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STUDIES ON 6-AZAURIDINE AND 6-AZACYTIDINE—V.

INFLUENCE OF 6-AZACYTIDINE ON PRENATAL DEVELOPMENT IN MICE

K. ČEREY, J. ELIS and H. RAŠKOVÁ

Institute of Pharmacology, Czechoslovak Academy of Sciences, Prague, Czechoslovakia

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Abstract—6-Azacytidine interrupts pregnancy in albino mice with an optimum effect between the 5th and 7th day; after the 15th day the administration is ineffective. Three consecutive doses are more effective than a single one. The efficacy of the compound undergoes seasonal variations. Malformations of the tails were observed in the 3rd pregnancy when the drug was administered during consecutive pregnancies. F_1-females without malformations from the first pregnancy mated with random males and given the drug during the whole gestation period had offspring with the same tail malformation after the first pregnancy.

6-AZACYTIDINE, a cytostatic compound prepared by Šorm and co-workers,[1] has been studied from several aspects by our group.[2–5] It has been described that 6-azauridine, another cytostatic agent, prepared by the same group, interrupts pregnancy of Swiss albino mice depending on the time of administration during the gestation period and the dosage.[6] The authors suggest that the drug, because of its apparent nontoxicity, might be a most useful tool in human pharmacology to interrupt pregnancy. Other cytostatic agents are known to interrupt pregnancy;[7, 8] some of them, like colchicine, have only abortive effects, while others, like the alkylating agents or certain antimetabolites, also have teratogenic effects.[8] It seemed worthwhile, therefore, to investigate 6-azacytidine from both points of view.

MATERIAL AND METHODS

All experiments were performed on non-inbred albino mice. The animals were kept at constant temperature and diet. Two types of experiments were performed. In one type (1) the effect of dosage and time of treatment on interruption of pregnancy was investigated. In another experiment (2) the possible teratogenicity of low doses of 6-azacytidine after chronic administration was investigated.

(1) In experiments designed to reveal the time of interruption of pregnancy, the drug was administered intravenously and mating was checked by vaginal smears. The drug was given either on one day or on three consecutive days in various doses. One experiment was performed in February and one in June. In the winter experiment, one-half of the females were killed, 1, 3 and 7 days, respectively, after the administration of the drug and investigated in the Department of Embryology of Charles University in Prague.

(2) In experiments aimed to reveal possible teratogenicity, the drug was given daily intraperitoneally for the duration of the pregnancy. After delivery the drug administration was discontinued and was repeated at the next pregnancy. The mating took place 3 weeks after weaning—i.e., after 6 weeks of life. The same procedure was repeated for the third and fourth matings. The female offspring of these mothers (F_1) were kept until they attained a weight of 18–20 g and were then mated.

RESULTS

(1) Pregnancy-interrupting results are summarized in Tables 1–5. In the first three tables the experiments performed in February are summarized. The tables present the results obtained with only one-half of the females, since the other half of the animals were killed for histological investigation.[9] Table 1 represents the control values; of 33 females, 26 had litters, with a total of 149 offspring. Table 2 shows the results of an experiment in which a single dose of 200 mg of 6-azacytidine per kg was administered intravenously to groups of mated females on various days after mating. Table 3 shows the results of experiments in which, under otherwise identical conditions, 6-azacytidine (200 mg/kg) was administered for three consecutive days.

TABLE 1. RESULTS OF PREGNANCY-INTERRUPTING EXPERIMENT IN WINTER*

Day of treatment after mating	No. of delivery	No. of offspring	Male	Female	Died	Average weight of offspring 21st day after birth (g)
0	3	16	4	7	5	7·6
1	1	8	6	2	0	7·2
2	2	13	6	6	0	8·0
3	2	9	5	4	0	6·8
4	3	17	9	8	0	9·3
5	3	17	9	7	1	9·9
6	2	11	6	5	0	7·8
7	3	15	7	8	0	8·9
9	2	13	7	4	2	7·8
12	3	18	8	7	3	9·0
15	2	12	7	5	0	8·3
Total:	26	149	74	64	11	8·2
	i.e., 5·7 young per mother		49·7%	43%	7·4%	

*Control values. (In every group there were three females)

If we compare the data from animals that received one dose or three doses of 6-azacytidine with controls, the reduction of the number of litters increases with the number of 6-azacytidine injections, i.e., after a single dose of 200 mg/kg, 12 of 33 mated females had 56 young, whereas three consecutive daily doses of the same amount of 6-azacytidine reduced the litters to 4 of 33 mated females, with a total of 20 young.

