Evaluation of Teratogenic Potential of Codeine Sulfate in CF-1 Mice

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Abstract □ The most prominent defect of codeine sulfate, 100 mg/kg sc, was delayed ossification of the supraoccipital bone, paws, xiphoid, and sternebrae as well as other sternebral defects such as checkerboard sternebrae and polysternebrae. Although these anomalies were similar to the minor defects seen in the fetuses of morphine sulfate-treated mice, the major anomalies such as exencephaly, cryptorchid testes, and rib and vertebral fusions produced by morphine were not present in the fetuses of mice challenged with codeine. Thus, codeine sulfate appears to be less teratogenic than morphine sulfate. A review of the incidences of the various defects in mice treated on a single day with codeine showed that there was a range of days on which the mouse fetus was susceptible to codeine's teratogenic effects, with the most critical days of gestation being Days 8–10. Furthermore, a comparison of the defects that occurred in those treated on both Days 8 and 9 with the defects that occurred in those treated on a single day of gestation reveals an additive or cumulative teratogenic response to codeine.

Keyphrases □ Codeine sulfate—teratogenic activity evaluated, mice □ Teratogenicity—codeine sulfate evaluated, mice □ Narcotic analgesics—codeine sulfate, teratogenic activity evaluated, mice

In 1968, Harpel and Gautieri (1), noting the lack of teratogenic studies involving narcotic analgesics, demonstrated that high subcutaneous doses of morphine sulfate administered to nontolerant CF-1 albino mice on the 8th or 9th day of gestation were capable of inducing various fetal malformations. The most prominent defect observed with drug challenge on Day 8 was exencephaly, while axial skeletal fusion was the most conspicuous malformation occurring with drug insult on Day 9 of gestation. In mice, exencephaly and axial skeletal fusions have been reported after many types of treatment, including the administration of insulin (2), galactoflavin (3), vitamins (4), and urethan (5), as well as with riboflavin deficiency (3), X-ray exposure (3), and anoxia (6).

Due to its close structural relationship to morphine, codeine was suspected of having teratogenic potential. Because the literature is devoid of studies concerning the effects of codeine on developing fetuses, the purposes of this study were: (*a*) to test the hypothesis that codeine is a potential teratogen and (*b*) to determine the day or range of days of gestation on which the fetus is most susceptible to any of its teratogenic effects.

EXPERIMENTAL

CF-1 albino mice[1], 25–30 g, were used. Females were placed in aggregate cages, each holding 25 mice. No attempt was made to breed these mice for at least 2 weeks following their arrival and unless they maintained a minimum weight of 25 g. Male mice were placed in individual metal cages, 12.5 × 15 × 10 cm, with a wire mesh front and bottom. All animals were maintained on laboratory food[2] and tap water *ad libitum*.

To produce timed pregnancies, two females were placed into the cage of a male at 4:00 pm and permitted to remain there until 8:00 am the following morning. At this time, the females were removed and examined for the presence of a vaginal plug. Females exhibiting this plug were regarded as being gravid, and the day on which the plug appeared was considered to be Day 0 of gestation. Pregnant mice were weighed to the nearest 0.1 g, and this weight was designated as the maternal starting weight. Gravid females were placed into individual cages, identical to those occupied by the males, and allowed to remain undisturbed until the morning of the 7th day of gestation, when they were weighed for a second time. A weight gain of 2 g or more at this time was accepted as a confirmation of pregnancy.

The confirmed pregnant females were assigned at random to one of eight experimental categories: six groups injected with 100 mg of codeine sulfate[3]/kg on a single day of gestation from Day 7 through Day 12, one group injected with 100 mg of codeine sulfate/kg on both Days 8 and 9 of gestation, and one group of untreated control mice which was examined to establish norms.

Since single saline injections administered subcutaneously on either Day 8 or 9 of gestation produced no significant change in the occurrence of fetal abnormalities compared to untreated controls in previous studies (7, 8), all treatment groups in this study were compared to one group of untreated controls. Arcuri and Gautieri (8) injected gravid CF-1 mice subcutaneously with 60 mg of sodium sulfate/kg on Day 9 of gestation and found no significant change in the number of fetal abnormalities. Because sodium sulfate contains approximately 68% sulfate or 40.8 mg/kg in the 60-mg/kg dose used in the latter study, while codeine sulfate contains only approximately 14% sulfate or 14.0 mg/kg in the 100-mg/kg dose used in the present study, sulfate-ion controls were not included.

