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TERATOGENICITY STUDY OF AMMONIUM GLYCYRRHIZINATE IN THE SPRAGUE–DAWLEY RAT

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Abstract—Ammonium glycyrrhizinate (AG), a commercially used salt of glycyrrhizic acid, was administered in the drinking-water to Sprague–Dawley rats on days 7–17 of pregnancy. The actual intakes were 0, 21.33 ± 1.22, 238.75 ± 17.50 and 679.94 ± 69.87 mg AG/kg body weight/day for groups 0, 1, 2 and 3, respectively. AG caused polydipsia in the dams. Foetuses from the treated litters did not present an increase in external malformations, a decrease in weight or a decrease in the degree of ossification. However, there was a slight but significant increase in embryolethality and in the prevalence of external haemorrhages. Skeletal examination revealed a dose-related increase in minor anomalies, especially in the sternebral variants. Renal ectopy also increased significantly at the highest dose. These results indicate that the possible embryotoxicity of aromatizing compounds should be considered.

INTRODUCTION

Food additives (of which aromatizing compounds are a major group) are of growing importance in human nutrition. However, the toxicological evaluation of aromatizers is far from satisfactory. Generally, the problems concerning their effects on reproduction and the conceptus *in vivo* have not been investigated, although there is evidence of an embryotoxic–teratogenic activity in *in vitro* systems for some compounds (Abramovici & Rachmuth-Roizman, 1983).

Glycyrrhizic acid (GA) is an aromatizing compound, derived from liquorice, *Glycyrrhiza glabra* (*Merck Index*, 1976). GA, which is present in *Glycyrrhiza glabra* root at a concentration of about 5–7%, is used as an anti-ulcer drug (e.g. for registration requirements in US, see US Food and Drug Administration, 1985). Furthermore, its industrial salt, ammonium glycyrrhizinate (AG) is widely employed as an aromatizer in sweets, drugs, beverages (such as pastis), chewing gums, tobacco chews and toothpastes. Liquorice may induce acute and severe signs of hypokalaemia and hypertension in humans (for a clinical description see Cumming *et al.* 1980): biochemical effects were observed in healthy volunteers who ate 700 mg liquorice/day for 1 wk (Epstein *et al.* 1980). Renal and cardiac lesions were present in rats treated with 1000 mg liquorice/kg body weight (Girerd *et al.* 1958). Considering the widespread use of AG, it appeared worthwhile to assess its teratogenic potential *in vivo*.

The animals in the study reported here were administered the compound in the drinking-water in order to simulate the human exposure (continuous consumption of small doses). They were treated from day 7–17 of pregnancy, according to the Japanese Guidelines of Drug Toxicity Studies (Ministry of Health and Welfare, 1984). This procedure enables the observation of the compound's effect on a few important endpoints of the late embryonal and early foetal periods that occur at days 16–18 of pregnancy: final final configuration of heart and vessels, cloacal membrane division by the urorectal septum, palate closure, eyelid closure, beginning of ossification (Brock & Von Kreybig, 1964; Butler & Jurlink, 1987; Shepard, 1980).

Biochemical parameters (such as serum levels of aldosterone, sodium and potassium) were also tested in the dams, as indicators of maternal toxicity; histological examination of the adrenals was carried out as it was thought that these glands may also be affected (Girerd *et al.* 1958).

MATERIALS AND METHODS

Animals. One hundred female virgin Sprague–Dawley rats (175–200 g) were purchased from Charles River (Calco). They were kept in solid plastic boxes and given water and Open Formula rat diet (D. ri Piccioni, Brescia) *ad lib.* They were kept in an environmentally controlled room at a temperature of 22 ± 0.5°C and relative humidity of 50–60% with 12/14 air changes/hr and a 12-hr light/dark cycle. After 1 wk of acclimatization, the animals were mated with adult sires of proven fertility (with a male:female ratio of 1:2) and the mating success was monitored by vaginal smear the following morning: if spermatozoa were found, the mating was considered successful (and the day was designated day 0 of pregnancy). The pregnant females were then isolated and randomly assigned to the different experimental groups; with 18, 19, 20 and 16 rats in groups 0 (control), 1, 2 and 3, respectively.

Material. AG (of 99% purity), the commercial salt of GA, was purchased from Inverni della Beffa (Milano).