Analysing the data in more detail, it can be seen that the size of litters does not differ significantly between controls and both groups of treated mice. There was no marked difference between sexes or weight in the offspring. It is also apparent that the maximum pregnancy-interrupting effect is between the 4th and 9th day after one single dose. Three administrations of the drug have effect when the first administration takes place between the 2nd and 12th day. Seen from this point of view, a single

dose of 200 mg of 6-azacytidine per kg, administered to 15 females on the 4th, 5th, 6th, 7th or 9th day after mating, reduced the number of litters from 12 of 15 in controls to 1 of 15 in the group treated with a single dose. Even more marked were the results from the group with three consecutive daily administrations. When the drug was given during the 2nd–4th, 3rd–5th, 4th–6th, 5th–7th, 6th–8th, 7th–9th, 9th–11th and

TABLE 2. RESULTS OF PREGNANCY-INTERRUPTING EXPERIMENT IN WINTER*

Day of treatment after mating	No. of delivery	No. of offspring	Male	Female	Died	Average weight of offspring 21st day after birth (g)
0	2	9	6	3	0	8·4
1	3	14	9	5	0	8·7
2	1	6	2	4	0	9·3
3	1	3	1	2	0	8·7
4	0	0	0	0	0	—
5	0	0	0	0	0	—
6	0	0	0	0	0	—
7	1	5	2	3	0	7·7
9	0	0	0	0	0	—
12	2	11	3	3	5	8·6
15	2	8	6	2	0	8·4
Total	12	56	29	22	5	8·5
	i.e., 4·6 young per mother		51·8%	39·3%	8·9%	

* 200 mg of 6-azacytidine per kg given intravenously. (In every group there were three females).

12th–14th days, respectively, there were no offspring among 24 mated females, whereas from 24 controls, 20 had litters. It can be concluded, therefore, that 6-azacytidine has a powerful inhibitory effect on reproduction; however, this seems to be an all-or-none effect, as the size of litters and the postnatal weight gain is the same in the treated groups as in the control group.

TABLE 3. RESULTS OF PREGNANCY-INTERRUPTING EXPERIMENT IN WINTER*

Day of treatment after mating	No. of delivery	No. of offspring	Male	Female	Died	Average weight of offspring 21st day after birth (g)
0–1–2	1	6	3	3	0	7·8
1–2–3	1	3	1	2	0	8·6
2–3–4	0	0	0	0	0	—
3–4–5	0	0	0	0	0	—
4–5–6	0	0	0	0	0	—
5–6–7	0	0	0	0	0	—
6–7–8	0	0	0	0	0	—
7–8–9	0	0	0	0	0	—
9–10–11	0	0	0	0	0	—
12–13–14	0	0	0	0	0	—
15–16–17	2	11	3	2	6	7·0
Total	4	20	7	7	6	7·8
	i.e., 5·0 young per mother		35·0%	35·0%	30·0%	

* 200 mg of 6-azacytidine per kg given three times intravenously. (In every group there were three females).

In the summer experiments, the effects were weaker than in winter; however, there is a definite dose-effect relationship both in the single dose and in the three-dose experiments. The maximum effect is apparently between the 5th and 7th day, which corresponds very well with the winter experiment. In the summer experiments, a single dose of 400 mg/kg was in no case sufficient to suppress reproduction completely. Three doses were more effective and the reproduction was completely suppressed between the 3rd and 7th day (Tables 4 and 5). In Fig. 1, the relation of dose

TABLE 4. RESULTS OF PREGNANCY-INTERRUPTING EXPERIMENT IN SUMMER*

Day of treatment after mating	Control values	Intravenous dose of 6-azacytidine, mg/kg 25	50	100	200	400
3	5/6– 24	4/6– 18	6/6– 36	5/6– 26	5/5– 21	3/6– 18
5	6/6– 32	6/6– 32	5/5– 32	3/5– 12	2/6– 12	4/6– 28
7	3/6– 18	4/5– 21	6/6– 31	2/3– 4	4/6– 19	2/6– 14
9	6/6– 29	3/6– 18	5/6– 29	5/5– 27	5/6– 24	5/6– 28
17	4/5– 23	5/5– 27	3/3– 15	6/6– 34	3/5– 17	6/6– 33
Total	24/29–126	22/28–116	25/26–143	21/25–103	19/28–93	20/30–121
Average of young per mother	5·2	5·2	5·7	4·9	4·9	6·0