An injectable solution of codeine sulfate[4], 3% (30.0 mg/ml), was prepared fresh weekly by dissolving the drug in triple-distilled water. All injections were made subcutaneously in the upper left abdomen using 1-ml tuberculin syringes equipped with 1.27-cm 26-gauge needles.

Following treatment, each mouse was permitted to proceed to Day 18, 1 day short of term in the gestation of the CF-1 mouse. The pregnant animal was weighed, and this weight was referred to as the terminal maternal weight. The animal was then sacrificed, and the abdominal wall was opened to expose the entire peritoneal cavity. Prior to actual opening, the uterine horns were examined grossly for fetal swellings and resorption sites, as indicated by the presence of small dark nodules (the metrial glands), which were counted and recorded.

The uterine horns were incised individually, and the exposed fetuses were removed by blunt dissection, blotted dry, and weighed to the nearest 0.01 g on a torsion balance. Each fetus was examined grossly for any externally visible soft tissue defects and determination of sex. Fetal viability was determined by reflex movement of the limbs in response to mechanical stimulus with a blunt probe.

Every second fetus was placed in either bone-staining solution or Bouin's fixative and processed for skeletal or soft tissue examination, respectively. Fetuses destined to be examined for soft tissue defects were fixed and decalcified in Bouin's fixative for a minimum of 2 weeks. These fetuses were reexamined for external defects under a low power binocular dissecting microscope and sectioned according to the method of Wilson and Warkany (9). Those fetuses designated from each litter for skeletal examinations were processed according to the method of Staples and Schnell (10).

The significance of observed variations among the experimental groups was determined by using the Student t test (11) and the uncorrected χ^2 test for binomial populations (11).

RESULTS AND DISCUSSION

The observations support the hypothesis that codeine is a potential teratogen. It is closely related structurally to morphine (12), which is known to produce soft tissue and skeletal anomalies in the fetuses of CF-1 mice when administered in high doses on Day 8 or 9 of gestation (1, 7, 8).

[1] Carworth Farms, New City, N.Y.
[2] Teklad rat and mouse diet (6% fat), Teklad Mills, Winfield, Iowa.
[3] Highest dose, as determined in a pilot study, where no maternal death or excessive excitement was observed.
[4] USP quality, Control No. 63375, Merck & Co., Rahway, N.J.

Table I—Mean Values of the Test Groups

Treatment[a]	Maternal Weight Ratio, S/T[b]	Fetal Ratio, Right Horn/ Left Horn	Resorption Ratio, Right Horn/ Left Horn	$\bar{X}$ Fetal Weight, g	Sex Ratio, M/F	Soft Tissue Abnormalities	Skeletal Abnormalities
Control (untreated)	25.3/50.2	5.4/5.5	0.5/0.5	1.38	5.3/5.6	0.75	1.63
Codeine on Day 7	26.1/50.3	6.3/4.7	1.1/0.6	1.26[c]	5.1/5.9	0.57	5.86[c]
Codeine on Day 8	25.4/48.3	6.9[c]/4.0	0.6/0.4	1.23[c]	5.6/5.3	0.13	6.00[c]
Codeine on Day 9	25.7/47.4	5.0/5.4	0.1/0.3	1.20[c]	6.1/4.3[c]	1.14	7.71[c]
Codeine on Day 10	26.4/55.1[c]	8.0[c]/6.0	0.2/0.2	1.18[c]	8.0/6.0	0.50	9.33[c]
Codeine on Day 11	26.7/50.1	4.3/5.2	0.3/0.2	1.32	4.7/4.8	1.00	6.33[c]
Codeine on Day 12	24.6/49.2	5.0/5.0	0.7/1.5	1.37	5.0/5.0	0.33	3.17
Codeine on Days 8 and 9	26.7/48.8	5.6/5.8	0.2/0.6	0.98[c]	5.4/6.0	0.80	15.60[c]