Treatment schedule. At 10.00 hr on day 7 of pregnancy, AG was administered in the drinking-water at

Abbreviations: AG = ammonium glycyrrhizinate; ETS = embryotoxicity score; GA = glycyrrhizic acid.

a concentration of 0, 10, 100 and 250 mg/100 ml to groups 0, 1, 2 and 3, respectively. Water consumption was recorded daily, and body weight and feed consumption were recorded every 3 days. At 10.00 hr on day 18 of pregnancy the treatment was suspended. At 16.00 hr on day 19 the feed was withdrawn. In the morning of day 20 the females were anaesthetized by an ip injection of sodium pentobarbital and subsequent exsanguination via cardiac puncture.

Parameters evaluated on the dams. Before cardiac puncture, the anaesthetized females were weighed. Blood was collected in tubes containing ethylenediaminetetraacetic acid and the following parameters were measured: levels of aldosterone (RIA, Packard gamma Counter, mod. 5130, Biodata kit), sodium and potassium (flame emission spectrophotometry, Perkin-Elmer mod. 5000). The adrenals were excised, weighed, fixed in Bouin's fluid and processed for haematoxylin and eosin histological staining.

Teratogenesis parameters. The ovaries were excised and the number of corpora lutea were counted. The uterus was removed and the following parameters were recorded: the total uterine weight; the number and position of implants, resorptions and dead foetuses in each uterine horn; the number, position, sex and weight of live foetuses; the presence of any external malformations and of haemorrhagic lesions and haematomas. The 'embryotoxicity score' (ETS) was evaluated for each litter as follows:

$$\frac{\text{No. of resorptions} + \text{no. of dead foetuses}}{\text{Total no. of implants}} \times 100$$

A foetus was defined as a 'runt' when it weighed $\leqslant 2$ SD compared with the average weight of the total live foetuses of the same sex of group 0. The foetuses from each litter were divided randomly into two groups of almost equal size. One group was fixed in 95% ethanol and underwent double staining with alcyan blue–Alizarin Red S (Whitaker & Dix, 1979) to evaluate: the variations of bone and cartilage structures; and the degree of skeletal ossification according to parameters chosen as indicators (namely: ossification of hyoid and pelvis, number of ossified sternebrae, metacarpi and coccygeal vertebrae). The other group was fixed in Bouin's fluid to be examined later for soft-tissue abnormalities according to Wilson's section method (Wilson & Warkany, 1965). Some haematomas were also examined histologically by means of the haematoxylin and eosin staining.

Data management and statistical analysis

Data for many of the parameters were collected both on a 'litter' basis and on a 'total foetuses' basis. The latter approach was preferred for infrequent and/or unevenly distributed parameters. The rate of litters showing a given abnormality, without regard to the prevalence inside the litter, was also taken into account.

Statistical analysis was performed using either parametrical (Lison, 1961; Senter, 1969) or non-parametrical tests (Siegel, 1967). Namely, one-way analysis of variance using Duncan's method of multiple comparisons was applied to the parameters evaluated on the dams and on the average weights/litter for male and female foetuses. The 2×2 chi-square test with the Yates' correction was performed on parameters of general embryotoxicity and on the data from the skeletal and the soft-tissue examination. The non-parametric Kruskal–Wallis test, equivalent to the analysis of variance, was applied to ossification parameters such as ossified sternebrae, coccygeal vertebrae and metacarpal bones. Finally, the parameters tested by means of the 2×2 chi-square test were also tested with the Armitage–Cochran trend test (Armitage, 1975) to assess whether a dose-response relationship was present. This test is more powerful than the chi-square test. Statistical significance was determined with regard to the actual intake of AG.

RESULTS

Parameters evaluated on the dams

No deaths or clinical signs attributable to the treatment were observed in any of the treated groups. Data on AG intake, body-weight increase (without the uterus), feed and water consumption and serum levels of aldosterone, sodium and potassium are summarized in Table 1. The average individual doses for the three treated groups were as follows (mean ± SEM); 21.33 ± 1.22 (group 1); 238.75 ± 17.50 (group 2); 679.94 ± 69.87 mg AG/kg body weight (group 3). No significant differences were found in the body-weight increase, either with or without the uterus, and feed consumption when compared with that of the

Table 1. Weight gain, food consumption and serum levels of potassium, sodium and aldosterone in dams given AG in the drinking-water on days 7–17 of pregnancy and killed on day 20

