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An Exploration of the Role of Hydroxyurea Injection Time in Fetal Growth and Teratogenesis in Rats

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ABSTRACT Pregnant Wistar rats were injected with hydroxyurea (HU) intraperitoneally (IP) at one of several 6-hour intervals on days 9-10.75 to study the role of circadian growth variations in the teratologic response of the fetuses. Two stocks of rats were studied and the results in each compared. The fetal response to HU, as observed at day 21, was not detectably modified by circadian fetal growth variation. No correlations between hour of HU administration and fetal weight, placental weight, resorption, or total malformation rates were found. Cyclic variations in the incidence of hydronephrosis and left umbilical artery was observed, but it was not clear that these were related to maternal light:dark cycles. Differences of response between two stocks of rats included marked variation in the incidence and type of malformations and variations in the timing of peak incidence for some but not all malformations.

Fetal growth of rats in late gestation shows a circadian rhythm in which rapid growth occurs during the dark phase of the daily cycle and slow growth during the light phase (Barr, '73b). The discovery of this phenomenon raised the question of whether or not circadian growth rhythms occur during organogenesis. If growth rhythms do occur during organogenesis, a corollary question is: Can they be detected by timed teratogen administration and examination of the near-term fetus? A teratogen with rapid onset of action and rapid elimination was sought to test the relations between the time of teratogen administration, fetal growth, and production of malformation. Hydroxyurea (HU) was chosen as an appropriate agent since it is (1) teratogenic in rats (Chaube and Murphy, '66), (2) rapidly acting (Scott et al., '71) and (3) rapidly eliminated (Adamson et al., '65). If cyclic variation in the fetal effects of HU could be demonstrated, it would support the hypothesis that circadian variations in fetal growth occur during early gestation.

MATERIALS AND METHODS

Virgin female Wistar rats (155-275 gm) were caged overnight with males and examined for sperm by vaginal smear. Pregnancy was timed for 6 AM (day 0.0) of the day sperm were found. Two stocks of rats were tested for their responses in parallel experiments because different stocks of the same strain of a species have been shown to respond variably to the same teratogen (Barr, '73a). A-stock rats were from a colony maintained by one of us (A.R.B.) derived from Wistar stock originally from Albino Farms (Red Bank, NJ): B-stock animals were CFN Wistar rats purchased for this study from Carworth (New City, NY).

Pregnant rats were caged individually and allowed Rockland rat diet and tap water ad libitum. They were exposed to light from 6 AM to 6 PM.

Hydroxyurea (HU) (Calbiochem, San Diego, CA) was prepared fresh weekly as a 5% (w/v) solution in sterile saline for injection and stored at 4°C until use. The pregnant animals were injected intraperitoneally (IP) at one of eight 6-hour intervals from day 9.0 (6 AM) to day 10.75 (12 PM). Published reports (Chaube and Murphy, '66) and preliminary experiments showed that progressively higher doses of HU are needed to produce malformation as gestation advances. Therefore, the dose was in-

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TABLE 1. Resorption rates, fetal and placental weights, and malformation rates of offspring of pregnant rats given hydroxyurea on days 9–10 of gestation.

Age treated (days)	HU dose (mg/kg)	Litters (no.)	Resorbed (%)	Fetuses (no.)	Fetal weight mean ± SE (gm)	Placental weight mean ± SE (mg)	Malformed (%)
A-stock rats							
9.00	200	9	10.6	110	4.91 ± 0.06	438 ± 6	71.8
9.25	225	8	11.5	100	5.09 ± 0.04	497 ± 8	95.0
9.50	250	9	9.4	115	5.26 ± 0.05	460 ± 8	71.3
9.75	275	8	4.2	91	5.51 ± 0.05	465 ± 7	64.8
10.00	300	9	7.3	102	5.39 ± 0.04	458 ± 5	67.6
10.25	325	9	7.7	108	5.15 ± 0.04	432 ± 8	62.0
10.50	350	10	11.1	120	5.35 ± 0.05	481 ± 11	62.5
10.75	375	9	8.8	114	5.16 ± 0.05	431 ± 6	41.2
B-stock rats							
9.00	200	11	12.9	128	4.26 ± 0.05	397 ± 5	78.1
9.25	225	10	15.9	122	4.10 ± 0.06	373 ± 5	91.8
9.50	250	10	9.4	116	4.29 ± 0.06	399 ± 6	97.4
9.75	275	10	11.8	127	4.45 ± 0.05	392 ± 6	93.7
10.00	300	9	9.9	100	4.41 ± 0.05	370 ± 6	86.0
10.25	325	10	12.9	122	4.44 ± 0.04	352 ± 5	77.9
10.50	350	10	15.8	128	4.35 ± 0.04	355 ± 5	62.5
10.75	375	8	10.2	97	4.29 ± 0.04	339 ± 5	57.7
Controls	–	27	6.1	340	4.89 ± 0.02	405 ± 3	3.2

creased with gestational age: The base dose was 200 mg HU/kg maternal weight at day 9.0 and this dose was increased by 25 mg/kg for each 6-hour interval beyond day 9.0. Thus, on day 10.75 the dose was 375 mg HU/kg maternal weight.