[a] The dose of codeine was 100 mg/kg sc in all cases. [b] S = starting weight and T = terminal weight. [c] Statistically significant in comparison with untreated control, $p < 0.05$

Table II—Occurrence of Delayed Ossification of the Supraoccipital Bone

Treatment[a]	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Control (untreated)	7	1	40	1
Codeine on Day 7	5	2	35	2
Codeine on Day 8	4	4	32	8[b]
Codeine on Day 9	3	4[b]	26	9[b]
Codeine on Day 10	2	4[b]	33	9[b]
Codeine on Day 11	3	3	18	9[b]
Codeine on Day 12	5	1	27	1
Codeine on Days 8 and 9	0	5[b]	7	22[b]

[a] The dose of codeine was 100 mg/kg sc in all cases. [b] Significance is indicated in p values of 0.05 or less when compared to control.

Although the teratogenic effects of codeine sulfate were similar to those of morphine sulfate, many distinct differences between the effects of codeine and morphine on gravid mice and their fetuses also were observed.

One paradoxical variation was the difference in the gross maternal effects of codeine sulfate and morphine sulfate. Although codeine is at least twice as toxic as morphine (13–15) when injected subcutaneously or intraperitoneally in mice, the maternal effects of 100 mg of codeine sulfate/kg sc were minimal compared to the stimulation followed by depression with a hyperactive recovery period with doses of 100–500 mg of morphine sulfate/kg sc (1). Slight excitation occurred in a few mice after 100 mg of the codeine sulfate solution/kg sc. However, a similar excitation also occurred in some untreated control mice from general handling and moving. A few mice seemed quiescent when returned to their cages postinjection, but most resumed the same level of activity as that prior to injection. None of the mice went through a period of great excitation followed by severe depression, which is typical with doses of codeine sulfate above 100 mg/kg. Furthermore, there was no variation in the maternal response of mice injected on a 2nd day with 100 mg of codeine sulfate/kg.

Another major variation between the effects of codeine sulfate and morphine sulfate on CF-1 mouse fetuses was in the production of soft tissue anomalies. While morphine sulfate shows a tendency to produce exencephaly and cryptorchid testes, especially when administered on Day 8 of gestation (1, 7), these defects were rare in the fetuses of codeine-treated mice. The only soft tissue defect that occurred with any frequency in the fetuses of codeine-treated mice was a small extra digit on the outside of one or both forepaws, but the incidence of this defect was not statistically significant. The one fetus of the codeine-treated mice that did have exencephaly was from the litter of a gravid mouse treated on Day 8 of gestation with 100 mg of codeine sulfate/kg.

Skeletal defects in the fetuses of codeine-treated mice were both similar and different from the defects seen in the fetuses of morphine-treated mice. Both morphine- and codeine-induced skeletal anomalies appear to be related to a reduction in the mean fetal weight (8). Mean fetal weights were decreased significantly in the groups of mice treated with

Table III—Occurrence of Sternebrae Defects

Treatment[a]	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Control (untreated)	5	3	34	7
Codeine on Day 7	0	7[b]	11	26[b]
Codeine on Day 8	2	6	25	15[b]
Codeine on Day 9	1	6[b]	17	18[b]
Codeine on Day 10	0	6[b]	18	24[b]
Codeine on Day 11	0	6[b]	14	13[b]
Codeine on Day 12	1	5	18	10
Codeine on Days 8 and 9	0	5[b]	8	21[b]

[a] The dose of codeine was 100 mg/kg sc in all cases. [b] Significance is indcated in p values of 0.05 or less when compared to control.

Table IV—Occurrence of Delayed Ossification of the Xiphoid

Treatment[a]	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Control (untreated)	8	0	41	0
Codeine on Day 7	7	0	37	0
Codeine on Day 8	8	0	40	0
Codeine on Day 9	4	3[b]	29	6[b]
Codeine on Day 10	5	1	41	1
Codeine on Day 11	6	0	27	0
Codeine on Day 12	5	1	27	1
Codeine on Days 8 and 9	0	5[b]	16	13[b]

[a] The dose of codeine was 100 mg/kg sc in all cases. [b] Significance is indicated in p values of 0.05 or less when compared to control.