		Values (mean ± SEM) for dams in groups:			
Parameter	No. of dams...	0 18	1 19	2 20	3 16
Actual dose (mg AG/kg body weight/day)		0	21.33 ± 1.22	238.75 ± 17.50	679.94 ± 69.87
Body weight increase (%)†		21.87 ± 1.76	19.33 ± 1.71	19.48 ± 1.87	18.96 ± 2.22
Food intake (g)‡		289.28 ± 8.09	294.26 ± 7.93	293.30 ± 8.57	289.44 ± 11.03
Water intake (g)‡		464.90 ± 30.20	590.60 ± 30.62	660.20 ± 38.07*	732.31 ± 75.26*
Serum levels					
—potassium (mEq/litre)		5.10 ± 0.15	4.94 ± 0.15	5.11 ± 0.07	4.63 ± 0.09
—sodium (mEq/litre)		142.18 ± 1.19	140.73 ± 0.31	139.75 ± 0.55	138.78 ± 0.79
—Aldosterone (pg/ml)		883.64 ± 82.74	1214.55 ± 104.97	939.17 ± 110.58	877.78 ± 126.42

†Body weight increases were measured without the uterus.

‡Food and water consumption were measured between days 7–17 of pregnancy. Values marked with an asterisk differ significantly (Duncan's test) from that of the control group 0: (*$P < 0.01$).

Table 2. Parameters of embryotoxicity for rats given AG in the drinking-water on days 7–17 of pregnancy and killed on day 20

Parameter	Values for rats in groups: 0	1	2	3
No. of litters...	18	19	20	16
No. of corpora lutea	280	290	315	268
Pre-implantation losses				
—% of corpora lutea affected	13.2	9.6	8.6	4.5
—% of litters with foetuses affected	50.0	40.0	60.0	50.0
No. of implants	243	262	288	256
Resorptions				
—% of implants affected	7.0	3.8	6.2	9.0
—% of litters with foetuses affected	55.5	42.1	70.0	75.0
Dead foetuses				
—% of foetuses affected	1.2	1.5	2.4	2.3
—% of litters with foetuses affected	16.7	15.8	30.0	31.2
No of live pups	223	248	263	227
Male:female ratio	0.78	0.82	0.96	0.69
Pup weight/litter (g)†				
— Male	3.43 ± 0.10	3.51 ± 0.07	3.70 ± 0.13	3.54 ± 0.12
— Female	3.30 ± 0.10	3.34 ± 0.06	3.49 ± 0.11	3.36 ± 0.12
Runts				
—% of foetuses affected	1.8	1.6	3.8	1.8
—% of litters with foetuses affected	22.3	21.0	20.0	18.7
Haemorrhages and haematomas‡				
—% of foetuses affected	3.1	8.5*	6.5	7.5*
—% of litters with foetuses affected	16.7	68.4**	40.0	68.7**
ETS (%)	8.23	5.34	8.68	11.33
Grossly malformed foetuses (%)	0.45	0	0.38	0.44

ETS = embryotoxicity score

†These values are means ± SEM.

‡The values for the prevalence of affected foetuses and litters marked with asterisks differ significantly (chi-square test) from those of control group 0 (*$P < 0.01$; **$P < 0.005$).

control groups. Water consumption increased significantly ($P < 0.01$) in groups 2 and 3. No variations were observed in the biochemical parameters tested: potassium levels were slightly, not significantly decreased in group 3.

Histological examination of the adrenals revealed occasional cortical-cell spongyosis which was not treatment related. Hyperplastic nodules were observed in one dam from group 2 and two dams from group 3: the significance of this finding was not assessed.

General parameters of embryotoxicity

The results of the embryotoxicity are summarized in Table 2. It appears that the number of corpora lutea, implants and live foetuses per litter were comparable or even slightly higher in the treated litters than in the control litters. No significant differences were found in the number of pre-implantation losses. The prevalence of resorptions was significantly related to the dose ($P < 0.03$) when tested by means of the Armitage–Cochran test. No significant differences were found in the number of dead foetuses; however, four dead embryos from treated litters were noted for their marked underdevelopment. The ETS was found to be significantly dose related ($P < 0.01$) by means of the Armitage–Cochran test.

The average weight of the foetuses in the treated groups was comparable, or even slightly higher than that of the controls of the same sex, even when there were a lot of foetuses. The prevalence of runts was similar for groups 0, 1 and 3; the higher prevalence in group 2 was due to a litter with a particularly low average weight. No significant differences were found in the male:female ratios.

No conclusions could be drawn concerning the presence of grossly malformed foetuses as their occurrence was rare. The prevalence of external haemorrhages and haematomas and the rate of affected litters were significantly higher ($P < 0.05$ and $P < 0.01$, respectively) in groups 1 and 3 compared with that of the controls.

