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THE EFFECTS OF ACETALDEHYDE AND 2,3-BUTANEDIOL ON RAT EMBRYOS DEVELOPING *IN VITRO*

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Abstract—The embryotoxicity of two ethanol metabolites, acetaldehyde and 2,3-butanediol, have been examined in cultured 10-day Albino Wistar rat embryos over a 2-day period. At acetaldehyde concentrations of 100 and 260 μM, no significant effects were observed on embryonic protein, DNA, somite development, gross morphology, or viability. 800 μM was overtly toxic causing rapid death and necrosis. 2,3-butanediol at a culture medium concentration of 25 mM had no effect on any of the above parameters. The significance of these results in relation to other animal experiments with these compounds and possible ramifications regarding the aetiology of the human Fetal Alcohol Syndrome are discussed.

The mechanism by which alcohol damages the developing human embryo to give rise to infants with what has been termed the Fetal Alcohol Syndrome (FAS) [1] is still poorly understood, despite much recent research [2, 3]. Animal models have demonstrated effects of ethanol on reproductive outcome [2], but teratogenicity has only consistently been demonstrated in mice [4, 5, 6].

Rat post-implantation embryo culture studies were able to demonstrate growth retardation by ethanol at concentrations that may be attained by chronic alcoholics [7, 8] but overt maldevelopment only occurred at unrealistically high concentrations [8]. Since these early embryos do not possess alcohol dehydrogenase, the observed effects could be attributed to the direct action of ethanol on the embryo.

The high concentrations of ethanol needed to cause effect could have been due to species differences in the susceptibility to ethanol or may have reflected the absence of a more toxic product of ethanol metabolism. The latter possibility is of particular importance in view of the hypothesis that proposes that acetaldehyde is the primary teratogen in the causation of the FAS [9, 10]. Recently it has been shown that when circulating acetaldehyde levels are raised there is a resultant increase in the circulating 2,3-butanediol (hereafter referred to as butanediol) concentration [11]. This is of interest because butanediol embryotoxicity has not previously been considered in relation to the FAS. Also, in developing the acetaldehyde toxicity hypothesis, importance was placed in experiments where rats were given ethanol, disulfiram or both together. Embryotoxicity was only observed in the latter group, which was ascribed to raised circulating acetaldehyde concentrations as a result of inhibition of the aldehyde dehydrogenase [9]. However, Veech and his colleagues have shown that in the rat, dosing with disulfiram or calcium carbimide together with ethanol also leads to the production of butanediol [11]. Since this compound has been found to be raised in a proportion of chronic alcoholics it should be considered for its potential embryotoxicity in relation to the FAS.

In this study the rat post-implantation embryo culture model has been further developed to study the embryotoxicity of acetaldehyde and butanediol.

MATERIALS AND METHODS

Albino Wistar rats were supplied by the Animal Resources Centre, Murdoch, W.A. Animals were mated overnight and the presence of sperm in the vagina taken as indication of pregnancy (day 0).

Embryos were explanted on day 10 by previously described methods [8, 12]. Briefly, uteri were aseptically removed and the decidua dissected free. The deciduum and underlying trophoblast and parietal yolk sac were then carefully removed leaving the visceral yolk sac intact.

The culture medium consisted of 70% rat serum, prepared by the 'immediate centrifugation' method [13], diluted with Dulbecco's modification of Eagles minimal essential medium (EDMEM), Flow Laboratories Ltd., North Ryde, Australia, to which was added 100 IU/ml benzyl penicillin and 100 μg/ml streptomycin sulphate. The medium was equilibrated with 20% O_2:5% CO_2:75% N_2 and regassed after 24 hr with 40% O_2:5% CO_2:55% N_2. The embryos were floated in the medium in 60 ml reagent bottles washed for tissue culture and rotated on a modified haematological roller [14].

After culture, embryos were evaluated for their morphological development using a dissecting microscope. The number of somites were counted and embryos homogenised by ultrasonication and the protein content measured by the method of Lowry [15], and DNA by the fluorometric method of Hinegardner [16].

Assay for acetaldehyde was done using a spectrofluorimetric method [17]. Samples were deproteinized with 4% v/v perchloric acid and neutralized with 0.85 M dipotassium carbonate. The reaction mixture consisted of 0.75 mg NAD^+,

0.45 mmole KCl, 90 μmole $Na_4P_2O_7$ (pH 8.0) and 7.5 μmole mercaptoethanol to which was added 100 μl of sample in quartz curvette. When the endogenous fluorescence value had stabilized, 15 μl of 0.5 units aldehyde dehydrogenase (ALDH, Sigma Chemical Co., St. Louis) was added and the fluorescence read in a Perkin-Elmer 3000 fluorescence spectrometer at 20°. Standard acetaldehyde concentrations were prepared in culture medium and the sample acetaldehyde concentrations extrapolated from the standard curve. The limit of detection was about 5 μM and the coefficient of variation (C.V.) of the assay was 0.14.