* In every column the number of deliveries per mated females and the total number of offspring are indicated.

and effect, both after one, and after three administrations, is represented. As the drug was effective only to the 9th day of administration, the relation of the percentage of litters among the total number of mated females was determined for both groups, in comparison with the respective controls. It is evident that for a single dose of 100 mg or more per kg, and for the repeated doses of 50 mg or more per kg, there is a linear relationship between the logarithmically increasing dose and the reduction of the number of young. In these experiments also the size of litters remained unaffected by the drug. A macroscopic inspection of all young in the described experiments did not reveal any signs of malformations.

TABLE 5. RESULTS OF PREGNANCY-INTERRUPTING EXPERIMENT IN SUMMER*

Day of treatment after mating	Control values	Intravenous dose of 6-azacytidine, mg/kg 25	50	100	200	400
3–4–5	3/5– 17	4/6– 23	3/6–16	2/5– 8	0/5– 0	2/4–12
5–6–7	4/6– 22	2/5– 12	2/6–17	0/6– 0	2/6– 8	06/– 0
7–8–9	4/5– 23	6/6– 33	5/5–25	4/6–22	4/6–20	2/6– 9
9–10–11	6/6– 29	5/5– 28	5/6–28	6/6–33	3/6–16	3/5–14
17–18–19	5/6– 28	6/6– 36	1/6– 4	6/6–35	6/6–37	3/5–17
Total	22/28–119	23/28–132	16/29–90	18/29–98	15/29–81	10/26–52
Average of young per mother	5·4	5·7	5·6	5·4	5·4	5·2

* In every column the number of deliveries per mated females and the total number of offspring are indicated.

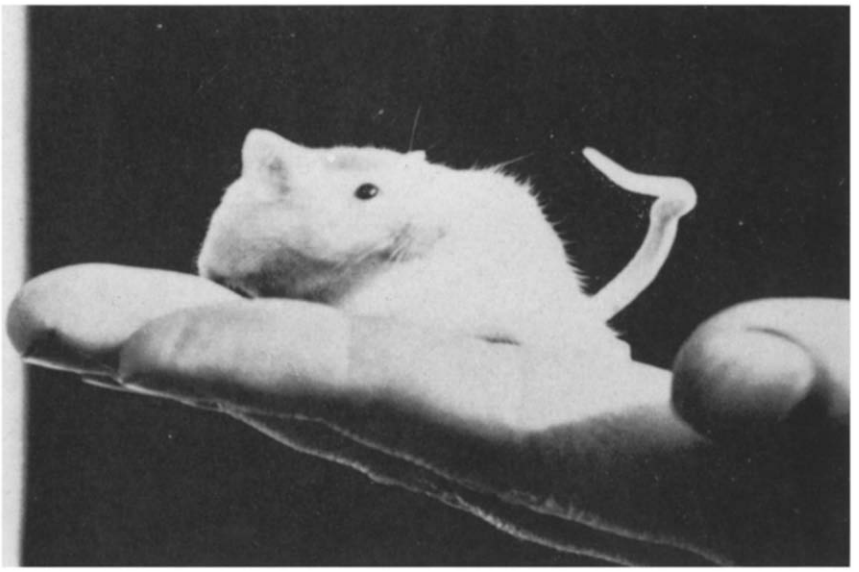

FIG. 2. Malformation of tail in F_1-generation.

FIG. 3. Malformation of tail in F_2-generation.

(2) Results of induced malformations are summarized in Tables 6–8. During the first mating period, 14 female mice were given 100 mg of 6-azacytidine per kg daily intraperitoneally. The offspring had no apparent malformations and the female offspring ($F_{1,1}$) were kept for further experimentation. The mothers were divided into 2 groups of seven each, one receiving the drug again during the second gestation period, the other receiving saline. In the offspring ($F_{1,2}$) no deformities were observed.

