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Early embryonic development in the rat following *in utero* exposure to alcohol and caffeine

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Summary. The influence of both alcohol and caffeine on early embryonic development was investigated in pregnant rats. Compared to the corresponding controls, a high incidence of resorptions and abnormal embryos was induced following treatment of the animals with alcohol (0.015 ml/g body weight, 12.5% v/v, i.p.) on gestational days 6 through 12 and with caffeine (25 mg/kg body weight, i.v.) on gestational day 10. In addition, embryonic growth was severely affected. Reduction of placental blood circulation and impairment of cellular proliferation may account for the observed deleterious effects on the embryo.

Key words: Embryopathy - Rat - Alcohol - Caffeine

lntroduction

The past few years have seen a surge of interest on the mutagenic and teratogenic potential of caffeine (Mulvihill, 1973; Thayer and Palm, 1975; Timson, 1977; Tarka Jr., 1982). Although no case of human malformations, attributed to caffeine, has been reported, the results of a large number of animal studies suggest that caffeine might be harmful to the fetus, so much so that the Food and Drug Administration of the United States urged recently that "pregnant women should avoid caffeine-containing food and drugs, if possible, or consume them sparingly" (Goyan, 1980; FDA, 1981).

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The fetal alcohol syndrome has been well described in humans and experimental studies in laboratory animals have confirmed the teratogenicity of ethanol (Abel, 1984; Rosett and Wiener, 1984).

Because of the widespread consumption of both caffeine and alcohol-containing beverages, we have studied their combined effects on the development of the early rat ernbryo.

Materials and methods

Albino Sprague Dawley rats of the Holtzman strain, weighing 250-300 g, were housed in wire mesh cages under controlled environmental conditions (temperature 72°F; 12 hour reversed day-night cycle) and maintained on standard rodent pellets and water ad *libitum*.

A male rat was caged together with two females overnight and allowed to mate. Vaginal smears were taken on the following rnorning and the presence of spermatozoa was considered to indicate day 1 of pregnancy. The animals were then randomly assigned to various treatrnent groups as shown in Table 1. The first group of animals was treated with a 12.5% v/v solution of alcohol in physiological saline (0.015 ml/g body weight), administered intraperitoneally, on gestational days 6 through 12. Caffeine (25 mg/kg) was administered intravenously via the tail vein, on gestational day 10, to a second group of pregnant animals. A third group of animals was treated with both alcohol and caffeine at doses as above. The control animals received physiological saline.

At 1400 hours on day 12 of pregnancy, embryonic age day 11.5, the animals were anaesthetized with ether and the uterine horns were removed and placed in Hank's balanced salt solution. In order to reveal the conceptuses, the uterine horns were opened along their

Table 1. Rat embryonic development following maternal treatment with alcohol and caffeine"

Groups of Animals	No. of Pregnant Animals	No. of Embryos**	Resorptions (%)***	Abnormal Ernbryos (%)
Untreated control	22	226	6 (2.6)	1 (0.44) (malrotation)
Alcohol-treated	12	150	15 (9.1) ($p<0.05$)	10 (6.7) ($p<0.01$)
Control to alcohol-treated	12	121	2 (1.6)	O
Caffeine-treated	12	143	23 (13.9) ($p<0.01$)	14 (9.8) ($p<0.01$)
Control to caffeine-treated	12	130	4 (3.0)	O
Alcohol and caffeine-treated	12	137	26 (16.0)**** ($p<0.01$)	21 (15.3) ($p<0.01$)
Control to alcohol and caffeine-treated	12	144	3 (2.0)	O

'Data subjected to Chi-square (X^2) test, p-values reflect significant differences between treatment and corresponding control groups.

** All ernbryos were morphologically scored, but for statistical analysis, 100 embryos were randomly selected frorn each group.

*** Resorption rates in all groups are expressed as a percentage of total implantation sites observed on gestational day 12 (ernbryonic age day 11.5).

**** One animal confirmed pregnant — resorbed all fetuses.

Table 2. Serum ethanol levels in pregnant rats

Groups of animals	Serum ethanol concentration (mmol/L) $\bar{X}$ + SDM
Alcohol-treated (N = 4)'	12.25 + 1.41
Control to alcohol-treated (N = 5)*	O ± 0
Alcohol and caffeine-treated (N = 8)*	10.36 + 2.84
Control to alcohol and caffeine-treated (N = 9)"	O + O

'Number of pregnant rats tested for serum ethanol.

antirnesornetrial border. Individual implantation sites were excised and transferred to a petri dish containing Hank's balanced salt solution. With fine Type 5 Durnont forceps and the aid of a stereo dissecting rnicroscope, the rnernbranes surrounding the ernbryo were removed to reveal the underlying visceral yolk sac. The diarneter of the yolk sac was rneasured using a rnicrorneter scale located in the eyepiece of the dissecting rnicroscope, after which the ernbryo was explanted and the following developmental' pararneters assessed (Brown and Fabro, 1981): crown-rump length, head length, degree of flexion (0 = ventrally convex, 1 = turning, 2 = dorsally convex, 3 = dorsally convex with spiral torsion), and the number of sornites present.