Assay for butanediol was done using a Shimadzu mini II gas chromatograph with a column of Chromosorb 750 (Alltech Associates, Summer Hill, N.S.W., Australia) coated with Triton X-405 (Technicon Chemicals, North Ryde, N.S.W., Australia). The column temperature was 160° and flame ionization detector temperature was 200°. The limit of detection for butanediol was about 5 μM.

The treatments were assessed for statistically significant differences by Students *t* test.

RESULTS AND DISCUSSION

Normal development

At the start of culture embryos were dorsally concave, had open neural tubes and between 5 and 12 somites (Fig. 1A). During the two days of culture the embryos turned to the ventrally concave position, had closed neural tubes with extensive head development, had formed about 33 somites and had good embryonic and vitelline circulations (Fig. 1B).

Acetaldehyde treatment

When embryos were exposed to acetaldehyde in the culture medium at concentrations of 100 and 260 μM there was no significant effect on the total embryonic protein, DNA, or the number of pairs of somites formed. Three of these embryos were seen to have a slightly abnormal curvature of the dorsal surface (Fig. 1C), probably associated with the process of axial rotation. This was the only morphological aberration in development seen (Table 1) and was judged to be of minor significance. A significant effect was seen only in the group exposed to 800 μM. This concentration caused a rapid cessation of growth and development such that at the conclusion of culture the embryos were no more than tiny unrecognisable necrotic masses (Table 1).

In developing an animal model it is important, as far as is possible, to choose experimental conditions that are likely to reflect the clinical situation under comparison. In the present context a number of preliminary experiments failed to find any acetaldehyde embryotoxicity in the range 10–100 μM. It was therefore decided to focus attention in the range 100–300 μM which was thought to include and, indeed, exceed the maximum acetaldehyde concentrations likely to be found in humans [18–20]. A smaller group of embryos was also exposed to a very high acetaldehyde concentration (800 μM) in order to document an effect, albeit at an unrealistically high concentration.

Thus, in the present study, the lack of any appreciable effect of acetaldehyde concentrations as high as 260 μM on embryonic growth or differentiation

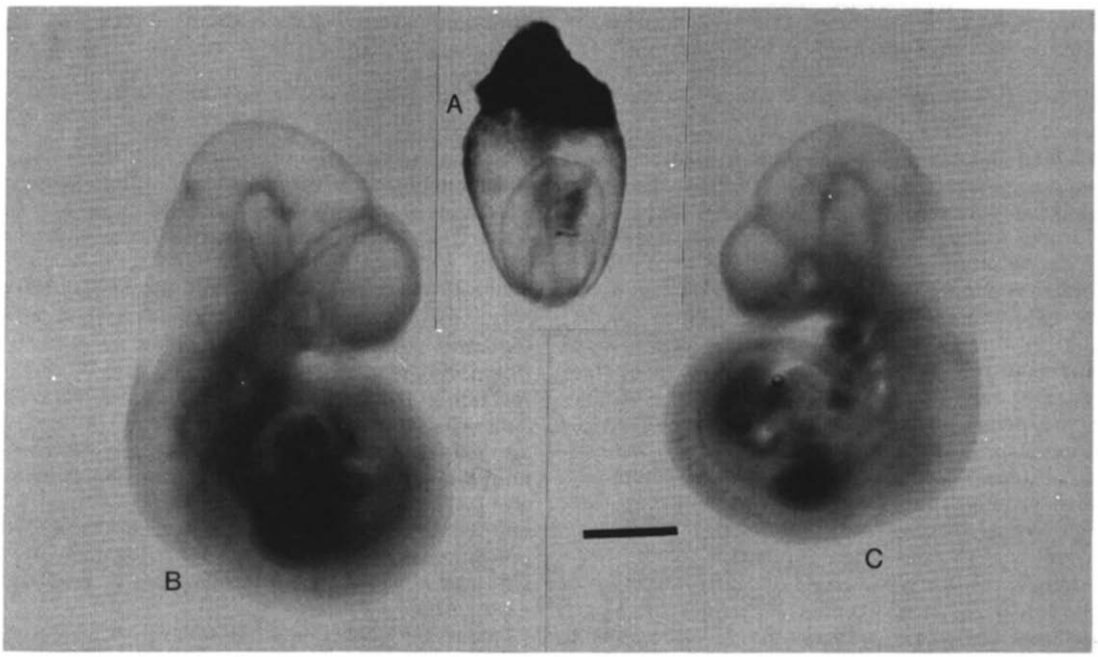

Fig. 1. Rat embryos before and after culture. (A) 10-day embryo prepared for culture shown with intact visceral yolk sac and ectoplacental cone; (B) control embryo after 48 hr culture with membranes removed; (C) embryo exposed to 260 μM acetaldehyde after 48 hr culture. Bar = 1 mm.