Skeletal examination

Results of the skeletal examinations are summarized in Table 3. The overall prevalence of minor skeletal variations was very high, especially with regards to sternebral anomalies (malaligned, asymmetric and bipartite sternebrae, hemisternebrae). In fact, these were so frequent in controls that they were considered as 'variants'. However, the prevalence of foetuses with some kind of skeletal anomaly and/or variant and that of foetuses with sternebral variants were significantly increased ($P < 0.001$) in the two higher dose groups with a dose-related pattern. The degree of ossification of the treated litters, as judged by the selected parameters, were equal or even higher than those of the controls. In fact, the average number of ossified coccygeal vertebrae was significantly higher ($P < 0.01$) in foetuses from treated litters; the presence of unossified hyoid bone was inversely related to the dose when tested by means of the Armitage–Cochran test ($P < 0.01$).

Soft-tissue examination

The results of the soft-tissue examinations are summarized in Table 4. A high background of minor anomalies in the controls was observed. These were mostly renal anomalies (renal pelvis dilatation, disappearance of papilla, dilatation of the ureters, rarely hydronephrosis): again, they were frequent enough to be considered as 'variants'. However, the prevalence of soft-tissue anomalies and variants was significantly dose related ($P < 0.03$) when tested by the

Table 3. Incidence of skeletal variations and anomalies, and ossification parameters in foetuses of rats given AG in the drinking-water on days 7–17 of pregnancy and killed on day 20

Parameter	Incidence of affected foetuses/litters in groups: 0 (No. of foetuses (litters) examined ...)	1	2	3
	113 (18)	127 (18)	134 (20)	118 (16)
Skeletal variants and anomalies				
—% of foetuses affected	15.0	24.4	39.5	32.2*
—% of litters with foetuses affected	61.1	100.0	100.0	100.0
Sternebral variants				
—% of foetuses affected	12.4	20.5	32.8*	29.7*
—% of litters with foetuses affected	55.5	94.4	95.0	93.7
Rib anomalies				
—% of foetuses affected	2.6	3.1	5.2	1.7
—% of litters with foetuses affected	5.5	22.2	35.0	12.5
Vertebral anomalies				
—% of foetuses affected	10.6	11.0	11.9	11.9
—% of litters with foetuses affected	27.8	33.3	40.0	37.5
Other anomalies (%)	0.9	0	0.7	0.8
Unossified hyoid				
—% of foetuses affected	15.0	13.4	7.5	6.8
—% of litters with foetuses affected	66.7	27.8	30.0	12.5
Unossified pelvis				
—% of foetuses affected	6.2	3.1	2.2	2.5
—% of litters with foetuses affected	22.2	11.1	15.0	12.5
No. of ossified†				
—sternebrae	4.60 ± 0.21	4.21 ± 0.22	4.92 ± 0.18	4.76 ± 0.23
—coccygeal vertebrae	2.75 ± 0.23	3.17 ± 0.16**	3.73 ± 0.19**	3.22 ± 0.06**
—metacarpi (one limb)	3.32 ± 0.09	3.46 ± 0.09	3.54 ± 0.09	3.46 ± 0.10

†These values are means ± SEM, those marked with asterisks are significantly higher (Kruskal–Wallis test) than those of control group 0 (**$P < 0.01$).

The values for the prevalence of affected foetuses marked with an asterisk differ significantly (chi-square test) from those of control group 0 (*$P < 0.001$).

Armitage–Cochran test. The presence of foetuses with renal ectopy and the rate of litters showing at least one foetus with this anomaly were significantly increased in group 3 ($P < 0.01$ and $P < 0.05$, respectively).

Some external haemorrhagic lesions (simple haematomas, without angiomatous or other more complex aspects) were observed histologically. Table 5 shows the parameters that exhibited a significantly dose-related trend.