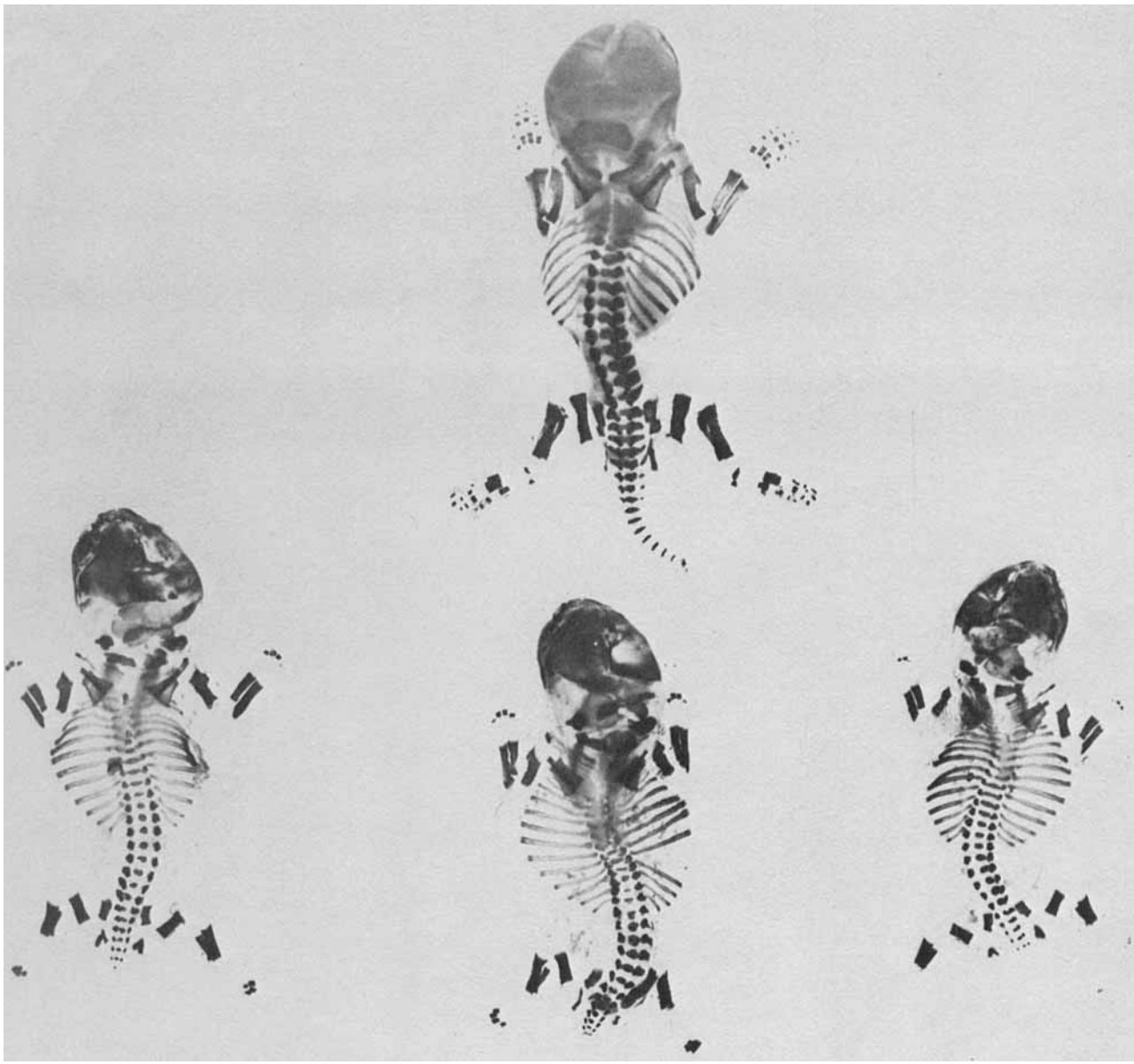

Figure 1—*Alizarin red S stained skeletal specimens of 18-day-old mouse fetuses, showing normal and delayed ossification of the supraoccipital bone and paws. Top: fetus of untreated control mouse with normal supraoccipital bone and paws. Bottom: fetuses of mice treated with 100 mg of codeine sulfate/kg on both Days 8 and 9 of gestation, showing delayed ossification of the supraoccipital bone and paws.*

100 mg of codeine sulfate/kg on Day 7, 8, 9, 10, or 11 or on both Days 8 and 9 of gestation. The most significant decrease occurred in the group treated on both Days 8 and 9 (Table I). Not only was the mean fetal weight lowest and the mean number of skeletal defects highest in this group, but the severity of the defects in this group was also greater than in any other codeine-treated group, suggesting an additive or cumulative effect of the drug and a range of days of gestation on which the fetus is susceptible to the effects of codeine sulfate.

