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THE EFFECTS OF ETHANOL ON RAT EMBRYOS DEVELOPING *IN VITRO*

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Abstract—A whole-embryo culture model has been developed as a means to study ethanol embryotoxicity. Ethanol exhibited a dose-related effect on Sprague–Dawley 10-day rat embryonic development over a 2-day culture period. Growth retardation was first detected at a concentration of 390 mg/100 ml and became more marked as the ethanol concentration was increased. At a concentration of 590 mg/100 ml there were signs of abnormal development and, since the embryos still appeared viable, that concentration was taken to be teratogenic. The possible significance of the high concentrations needed to cause effects in the present study in relation to rodent models of human ethanol embryotoxicity are discussed.

The current interest in alcohol as a teratogen was stimulated by reports from France [1] and the U.S.A. [2–4]. These reports described a pattern of malformations in the offspring of alcoholic mothers which was termed the Fetal Alcohol Syndrome (FAS).

The difficulty in studying the pregnant human population has created a need to develop animal models for the FAS. Such studies have helped our understanding of the role of alcohol in producing birth defects but, so far, have not helped to understand the mechanism by which ethanol affects the developing fetus. The present study was initiated to try and develop an *in vitro* embryo culture model for the study of FAS pathogenesis.

MATERIALS AND METHODS

Sprague–Dawley rats were supplied by the breeding unit of the Clinical Research Centre, Harrow. Animals were boxed overnight in pairs and the presence of a copulation plug on the floor of the cage was taken as evidence of mating (day 0).

The embryo culture technique was that of New *et al.* [5] as modified by Priscott [6] using roller bottles. Embryos, together with visceral yolk sacs and ectoplacental cone regions, were explanted on day 10, as described by New [7, 8]. The embryos had between 5 and 12 somites and were dorsally concave before culture (Witschi stage 15 [9]).

The serum for the culture medium was prepared by immediate-centrifugation [10] and frozen at −30° until use. The serum was also heat-treated at 56° for 30 min before use in culture media. For use, serum was diluted 50 per cent with Dulbecco's modification of Eagle's minimal essential medium and 100 I.U./ml benzyl penicillin and 100 μg/ml streptomycin sulphate (EDMEM).

Four or five embryos were placed in 30 ml all-glass reagent bottles containing 4 ml of culture medium. The cultures were gassed initially with 20% O_2/5% CO_2/75% N_2 and regassed at 24 hr with 35% O_2/5% CO_2/60% N_2. The medium was not replaced during the 48-hr culture period.

Ethanol (BDH, Poole) was assayed using a Pye 104 Gas Chromatograph (Pye Unicam Ltd., Cambridge) with a Chromosorb 101 column. The results were determined by triangulation of sample and internal standard *n*-propanol peaks and subsequent reference to a standard ethanol preparation (Sigma Chemical Co., London). Concentrations were calculated as mg/100 ml.

Treatment groups were compared for significant differences using Students' *t*-test.

RESULTS AND DISCUSSION

In an attempt to confirm that ethanol was directly teratogenic to the mammalian embryo, 10-day rat embryos were exposed to different concentrations of the drug during two days *in vitro* culture.

The lowest dilution in which an effect was detected was 390 mg/100 ml (Table 1). At that dilution, embryonic growth was significantly retarded as judged by crown–rump lengths ($P = < 0.001$ by Student's *t*-test, compared with the untreated control group). The numbers of somites formed were fewer

Table 1. Effects of ethanol treatment on rat embryo development *in vitro*

Ethanol concentration (mg/100 ml)	*N*	Crown–rump length (mm) ± S.E.M.	No. of somites ± S.E.M.
0	57	4.4 ± 0.05	32.0 ± 0.2
80	21	4.3 ± 0.07	31.4 ± 0.2
200	9	4.2 ± 0.07	31.7 ± 0.4
390	16	3.8 ± 0.09*	30.3 ± 0.4
590	13	3.2 ± 0.16*	28.1 ± 0.8*†

* $P < 0.001$ compared with untreated group by Student's *t*-test.
† $n = 12$, one not countable.

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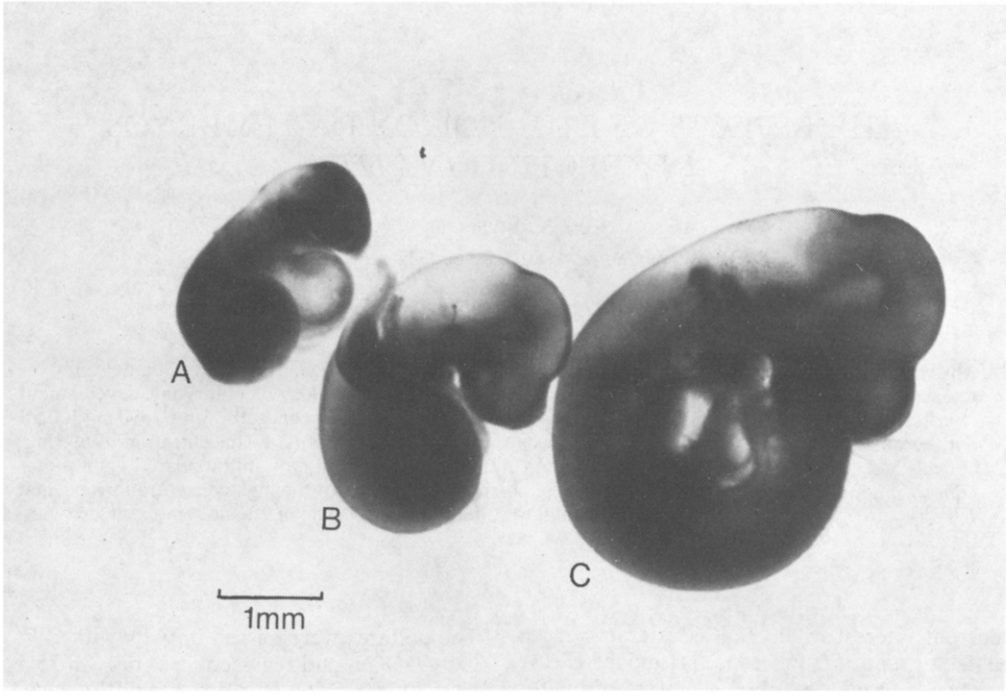