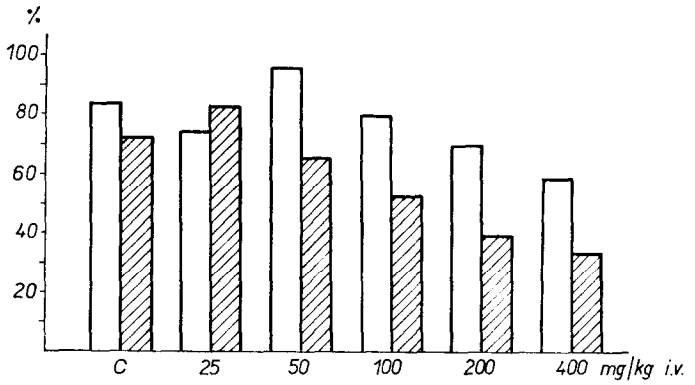

FIG. 1. Relation of dose and effect after either one application (white column) or three application (striated column) of 6-azacytidine.

The seven females, which had received 6-azacytidine twice during the two preceding gestation periods, were given the drug again during the third pregnancy. Only one had a litter ($F_{1,3}$) of 4 young: one died, the others (2 females and one male) had deformed tails (Fig. 2). The group that was treated with 6-azacytidine only during the first pregnancy and then twice with saline had a normal birth rate, 7 of 7 had normal litters ($F_{1,3}$). The drug was then administered a fourth time to the 7 females, which had had it three times during the previous gestation periods, for a fourth gestation period, none had a litter, whereas the controls, which had the drug for the first gestation period only and then were given saline, had again 7 litters for 7 females ($F_{1,4}$), (Table 6).

TABLE 6. MALFORMATIONS INDUCED AFTER APPLICATION OF 100 mg OF EITHER 6-AZACYTIDINE PER kg (A) OR SALINE (S) GIVEN INTRAPERITONEALLY DURING THE ENTIRE GESTATION PERIOD*

Treatment during pregnancy				No. of females	No. of delivery	No. of off-spring	Male	Female	Died	Malformations
1st	2nd	3rd	4th							
A	A			7	2	14	6	6	2	
A	S			7	4	18	5	7	6	
A	A	A		7	1	4	1	2	1	2 young with deformed tails
A	S	S		7	7	34	9	7	8	
A	A	A	A	7	0	0				
A	S	S	S	7	7					

* See text for further details.

The possible teratogenicity of 6-azacytidine was confirmed also with the dose of 200 mg/kg. In this case a group of 4 females, which had been given, during the first gestation period, 200 mg of 6-azacytidine per kg, and saline during the second gestation period, again received 200 mg of the drug per kg during the third pregnancy. Two litters were born, both with malformations; one litter consisted of 5 young, of which 4 had malformed tails, the others of 3 young, of which one had a malformed tail (Table 7).

TABLE 7. MALFORMATIONS INDUCED AFTER APPLICATION OF 200 mg OF EITHER 6-AZACYTIDINE PER kg (A) OR SALINE (S) GIVEN INTRAPERITONEALLY DURING THE ENTIRE GESTATION PERIOD*

Treatment during 1st pregnancy	2nd	3rd	No. of females	No. of delivery	No. of offspring	Male	Female	Died	Malformations
A	S	A	4	2	8	5	3	0	5 young from both mothers with deformed tails
A	S	S	4	3	17	8	7	2	

* See text for further details.

Results with the F_1 generation females are shown in Table 8. Six females from mothers that had received 200 mg of 6-azacytidine per kg during the first pregnancy were mated at random with non-inbred males and the same amount of 6-azacytidine

TABLE 8. MALFORMATIONS INDUCED IN F_1-GENERATION AFTER APPLICATION OF EITHER 200 mg (A-200) OR 100 mg (A-100) OF 6-AZACYTIDINE PER kg OR SALINE (S) GIVEN INTRAPERITONEALLY*

Treatment of mothers during 1st pregnancy	2nd	Treatment of F_1-females during 1st pregnancy		No. of females	No. of delivery	No. of offspring	Malformations
A-200		$F_{1,1}$	A-200	6	2	8	5 young with deformed tails
A-200		$F_{1,1}$	S	4	1	15	
A-100	A-100	$F_{1,2}$	A-100	6	4	6	2 young with deformed tails
A-100	S	$F_{1,2}$	A-100	6	5	16	

* See text for further details.

was administered during their first pregnancy period. Two females had 8 young, five of which had deformed tails (Fig. 3). Four females of the same background served as controls (saline) and the offspring revealed no deformities. An analogous experiment was performed with females ($F_{1,2}$) from litters whose mothers were treated during the first and second pregnancy period with 100 mg of 6-azacytidine per kg. Of six, four females had litters with a high perinatal mortality. From the six young that survived, 2 had a tail deformation.

