methadone treated[d]	14	3	4.21 ± 0.41	34.5	
Injected controls	28	3	3.60 ± 0.52	31.9	NS[e]
Methadone treated	28	3	3.34 ± 0.50	31.9	

[a] Number of young (less than five nuclear lobes) megakaryocytes per high power field.
[b] Percentage of young megakaryocytes per high power field.
[c] SEM.
[d] Methadone was administered to the mothers s.c. at a dose of 2.5 mg/kg per day.
[e] Not significant.

normal adult platelet counts (19). Further, the thrombocytosis observed in the offspring of methadone-treated mice lasts 3 to 4 weeks, while in human infants the thrombocytosis was observed to last for over 16 weeks (19). Of note is the fact that thrombocytosis does not occur in adult humans or mice maintained on methadone. Thrombocytosis in adult humans or mice following withdrawal of methadone has not been documented.

A number of factors may have contributed to the findings of the present study. The pups were breast fed and therefore could have received small amounts of methadone via maternal milk. The concentration of methadone in human maternal milk is less than one-tenth that seen in their blood (26). Methadone has been noted to increase the synthesis of certain proteins (10, 14), one of which might contribute to thrombocytosis in the offspring of mothers receiving the drug. The findings in the bone marrow specimens indicating an increase in the total number of megakaryocytes are consistent with the hypothesis that there is an increase in a substance which stimulates platelet production. The selective vulnerability of the infants to thrombocytosis in the absence of an increased platelet count in the mothers remains to be explained. Additional evidence in support of an increased amount of a thrombocyte-stimulating substance is provided by the observation that the urine of the offspring of human polydrug abusers stimulated thrombocytosis to a greater extent than control urine when injected into mice (27).

The mouse provides a useful model for the study of the effect of methadone on the hematological system of newborns whose mothers have received the compound. The documentation of increased platelet counts and increased marrow megakaryocytes in neonates of mice maintained on methadone lends credence to the hypothesis that methadone was responsible for the increased platelet counts noted in the human studies. The mouse system also provides a means whereby the mechanism of these neonatal hematological changes can be further elucidated.

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