Skeletal defects consisted of delayed ossification of the supraoccipital bone, split supraoccipital bone, missing supraoccipital bone, delayed ossification of the sternebrae, split sternebrae, checkerboard sternebrae, polysternebrae, missing sternebrae, delayed ossification of the xiphoid, split xiphoid, and delayed ossification of the paws (Figs. 1 and 2). When these defects were summarized into four categories (delayed ossification of the supraoccipital bone, sternebrae defects, delayed ossification of the xiphoid, and delayed ossification of the paws) and compared to the control group on a litter and fetal basis (Tables II–V), the range of days on which the fetus is susceptible and the additive or cumulative effect of codeine sulfate can again be seen.

One common defect occurring from treatment with codeine was delayed ossification of the paws. To set a standard for the determination of this defect, eight litters of alizarin red S bone stained fetuses from untreated mice were examined. The forepaws had 12 ossification sites each, while the hindpaws had 14 ossification sites each (excluding the toenails). In the forepaws, the ossification sites were arranged in three rows containing four sites each. The ossification sites in the hindpaws were also arranged in three rows, except that the first and second rows each contained five sites, counting from the metatarsals toward the toenails. Also, each hindpaw had two ossification sites in the lower ankle or heel. Thus, the total number of ossification sites in the forepaws and hindpaws, excluding the toenails, was 56.

However, due to the lateness of the ossification of the paws in gestation and minor variations in the exact time of conception, the third set of ossification sites nearest the toes was often missing, even in the fetuses of untreated mice. Therefore, eliminating the latter set of sites from the overall count of ossification sites in the four paws left 40 sites present in normally developing fetuses at Day 18 of gestation. Thus, any fetus having 40 or more ossification sites in its four paws, excluding the toenails, was considered to have normal ossification of the paws; any fetus having less was considered to have delayed ossification of the paws.

On this basis, there was a significant increase in the litter incidence of delayed ossification of the paws in all treated groups of gravid mice except the group treated with 100 mg of codeine sulfate/kg on Day 12 of gestation (Table V). The fetal incidence of this defect was significantly