A sarnple of maternal blood was taken at the time of recovery of the ernbryos and the concentration of ethyl alcohol in serurn was deterrnined using the DuPont autornatic clinical analyzer.

Statistical analyses were perforrned using the chi-square test and Duncan's new multiple range test.

Results

The concentration of ethanol in maternal serum is shown in Table 2.

Ernbryotoxic and teratogenic effects of ethanol, caffeine, and ethanol cornbined with caffeine are shown in Table 1. The highest incidence of resorptions and abnorrnal ernbryos was induced in anirnals treated with both alcohol and caffeine.

In the alcohol-treated group of anirnals, ernbryonic growth was severely affected. The ernbryos displayed a significant decrease in yolk sac diameter, crown-rurnp length and head length, when cornpared to the corresponding control and the untreated control groups (Table 3). Two embryos were grossly underdeveloped for ernbryonic age day 11.5, lagging approxirnately 24 hours behind, and hence were of a developrnental stage characteristic of embryonic age day 10.5. One ernbryo failed to rotate. Another ernbryo was noticeably rnalrotated and did not achieve a dorsally convex orientation, but rather rernained concave and twisted, giving it a characteristic "sea-horse" type of appearance.

In the caffeine-treated group of anirnals overall ernbryonic growth was also retarded. The ernbryos showed a significant decrease in yolk sac diameter,

Table 3. Rat embryonic growth following maternal treatment with alcohol and caffeine*

Treatment group	Yolk sac Diameter (mm) $\bar{X}$ + SDM	Crown-rump Length (mm) $\bar{X}$ + SDM	Head length (mm) $\bar{X}$ + SDM	Flexion (0-3) $\bar{X}$ + SDM	Somite number (0-41) $\bar{X}$ + SDM
Untreated control	4.59 + .007	3.57 + .007	1.90 + .008	2.98 + .020	28.2 + .071
Alcohol-treated	4.35 + .018**	3.43 + .011**	1.71 + .010**	2.84 + .033**	27.9 + .080**
Control to alcohol-treated	4.58 + .009	3.54 + .008	1.81 + .080	3.00 + 0	28.7 + .073
Caffeine-treated	4.28 + .018**	3.38 + .011**	1.73 + .008**	2.88 + .027**	28.0 + .096**
Control to caffeine-treated	4.57 + .006	3.55 + .006	1.85 + .005	3.00 + 0	28.8 + .067
Alcohol and caffeine-treated	4.09 + .022**	3.17 + .018**	1.60 + .014**	2.78 + .040**	27.5 + .099**
Control to alcohol and caffeine-treated	4.55 + .008	3.54 + .008	1.82 + .008	2.98 + .010	28.6 + .084

*Statistical differences between treated and control groups analyzed by Duncan's New Multiple Range Test.
**$p < 0.01$

crown-rump length and head length when compared to the corresponding control and the untreated control groups (Table 3). One embryo was grossly underdeveloped for embryonic age day 11.5 and it appeared as a saccule-like configuration within the visceral yolk sac. According to normal developmental charts, this embryo appeared to be at embryonic age day 9.5. Malrotation was evident in three embryos. These embryos were at the "turning stage" (morphological score = 2), a stage between being originally convex ventrally and definitively convex dorsally. Only in one ernbryo was the number of somites substantially decreased. Instead of having a range of 28-34 somites, the embryo had only 24. This reduction in the number of somites was responsible for the lower overall morphological score.

In those animals treated with both alcohol and caffeine, embryonic growth was significantly affected compared to the corresponding control group (Table 3). Yolk sac diameter, crown-rump length and head length in the alcohol and caffeine-treated animals were reduced further than if alcohol or caffeine had been administered alone. These three developmental parameters were found to be significantly different when compared to all the control groups. Eight embryos were growth-retarded and their developrnent did not correspond to their gestational age. Of these embryos, three were estimated to be at a stage of development equivalent to ernbryonic age day 9.5, while the other five displayed developmental features characteristic of a stage equivalent to embryonic age day 10.5.