Control animals, of the B-stock only, received IP injections of sterile saline at various times. Since there was no evidence of deviation of fetal or placental weight by time of saline injection, and no malformations, they were combined as a single control group regardless of time of injection. Controls for the A-stock were not included since the purpose of the study was not to establish the teratogenicity of HU, but only to determine if there was a relation between time of dosage and fetal outcome.

The fetuses were delivered by cesarean section between 9 and 10 AM on day 21. Fetal blood loss was prevented by electocautery of the umbilical cord. The fetuses and placentas were cleaned, blotted free of surface moisture, weighed, and examined for externally evident malformations. After fixation in Bouin's solution, the fetuses were examined for soft tissue malformations by a modification of Wilson's ('65) razor-blade dissection technique.

Mean fetal and placental weights were calculated for each litter and experimental subgroup. Comparisons of mean weights were made by Student's t-test. Resorption rates were calculated on the basis of experimental subgroups, e.g., the percentage of resorptions among the total number of implantations in the subgroup. The incidence of malformations was also calculated on the basis of subgroup totals. Interstock comparisons of resorptions and malformations were made by χ^2 analysis. Dose-dependent effects of placental weight were measured by regression-correlation analysis, least squares method, assuming linearity of regression.

RESULTS

Weights

HU produced marked depression of fetal and placental weights below control values in the B-stock rats (Table 1). Whether or not HU produced growth retardation in the A-stock is unknown since untreated controls were not studied. There were significant differences between the treated stocks in both fetal and placental weights, with the A-stock being a great deal heavier than the B-stock (Table 1). In neither stock was there any evident association between the hour of HU administration and fetal weight on day 21. Fetuses were classified by malformation and reevaluated for variations in weight but no time-dependent associations were found. There was a dose-dependent depression of placental weight in the B-stock (r 0.855, df7, $P < 0.01$) but not in the A-stock (r 0.272, df7, $P > 0.10$).

TABLE 2. Percentage of malformations produced by IP hydroxyurea in two stocks of Wistar rats[1]
(values in boldface indicate 50% or more of treated litters contained affected fetuses)

	A-stock rats								B-stock rats							
Age treated (days):	9.00	9.25	9.50	9.75	10.00	10.25	10.50	10.75	9.00	9.25	9.50	9.75	10.00	10.25	10.50	10.75
Number of fetuses:	110	100	115	91	102	108	120	114	128	122	116	127	100	122	128	97
Anopthalmia Microphthalmia	**55.5**	**87.0**	**51.3***	**53.8***	**35.3***	5.6*	1.7*	–*	**66.4**	**91.0**	**94.0***	**90.6***	**82.0***	**47.5***	**18.7***	**6.2***
Hydrocephaly	**13.6***	**30.0**	**18.3***	6.6*	1.0*	1.9	3.3	–	**25.0***	**23.0**	**34.5***	**32.3***	**14.0***	6.6	4.7	3.1
Encephalocele	3.6	**7.0**	3.5	–	–	–	–	–	**10.2**	**13.1**	**9.5**	**4.7**	–	–	–	–
Exencephaly	**8.2**	4.0*	2.6*	–	–	–	–	–	**8.6**	**32.0***	**25.0***	4.7	–	–	–	–
Ear dysplasia.	6.4	3.0*	1.7*	–*	–	0.9	–	–	8.6	**16.4***	**29.3***	**12.6***	4.0	4.1	1.6	–
Micrognathia	**7.3**	**5.0**	2.6	1.1	–	–	–	–	7.8	**7.4**	4.3	1.6	–	0.8	–	–
Maxillary hypoplasia	**11.8**	**12.0***	3.5*	–*	–	–	–	–	**8.6**	**41.0***	**27.6***	**11.0***	2.0	–	–	–
Facial asymmetry	5.5	2.0*	1.7	–	–	–	–	–	3.1	**11.5***	5.2	4.7	1.0	–	–	–
Pointed mandible	3.6	4.0	**7.0***	–	–	–	–	–	3.9	**10.7**	**18.1***	6.3	1.0	0.8	0.8	–
Protruding tongue	0.9*	5.0*	2.6*	–	–	–	–	–	**7.8***	**27.0***	**20.7***	5.5	1.0	–	–	–
Cleft lip	5.5	3.0*	0.9	–	–	–	–	–	3.9	**11.5***	3.4	1.6	–	–	–	–
Cleft palate	5.5	1.0	0.9	–	–	–	–	–	6.2	3.3	4.3	0.8	–	0.8	1.6	1.0
Diaphragmatic hernia-right	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
Hydronephrosis	**17.3***	**42.0***	**37.4***	**28.6***	**34.3***	**49.1***	**58.3***	**34.2**	**5.5***	**7.4***	**12.9***	**11.8***	**7.0***	**21.3***	**21.1***	**23.7**
Left umbilical artery	**8.2**	**14.0**	**11.3**	**4.4**	**11.8**	**13.0**	**9.2**	7.0	**17.2**	**20.5**	**16.4**	**11.0**	**9.0**	**13.9**	**13.3**	**14.4**
Hindlimb dysplasia	3.6	–	–	–	–	–	–	–	3.1	–	2.6	–	1.0	3.3	3.9	**4.1**
Tail dysplasia	4.5	1.0	–	–	–	2.8*	1.7*	–*	2.3	5.7	4.3	1.6	4.0	**27.9***	**29.7***	**27.8***
Anal atresia	0.9	–	–	–	–	– *	0.8*	–	–	0.8	1.7	–	2.0	**14.8***	**19.5***	2.1