DISCUSSION AND CONCLUSIONS

AG, the commercial salt of GA, proved to be somewhat embryotoxic in the study reported here. Signs of toxicity in the mothers were not evident, judging from the body-weight increase (without the uterus), feed consumption and biochemical and histological parameters. The decrease in serum potassium, observed at the highest dose, was not significant, but the water consumption increased significantly

Table 4. Soft-tissue examination by Wilson's method of foetuses of rats given AG in the drinking-water on days 7–17 of pregnancy and killed on day 20

Parameter	Incidence of affected foetuses/litters in groups: No. of foetuses (litters) examined ... 0	1	2	3
	113 (18)	123 (19)	135 (20)	115 (16)
Soft-tissue variants and anomalies				
—% of foetuses affected	48.7	41.5	54.0	55.6
—% of litters with foetuses affected	100.0	73.7	95.0	100.0
Renal variants†				
—% of foetuses affected	33.6	19.5**	27.4	25.2
—% of litters with foetuses affected	94.4	57.9	60.0	100.0
Ectopic or hypoplastic kidney				
—% of foetuses affected	6.2	13.8*	11.1	25.2**
—% of litters with foetuses affected	27.7	52.6	45.0	75.0*
Cerebral ventricular dilatation				
—% of foetuses affected	4.4	1.6	0.7	2.6
—% of litters with foetuses affected	27.8	10.5	5.0	12.5
Microphthalmia and microphakia				
—% of foetuses affected	3.5	1.6	0	2.6
—% of litters with foetuses affected	11.1	10.5	0	18.7
Internal haemorrhages				
—% of foetuses affected	9.7	16.3	23.0*	17.4
—% of litters with foetuses affected	50.0	52.6	80.0	62.5
Other anomalies				
—% of foetuses affected	2.6	0	3.7	0.9
—% of litters with foetuses affected	11.1	0	20.0	6.2

†Renal variants include pelvis dilatations, reduced or absent papilla, and dilated ureters.

The values for the prevalence of affected foetuses and litters marked with asterisks differ significantly (chi-square test) from those of control group 0 (*$P < 0.05$; **$P < 0.01$).

Table 5. The dose-response relationship for various foetal and implant parameters

Parameters	Significance of the trend
Resorptions*	$P < 0.03$
ETS*	$P < 0.01$
Skeletal variants and anomalies†	$P < 0.001$
Sternebral variants†	$P < 0.001$
Unossified hyoid†	$P < 0.01$ (negative)
Soft-tissue variants†	$P < 0.03$

ETS = embryotoxicity score
The parameters reported show a significant (Armitage–Cochran test) dose-response relationship with regard to the incidence on implants* and foetuses†.

at the two higher doses. Girerd *et al.* (1958) had previously observed polydipsia in liquorice-treated rats.

The evaluation of the embryotoxicity parameters was carried out either by means of the 2×2 chi-square test to compare the individual treated groups and the control group, and by means of the Armitage–Cochran test to highlight a significant dose–response relationship. Trend tests are currently used in carcinogenesis evaluation, but they are considered valuable in classical toxicological studies as well (Ludin, 1985).

In general, foetuses from the treated litters were neither smaller nor less mature (as judged by the ossification parameters) than those from the controls: there were even some indications that the reverse happened (a greater number of ossified hyoid).

Statistically significant differences were found for the parameters of embryonal and foetal toxicity (the prevalence of haemorrhages, skeletal anomalies in general, sternebral anomalies, ectopic kidney) when the values for the treated groups were compared to those of the control groups. A dose-related effect of treatment was identified with respect to the number of resorptions, ETS, skeletal anomalies, sternebral anomalies and soft-tissue anomalies in general.

No major external or internal anomalies were detected, while the increase in embryolethality (as indicated by the number of resorptions and ETS) was slight and it was only detected by means of a trend test. The significantly altered parameters are endpoints either indicating a potentially reversible effect, such as the sternebral variants (Collins *et al.* 1987) or partly subjective, such as renal ectopy, while the actual significance of foetal haemorrhages–haematomas is not satisfactorily assessed. Furthermore, sternebral anomalies had to be considered as 'variants', due to their high prevalence in the control group. The same applies to renal pelvis dilatation and related anomalies, for which no significant differences were detected: the significant decrease in the low-dose group was considered incidental.

However, our data give a clear indication of an undesirable, though slight, action of AG on the conceptus in the absence of any significant maternal toxicity, besides polydipsia.

The existing data concerning the embryotoxic potential of GA and related compounds, indicate that high concentrations of AG increased the resorption rate in a dominant-lethal test carried out on the mouse (Jorgeson & Rushbrook, 1978), but no significant effects were observed by Itami *et al.* (1985) in a reproductive study on the rat with disodium glycyrrhizinate.

An epidemiological study performed in Australia failed to show a relationship between the use of liquorice-based cough mixtures and the occurrence of abnormal neonatal outcomes (Colley & Gibson, 1982). Nevertheless, according to the data reported here, a dose-related increase in embryotoxicity and minor anomalies are associated with the oral administration of AG to the rat.

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