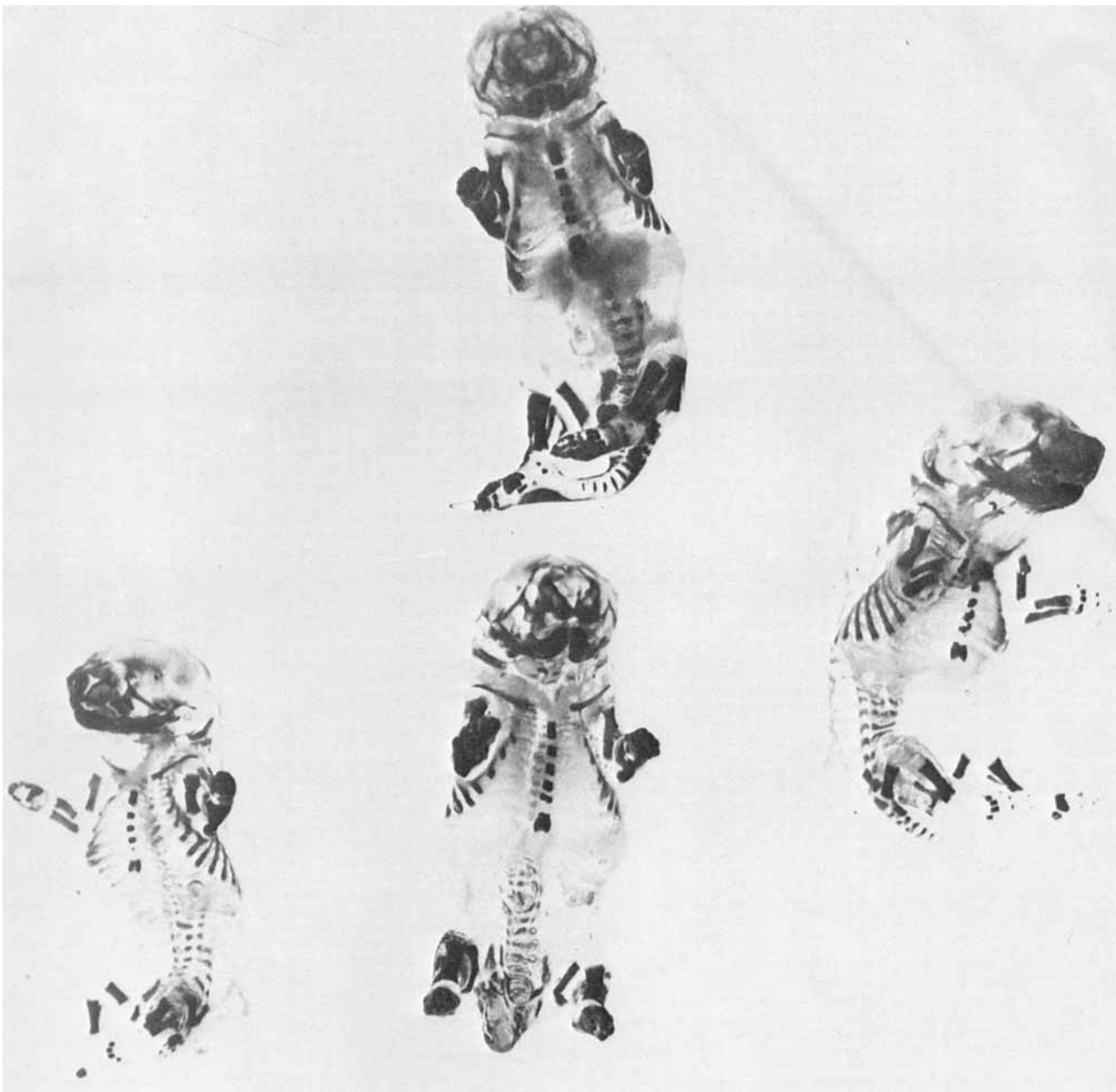

Figure 2—*Alizarin red S stained skeletal specimens of 18-day-old mouse fetuses, showing normal and abnormal sternebrae and xiphoids. Top: fetus of untreated control mouse, showing normal sternebrae and xiphoid. Bottom (left to right): fetuses of mice treated with 100 mg of codeine sulfate/kg on both Days 8 and 9 of gestation, showing delayed ossification of the sternebrae and split xiphoid, misaligned fifth sternebra, slightly delayed ossification of the xiphoid (note butterfly shape), checkerboard sternebrae, and split xiphoid.*

higher than the control group in all treated groups. The least-significant increase occurred in the group treated with a single dose of codeine on Day 12 of gestation, and the most significant increase occurred in the group treated with codeine on both Days 8 and 9 of gestation (Table V). With a single dose of codeine on a specific day of gestation from Days 7 to 12, the most significant increases in the incidence of delayed ossification of the paws on both a litter and fetal basis occurred with treatment on Day 8, 9, or 10, suggesting that these are the days of gestation on which the fetus is most susceptible to this effect of codeine (Table V).

Similarly, the highest incidence of sternebrae defects on a fetal basis, as well as the most severe defects such as split or missing sternebrae, occurred with injection of 100 mg of codeine sulfate/kg on both Days 8 and 9 of gestation. Significant increases in the incidences of sternebrae defects occurred in all treated groups except the group treated on Day 12 of gestation (Table III). On a litter basis, significant increases in the incidence of sternebrae defects occurred in the groups treated with a single dose of codeine on Day 7, 9, 10, or 11 of gestation as well as in the group treated on both Days 8 and 9 of gestation (Table III). Thus, much like the defect of delayed ossification of the paws, the most critical days for the production of sternebrae defects by codeine sulfate appear to be centered around Days 8–10 of gestation.