*Significant difference between A and B stocks ($\chi^2 > 3.84$, $P < 0.05$).

[1]In addition to other less common anomalies including: cardiac defects, left diaphragmatic hernia, pulmonary hypoplasia, thymic agenesis, gut malrotation, omphalocele, abdominal wall hypoplasia, renal agenesis, gonadal agenesis, undescended testis, forelimb dysplasia, spina bifida, and several unclassified defects of craniofacial structures.

Mortality

Among the A-stock, previous experience led us to expect a normal resorption rate of about 3%. The observed resorption rate ranged from 4.2% to 11.5%. The B-stock controls resorbed 6.1% of implantations, a rate close to that previously found with animals from the same source. Resorptions in the HU-treated B-stock rats varied from 9.4% to 15.9%. No statistically significant association between resorption rate and hour of injection was found in either stock. Incidence curves for resorptions by time of HU injection for the two stocks had the same shape, with peaks after injection at days 9.25 and 10.50.

Malformation

HU was teratogenic in both stocks of rats. However, there were some notable differences between the stocks in the types, incidence, and timing of the defects produced (Table 2). In addition to fetal malformations, several instances of placental abnormality were seen, including gross cysts of the placenta and extremes of placental size, both large and small. These abnormalities of the placenta had no obvious correlation with fetal malformations or fetal size.

Normally in the rat the right umbilical artery persists and the left becomes obliterated during development. In 2.9% of the B-stock control fetuses the situation was reversed and the left artery persisted. Experience in other studies with Wistar rats from several sources suggests that a left umbilical artery can normally be expected in 2–3% of fetuses regardless of the source of the stock (unpublished data). Of the different subgroups of HU-treated rats 6/8 of the A-stock and 8/8 of the B-stock had an incidence of left umbilical artery that was significantly ($P < 0.05$) higher than in controls.

With the notable exception of hydronephrosis, the incidence of each malformation was higher in the B-stock than the A-stock (Table 2). With a few exceptions, the higher incidences in the B-stock were found at every injection time compared. Several defects were common in the B-stock but uncommon in the A-stock. These were protruding tongue, hindlimb dysplasia, tail malformations, and anal atresia. A-stock rats had a significantly ($P < 0.05$) higher incidence of hydronephrosis, except from day − 10.75 HU injection, when the difference between the stocks did not reach statistical significance.

In most instances a particular malformation was found after a wider range of injection times in the B-stock than in the A-stock. For example, maxillary hypoplasia was found after day 9.0 - 9.5 injection in the A-stock and day 9.0 - 10.0 in the B-stock. Another difference between the stocks was that the peak incidence of several malformations occurred later, by about 6 hours, in the B-stock. Of those malformations for which there were sufficient data for interstock comparisions, the peak incidence of an-/microphthalmia, hydrocephaly, and hydronephrosis was produced by HU injection 6 hours earlier in the A-stock than in the B-stock.

Data for other malformations were insufficient for detailed comparison but were suggestive of a 6-hour advance in peak incidence in the A-stock for facial asymmetry, maxillary hypoplasia, micrognathia, clefts of the lip/anterior palate, and exencephaly. Incidence curves for pointed mandible and encephalocele appeared to be symmetrical between two stocks. Occurrences of ear dysplasia, cleft palate, right diaphragmatic hernia, hindlimb dysplasia, tail malformation, and anal atresia were too rare in the A-stock to permit interstock comparisons of peak incidence timing.