Table 1. The effects of acetaldehyde and 2,3-butanediol on 10-day rat embryo development *in vitro*

Treatment*	No.	Somites*	Protein (μG)*	DNA (μG)*	No. with heart beat	Normal gross morphology (%)
Control	18	33.6 ± 0.3	738 ± 49	73 ± 4	18	18 (100)
A_cH [μM]						
102 ± 6.7	17	33.8 ± 0.3	662 ± 31†	74 ± 4	17	16 (94)
260 ± 26.3	21	33.6 ± 0.2	628 ± 20†	73 ± 4	21	19 (91)
815 ± 24.6	6	Uncountable	67 ± 19	<5	0	0 (0)
2,3 Bd [mM]						
25	12	33.6 ± 0.3	698 ± 24†	78 ± 3	12	12 (100)

* ±S.D.
† $P > 0.5$ compared with control values.

argues against a role for acetaldehyde in the genesis of the FAS. In contrast to this, other workers have found dose-related embryotoxicity of acetaldehyde at similar developmental stages in rat embryo cultures [21]. Embryotoxicity was found at concentrations as low as 25 μM. Although these results seem to be at variance with those of the present study it is noteworthy that Sprague–Dawley rats were used rather than Wistar rats as described herein. We have taken care to document the amount of acetaldehyde present at the start of culture so that we can exclude the possibility that the compound had evaporated before the embryos were exposed. Thus, the simplest explanation is that the different findings are the result of the different strains of animals used in the two studies. Strain differences in the embryotoxicity of ethanol have been found in the mouse [4] that resulted from differences in the activity of alcohol dehydrogenase in the mother [5]. However, no such strain differences have yet been described in the rat with respect to either ethanol or acetaldehyde.

Three *in vivo* studies have investigated the effects of acetaldehyde on development. O'Shea and Kaufman [22, 23] injected acetaldehyde into pregnant CFLP mice and produced neural-tube defects in the offspring. The concentration of acetaldehyde reached in the circulation, however, was not documented, so it is possible that the observed effects were the result of unrealistic exposure to the agent. Using C57B1/6J mice Webster *et al.* [24] found acetaldehyde to be only weakly teratogenic at doses approaching the LD_{50} for mice. The only *in vivo* study in the rat demonstrated an effect of low blood acetaldehyde concentrations on DNA synthesis, especially in the central nervous system [25]. These workers did not find any excess of gross malformations at term, although there was a significant increase in the number of resorptions, suggesting a transient embryotoxicity. It can thus be seen that studies to date have argued both for and against a role for acetaldehyde in the genesis of the FAS. The species and strain of animal used for experiments have probably played a significant role in the conclusions drawn from the data.

2,3-Butanediol treatment

When embryos were exposed to butanediol in the culture medium at a concentration of 25 mM there was no significant effect on the total embryonic protein, DNA or the number of pairs of somites found (Table 1). There were no alterations in the gross morphology or viability of the embryos observed with a dissecting microscope.

Recent work with alcoholics indicates that butanediol may reach circulating concentrations in the range of 10–800 μM [26] although values as high as 8 mM have been recorded (Veech, personal communication, 1983). The compound was therefore examined for its embryotoxicity since no previous studies appeared to have addressed the question. No effects were seen on growth and development at up to 25 mM, a dose considerably greater than is ever likely to be encountered in the human alcoholic. A chemically similar compound, 1,3-butanediol, was not teratogenic in rats or rabbits [27]. It therefore seems unlikely, on the basis of the present results, that butanediol, *per se* plays a significant role in the genesis of the FAS.

In this paper, the normal development of rat embryos in the presence of high concentrations of acetaldehyde and butanediol is described and discussed in relation to other embryotoxicity data in rodents. That acetaldehyde can be toxic at a high enough concentration is beyond doubt. The essence of the problem is therefore, 1, what concentrations of acetaldehyde are reached in the pregnant woman, and, 2, what is the sensitivity of the human embryo to these concentrations?

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