Another defect showing the most significant increase in incidence on both a fetal and litter basis with treatment on both Days 8 and 9 of gestation with 100 mg of codeine sulfate/kg was delayed ossification of the supraoccipital bone (Table II). Moreover, the degree of delayed ossification of the supraoccipital bone varied throughout the affected groups but was most severe in the fetuses of gravid mice treated on both Days 8 and 9 of gestation, often resulting in a split or missing supraoccipital bone. However, the range of days of gestation on which the mouse fetus was susceptible to the production of this defect by codeine was slightly smaller than the ranges for sternebrae defects and delayed ossification of the paws (Tables III and V). On a fetal basis, significant increases in the incidence of delayed ossification of the supraoccipital bone occurred with treatment on Day 8, 9, 10, or 11 of gestation; on a litter basis, only treatment on either Day 9 or 10 caused a significant increase in the incidence of this defect (Table II). Thus, the most susceptible period of

Table V—Occurrence of Delayed Ossification of the Paws

Treatment[a]	Litters Absent	Litters Present	Normal Fetuses	Abnormal Fetuses
Control (untreated)	7	1	40	1
Codeine on Day 7	3	4[b]	27	10[b]
Codeine on Day 8	0	8[b]	17	23[b]
Codeine on Day 9	1	6[b]	17	18[b]
Codeine on Day 10	0	6[b]	23	19[b]
Codeine on Day 11	1	5[b]	17	10[b]
Codeine on Day 12	4	2	22	6[b]
Codeine on Days 8 and 9	0	5[b]	7	22[b]

[a] The dose of codeine was 100 mg/kg sc in all cases. [b] Significance is indicated in p values of 0.05 or less when compared to control.

gestation of the CF-1 mouse fetus to the effects of codeine sulfate in the production of delayed ossification of the supraoccipital bone centers around Days 9 and 10.

The least frequent of the defects that increased significantly in a treated group was delayed ossification of the xiphoid. The most significant increase in the incidence of this defect on a litter and fetal basis occurred with treatment on both Days 8 and 9 of gestation (Table IV). However, with a single 100-mg/kg dose of codeine sulfate on a single day of gestation from Days 7 through 12, the only day of treatment showing a significant increase in the incidence of delayed ossification of the xiphoid on either a litter or fetal basis was Day 9 of gestation (Table IV). Although treatment on Day 7, 8, 10, 11, or 12 of gestation did not significantly increase the incidence of delayed ossification of the xiphoid and, in fact, treatment on Day 8 caused no incidence of delayed ossification of the xiphoid, treatment on both Days 8 and 9 of gestation with 100 mg of codeine sulfate/kg more significantly increased the incidence of this defect than treatment on Day 9 alone (Table IV). Thus, this finding again suggests that there is an additive or cumulative effect of the drug in the tissues that accounts for the increase in the teratogenic response.

In conclusion, the effects of codeine sulfate on the development of the CF-1 mouse fetus parallel the effects of morphine sulfate with regard to mean fetal weight reduction and the production of skeletal defects such as delayed ossification of the supraoccipital bone, sternebrae, and paws. However, the gross soft tissue defects, such as exencephaly and cryptorchid testes, and the gross skeletal defects, such as vertebral and rib fusions, present in the fetuses of mice treated on Day 8 or 9 of gestation with morphine sulfate (1, 7, 8) were not present in the fetuses of codeine-treated mice.

The major defects caused by codeine sulfate appear to parallel closely the minor defects caused by morphine sulfate. Consequently, the effects of codeine sulfate on fetuses of CF-1 mice appear to be those of a weakened morphine, which is not unexpected since about 10% of a dose of codeine is metabolized to morphine (16). Therefore, although the toxicity of codeine in mice when injected subcutaneously is at least twice that of morphine (13), the teratogenic potential of codeine sulfate in pregnant mice apparently is much lower than that of morphine sulfate. On the other hand, considering the additive or cumulative effect of codeine in regard to the teratogenicity demonstrated in this study and the known potentiation of the teratogenic effects of morphine by antihistamines and anticholinergics (7, 8), one cannot rule out the possibility of an enhanced teratogenic response to codeine when given in combination with either or both of the latter types of agents during early pregnancy.

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