Inspection of incidence graphs for resorptions, left umbilical artery, and hydronephrosis suggested a cyclic factor in their occurrence. The data were summed for hour of injection, disregarding day of injections, and χ^2 analyses were performed. In a 4×2 format (6 AM, 12 AM, 6 PM, 12 PM) there were no significant deviations from expected distributions for resorptions in either stock. For left umbilical artery the difference between the observed and expected distribution approached but did not reach statistical significance in both stocks ($0.10 > P > 0.05$). In both stocks the distribution of fetuses with hydronephrosis by hour of HU injection was significantly different from expected values (A-stock, $P < 0.0005$; B-stock, $P < 0.01$). When the data were analyzed in a 2×2 format, by combining adjacent hours of injection, it was found that the A-stock had a significant excess of fetuses with hydronephrosis after HU injection at 12 AM + 6 PM ($P < 0.0005$). The B-stock had a significant excess of fetuses with hydronephrosis following injections at 6 PM + 12 PM ($P < 0.01$). As was suggested from the data for other malformations, there appeared to be a 6-hour advance in the time of maximum sensitivity to HU-induced hydronephrosis in the A-stock compared to the B-stock.

DISCUSSION

Fetal rats have been shown to exhibit a circadian variation in growth rate during late gestation (Barr, '73b). A phase of rapid growth occurs during the night, followed by a phase of slow growth during the day. Marked fluctuations of body weight in adult rats, pregnant

and nonpregnant, during a 24-hour period correlate well with feeding and activity cycles which in turn seem to be regulated by environmental lighting (Slonaker, '12; Barr, '73b). It is not yet known how early in gestation diurnal fluctuations in fetal growth occur. We are unaware of any published data on the specific question of circadian variations of cell proliferation in the normal embryo in vivo. Scott et al. ('71) measured DNA synthesis sequentially for 29 hours in normal rat embryos as controls for HU-treated embryos. Their data indicate that the rate of DNA synthesis had a diurnal fluctuation in the day-12-13 embryo.

Hydroxyurea inhibits DNA synthesis, producing mitotic arrest or cell death (Philips et al., '67). When HU levels drop below threshold values, DNA synthesis and division resume in the surviving cells which appear to be synchronized (Scott et al., '71). HU is an appropriate agent to test for the presence of diurnal variations in embryonic cell division since it produces maximal DNA synthesis inhibition in the rat fetus within 5 hours after IP administration with a rapid return toward normal by 11 hours (Scott et al., '71).

If diurnal variations in cell division rates occur in the rat embryo, it would be expected that HU given during a phase of rapid cell proliferation would have more deleterious effects than when given during a phase of slower cell proliferation. Such effects could be manifest as fetal death, malformations, and/or intrauterine growth retardation. The search for modification of HU teratogenesis by circadian rhythms had no clear-cut results. Inspection of the incidence curves for left umbilical artery suggested a daily rise and fall for this anomaly, but when tested for time-dependent effects the variation failed to reach a level of statistical significance ($0.10 > P > 0.05$). In the case of HU-induced hydronephrosis, there was a cyclic rise and fall in incidence during a 24-hour period. However, the timing of the rise varied by 6 hours between the two stocks, despite the fact environmental lighting and presumably feeding/activity cycles were the same. This suggests that some phenomenon other than light-controlled maternal circadian rhythms operates to produce the variation.

The data from this study do not answer the question of whether circadian growth rhythms occur during organogenesis. No correlation between hour of HU administration and fetal weight, resorptions, or total malformation rates were found. If circadian embryonic growth has an effect on the response to teratogens we were unable to detect it in near-term fetuses. We think that if a search for circadian growth rhythms in the early embryo is to be made it will have to be by direct means and not by the indirect means that we tried.

The data from this study provide some further insights into the nature of interstock variability of response to teratogens. There were rather great differences in the incidence of malformations produced in both stocks and some malformations were found almost exclusively in one stock. There were also differences between stocks in the duration of the period during which several of the malformations could be produced. It may be that the sensitive period was longer in one stock of rats or that there was a shift upward in the teratogenic threshold in the other.

Another difference in the response of the two stocks of rats was an apparent shift in the hour of maximum sensitivity to HU teratogenesis. A-stock animals appeared to reach the time of maximum sensitivity 6 hours before the B-stock. In exploiting a teratologic model to study a particular malformation, it would be advisable to give the teratogen at various times of day to determine the time of maximum response. The time of maximum response in this study varied from 6 AM to 6 PM. Of course the need for such "fine tuning" of dosing times would depend greatly on the mode of action of the teratogen; those agents with a rapid onset of action and prompt elimination/deactivation would require more precise timing.

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