Flexion of the embryo was affected. Six embryos were grossly malrotated, as well as being malformed. Four of the malrotated embryos showed central nervous system developmental defects coupled with a significant reduction in the absolute number of somites. With respect to this absolute number, the alcohol and caffeine-treated embryos differed significantly ($p < 0.01$) from those of the control groups (Table 1).

Discussion

Treatment of pregnant rats with ethanol significantly disturbed embryonic growth and induced a high incidence of resorptions and abnormal embryos. Because the embryos were recovered at day 12 of gestation, it is difficult to predict what the final outcome or extent of these developmental disturbances would have been at terrn. Compensatory repair and growth in the embryo has been suggested to explain why only few embryos from each litter of the ethanol-treated mothers are damaged (Anders and Persaud, 1980).

The number of resorptions and abnormal embryos found in the caffeine-treated group significantly differed from the corresponding control group. Kimmel et al. (1984) also reported a significant increase in resorptions following oral administration of caffeine to pregnant rats at a dose level of 120 mg/kg on day 12 of gestation. Even though epidemiological studies have found no real association between coffee consumption during pregnancy and adverse fetal outcome (Linn et al., 1982), the United States Food and Drug Administration still advised pregnant women to avoid caffeine-containing foods and drugs. The rationale for this is that caffeine is a methylxanthine which resembles the purines found in genetic material. Thus, caffeine possesses the potential to derange the processes involved in cell proliferation. Because it has been known for some time that caffeine readily crosses the placenta and reaches the fetus (Goldstein and Warren, 1962), the warning of the Food and Drug Adrninistration merits serious consideration.

ln the present study, yolk sac diameter, crown-rump length, head lengths, flexion and the number of somites were used to assess the growth of the embryo (Brown and Fabro, 1981).

Embryos from mothers treated with ethanol revealed a significant reduction in overall crown-rurnp length.

Head length was also affected in the ethanol exposed embryos. A large number of these embryos showed distortions in head shape. Such changes may account for some of the variations seen in craniofacial (Sulik et al., 1981) and central nervous system morphogenesis (Abel, 1984).

Other investigators have noted exencephaly (Fernandez et al., 1983) and disturbances in gastrulation and neurulation (Nakatsuji and Johnson, 1984) in the offspring of laboratory rodents following ethanol treatment. In monkeys, Scott and Fradkin (1984) discovered a peculiar head shape in one offspring of mothers treated with 5 g/kg/day of ethanol. X-ray of the skull revealed that the cranial bones were not orientated properly and that the peculiar head shape was due to a modelling effect caused by autolysis and intrauterine pressure.

The retarded embryonic growth of ethanol exposed embryos, as evidenced by the decrease in crown-rump length, head length and yolk sac diameter, is in agreement with the clinical observations of intrauterine growth retardation seen in children born to alcoholic mothers. Jones et al. (1982) believed intrauterine growth retardation to be caused by a reduction of blood flow from the maternal circulation through the placenta. Such a reduction in placental circulation was thought to severely reduce oxygen level and nutrients in the fetal circulation. Marquis et al. (1984) have reported that maternal alcohol consumption produces a significant reduction in fetal plasma glucose levels coupled with changes in fetal and maternal plasma amino acid concentration. These changes may ultimately limit the growth of the embyro.

The degree of flexion achieved by the ethanol-treated embryos varied. These changes ranged from failure to turn, to mild alterations, in flexion. It is ultimately through the state of flexion that one could predict the growth pattern of that particular embryo. Normally, proper flexion is reflected by orderly embryonic growth.

The developmental characteristics of embryonic growth following exposure to caffeine alone were all significantly affected in the present study. It is believed that maternal treatment with caffeine alters utero-placental circulation to such an extent that normal embryonic development is impaired (Adamsons et al., 1971; Chernoff and Grabowski, 1971).

Treatment of the pregnant animals with both ethanol and caffeine caused severe disturbances in embryonic growth. Yolk sac diameter, crown-rump length, head length, flexion and absolute somite number, were all significantly reduced compared to the corresponding control group as well as to the untreated control group.

Weathersbee and Lodge (1979) found that alcohol and caffeine both possess the biochemical ability to affect fetal levels of cAMP and, in doing so, potentially alter the course of fetal development. It seems that the complex and diverse cellular mechanisms which apparently operate through various cyclic nucleotides are able to become niodified upon the administration of alcohol and caffeine. For rhe fetus this is of crucial importance, because at this developmental stage cellular division and differentiation are the major events occurring. It is possible that these indirect effects in the mother, coupled with the direct effects of the agent on the embryo, jeopardize the normal completion of intricate developmental processes.