Fig. 1. Ten-day rat embryos after two days' growth *in vitro* in medium containing different ethanol concentrations. A, 790 mg/100 ml; B, 590 mg/100 ml and C, 80 mg/100 ml ethanol.

although the reduction was not significant ($P = > 0.10$, compared with the untreated control group). There were no apparent abnormalities of embryonic form but most were visibly smaller than control embryos. At higher medium concentrations of ethanol the embryotoxicity became more marked in a dose-related manner. Thus at 590 mg/100 ml there was a further reduction in crown–rump lengths and number of somites formed (Table 1), both reductions being significant ($P = <0.001$, compared with the untreated group). Furthermore, at this concentration there were signs of abnormal development. Six embryos had smaller mesen- and telencephalons (46%), 3 had abnormally large expansion of the roof of the hind brain (23%) and 2 had abnormal swelling of the pericardium. Representative embryos from 790 mg/100 ml, 590 mg/100 ml and 80 mg/100 ml ethanol treatment groups are shown in Fig. 1.

It was concluded that ethanol was growth-retarding at medium concentrations above 390 mg/100 ml and the effect was dose-related. Since all the embryos that received 590 mg/100 ml ethanol had beating hearts and appeared viable in spite of abnormal brain development, this concentration was considered to be teratogenic.

It was decided to attempt to determine whether or not ethanol was metabolically inert in the presently described culture system by determining the concentrations of ethanol during the culture period. The ethanol concentrations in the culture media declined gradually over the 48 hr period (Table 2). After 48 hr there was a significant reduction in the ethanol content both with ($P = <0.001$) and without ($P = <0.02$) embryos when compared with the starting concentrations. The ethanol concentration was lower when embryos were present but the difference was not significant ($P = >0.10$). The coefficient of variation of 6 replicate samples was 0.038. It therefore seemed doubtful that ethanol was metabolised to any significant extent by embryos or visceral yolk sacs in the culture system.

Until recently the only unequivocal evidence that ethanol itself was teratogenic came from studies of chicken embryos [11]. The present study extends this

Table 2. Ethanol concentrations during the culture of rat embryos

Culture bottle	Ethanol concentration (mg/100 ml)* at 0 hr	24 hr	48 hr
With embryos	875	785	745†
Without embryos	865	835	790‡

* Determined from two to six samples; coefficient of variation of six replicate determinations was 0.038.

† $P < 0.001$ compared with start of culture.

‡ $P < 0.02$ compared with start of culture.

evidence to the mammalian embryo, cultured *in vitro*, away from the confounding influences of the mother. A recent report that also used cultured rat embryos to study ethanol toxicity also demonstrated a growth-retarding effect [12]. The concentrations of ethanol incorporated into the medium were chosen to reflect the concentrations of ethanol found in human chronic alcoholics. Interestingly, their highest concentration (300 mg/100 ml) was similar to the lowest having a detectable effect (390 mg/100 ml) in the present study. The mean lethal blood concentration in man is reported as 420 mg/100 ml whereas in the rat it is 930 mg/100 ml, approximately twice as much [13]. If the lethal blood concentration was related to the embryotoxic concentration in both species, then the concentrations required in the rat embryo culture system may be twice as much as those required to cause effects in man.

The highest blood alcohol concentration reported in rats during *in vivo* studies was 267 mg/100 ml in animals given 6.0 g/kg ethanol daily by intubation [14]. The offspring were retarded but no increase in the malformation rate occurred when compared with control treatment animals that were pair-fed isocalorically. Of seven rat studies reported in the literature, only one found any malformations associated with ethanol treatment [15] while four found retardation only [14, 16–18]. On the other hand, of four studies in the mouse [19–22], only one [18] failed to detect an increase in the number of malformations at the end of gestation after ethanol administration. Blood ethanol concentrations tended to be greater in the mouse, which may be significant. The rat is more tolerant to ethanol [13] and teratogenic insult [23] than the mouse, so that the moderately higher blood ethanol concentrations in the mouse represents a much greater dose for that species; perhaps more than the threshold for teratogenicity. This contention is supported by recent studies involving cross-breeding experiments with several strains of mice having differing susceptibilities to ethanol embryotoxicity. It was demonstrated that blood ethanol concentration alone determined whether or not the offspring would be affected [21].

It should be noted that the present study and that of Brown *et al.* [12] have related to a more or less constant exposure of the embryo to ethanol and therefore bear no relation to the effects of a single exposure, although the studies may be of relevance to the human chronic alcoholic.

The present study failed to demonstrate any metabolism of ethanol by the 10-day rat embryo-yolk sac complex during two days of culture which was in accordance with existing experience [24]. The first metabolite of ethanol, acetaldehyde, was not measured in the present study but one would expect its concentration in the culture medium to have remained very low; partly because of a lack of alcohol dehydrogenase and also because any that formed spontaneously would have been rapidly metabolised or evaporated to the gas phase of the cultures.

Nevertheless, the idea that acetaldehyde played little part in the genesis of the ethanol effect needs more study since Véghelyi and his colleagues [25] have proposed that acetaldehyde is the primary teratogen. Such an approach is currently under investigation using the above culture system.

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