DISCUSSION

In the search for substances that would interrupt pregnancy and cause a complete resorption of the foetus, antitumorous substances that act on quickly growing tissue and therefore come, *a priori*, into consideration, have received considerable attention. Such properties have been ascribed for instance to 6-mercaptopurine, which according to Thiersch[10] leads to 90 per cent, and according to Tuchmann-Duplessis[8] to 50 per cent of resorptions in rats, without any serious malformations. All tested antitumorous agents had in common a comparatively great toxicity for the mother. The results described for 6-azauridine by Sanders and co-workers[6] are of special interest because of the low toxicity of the drug, given either a single or in a few consecutive doses. As 6-azacytidine is under these conditions even less toxic than 6-azauridine, the demonstrated effects on reproduction are very interesting. On the material from killed females during pregnancy from the group in which three administrations of the drug between the 2nd and 12th day stopped the reproduction completely, histological examination has shown a complete resorption of the embryos. The significantly larger size of the tubes and the leukocyte agglomeration indicated the previous implantations.[9]

The quantitative difference between the winter and summer experiments demonstrates that seasonal and environmental factors have to be taken into consideration. Whereas the dose of 200 mg/kg was sufficient in winter to interrupt pregnancy even after a single dose on the 4th, 5th and 6th day after mating, and three doses were fully effective between the 2nd and 12th day, in the June experiment 200 mg/kg and even 400 mg/kg in a single dose were not high enough to stop reproduction completely and three doses were fully effective only within the most susceptible period, i.e., around the 5th day. Sanders *et al.*[6] have administered comparatively high oral doses, as high as 1 g/kg, in their experiments. Without consideration of the dangers of oral administration of 6-azauridine (formation of azauracil and the hazard of neurotoxicity),[11] we think that it is necessary to avoid for reproduction and fertility-inhibiting experiments doses in which general toxicity, i.e., non-specific toxicity could be encountered. Therefore, we have not given more than 400 mg of 6-azacytidine per kg in the pregnancy-interrupting experiments. For the experiments in which the drug was administered during the whole gestation period, the dose of 200 mg/kg was never surpassed, as 250 mg/kg after three weeks already caused some mortality of experimental animals.[2]

Teratogenicity and impairment of reproduction has been described for many cytostatic drugs.[8, 10, 12–17] A strong correlation is found between teratogenicity and mutagenicity and incorporation of antimetabolites into nucleic acids.[18, 19] Accordingly, we were much interested in recent studies of 6-azauridine and 6-azacytidine, since it had been reported originally that neither of these compounds is incorporated into nucleic acids.[20, 21] Recently, however, it has been reported that 6-azauridine is incorporated to a small extent into RNA of feline midbrain[22] and 6-azacytidine-5′-diphosphate into co-polymers of natural nucleotides and 6-azacytidylic acid *in vitro*.[23]

The antifertility effect of 6-azauridine described has been previously observed in this laboratory.[5] 6-Azacytidine in apparently non-toxic doses induces interruption of pregnancy in the same optimum period as 6-azauridine, i.e., between the 5th–7th day

and becomes completely ineffective after the 14th day. The teratogenic action of 6-azacytidine described diminishes its value for possible human use. In preliminary experiments, deformations of the tails also have been found after the administration of 6-azauridine.[24] But the most interesting fact is the necessity to give the drug during several gestation periods before malformations appear. It seems that more is needed for the horizontal, i.e., in the consecutive administration of 6-azacytidine to mothers, than for the vertical production of malformations, in which apparently healthy animals from mothers, which had received the drug for one gestation period, had malformed offspring when the drug was administered during the first pregnancy. This seems to be very important for further studies, not only with antimetabolites, but with malformation in general. As 6-azacytidine produces in low doses stimulating effects which resemble those seen with X-ray irradiation,[2] and we now know that 6-azauridine and 6-azacytidine are both incorporated into nucleic acids, possible carcinogenicity, as described for other metabolic antagonists,[7] should be studied. It has been reported that 6-azauridine affects spermatogenesis in rats[25] and that the treatment of males with 6-azauridine during mating reduces the number of offspring in mice.[5] Therefore, further studies of spermatogenesis, as well as on the ovaries are indicated. No malformed offspring was hitherto found when 6-azacytidine-malformed mice were mated in our laboratory; however, the numbers are too small to allow definite conclusions and further work is necessary.

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