Short-term ingestion of ethanol impairs the elimination of caffeine in human subjects and dogs (Mitchell et al., 1983), as well as in rodents (Mitchell et al., 1982). The ability of caffeine to remain non-metabolized increases its transfer across the placenta and its accumulation in fetal tissues. Because caffeine is a mitotic poison, it may alter the development of the embryo by inhibiting or even arresting cell division. Cell division and cell proliferation contribute to the growth of the embryo. lmpairment of these cellular events could account for the lack of development or developmental delay observed in the alcohol and caffeine exposed embryos.

References

Abel E.L. (1984). Fetal Alcohol Syndrorne and Fetal Alcohol Effects. Plenurn Press, New York.

Adarnsons K., Mueller-Heuback E. and Myers R. (1971). Production of fetal asphyxia in the rhesus rnonkey by adrninistration of catecholamines to the mother. Arn. J. Obstet. Gynecol. 109, 248-262.

Anders K. and Persaud T.V.N. (1980). Compensatory ernbryonic developrnent in the rat following maternal treatrnent with ethanol. Anat. Anz. 148, 375-383.

Brown N.A. and Fabro S. (1981). Quantitation of rat ernbryonic developrnent *in vitro:* A rnorphological scoring systern. Teratology 24, 65-78.

Chernoff N. and Grabowski C.T. (1971). Responses of the rat foetus to maternal injections of adrenaline and vasopressin. Brit. J. Pharrnacol. 43, 270-278.

FDA (1981). Caffeine and pregnancy. U.S. Departrnent of Health and Hurnan Services. HHS Publication No. (FDA). 81-1081.

Fernandez K., Caul W.F., Boyd J.E., Henderson G.I. and Michealis R.C. (1983). Malforrnations and growth of rat fetuses exposed to brief periods of alcohol *in utero.* Terat. Carcino. and Mutag. 3, 457-460.

Goldstein A. and Warren R. (1962). Passage of caffeine into hurnan gonadal and fetal tissue. Biochem. Pharrnac. 11, 166-168.

Goyan J.F. (1980). Food and Drug Adrninistration News Release No. P 80-36.

Jones P.J.H., Leichter H.J. and Lee M. (1981). Placental blood flow in rats fed alcohol before and during gestation. Life Sciences 29, 1153-1159.

Kirnrnel C.A., Kirnrnel G.L., White C.G., Grafton T.V., Young J.F. and Nelson C.J. (1984). Blood flow changes and conceptual developrnent in pregnant rats in response to caffeine. Fund and Appl. Toxicol. 4, 240-247.

Linn S., Schoenbaurn S.C., Monson R.R., Rosner G., Stubblefield P.G. and Ryan K.J. (1982). No association between coffee consurnption and adverse outcornes of pregnancy. New Eng. J. Med. 306, 141-144.

Marquis S.M., Leichter J. and Lee M. (1984). Plasma arnino acids and glucose levels in the rat fetus and darn after chronic maternal alcohol consurnption. Biol. Neonat. 46, 36-43.

Mitchell M.C., Hoyumpa A.M., Schenker S. and Patwardhan R.V. (1982). Differential effects of chronic ethanol feeding on cytochrome P-448 and P-450-mediated drug metabolism in the rat. Biochem. Pharmacol. 31, 695.

Mitchell M.C., Hoyumpa A.M., Schenker S., Johnson R.F., Nichols S. and Patwardhan R.V. (1983). Inhibition of caffeine elirnination by short-term ethanol administration. J. Lab. Clin. Med. 101, 826-834.

Mulvihill J.J. (1973). Caffeine as teratogen and mutagen. Teratology 8, 69-72.

Nakatsuji N. and Johnson K.E. (1984). Effects of ethanol on the primitive streak of mouse embryo. Teratology 29, 369-375.

Rosett H.L. and Wiener L. (1984). Alcohol and the Fetus. A Clinical Perspective. Oxford University Press, New York.

Scott W.J. and Fradkin R. (1984). The effects of prenatal ethanol in cynomolgus monkeys. Teratology 29, 49-56.

Sulik K.K., Johnston M.C. and Webb M.A. (1981). Fetal alcohol syndrome: Embryogenesis in a mouse model. Science 214, 936-938.

Tarka Jr. S.M. (1982). The toxicology of cocoa and rnethylxanthines: a review of the literature. CRC Critical Reviews in Toxicology 9, 275-312.

Thayer P.S. and Palm P.E. (1975). A current assessment of the mutagenic and teratogenic effects of caffeine. CRC Critical Reviews in Toxicology 3, 345-369.

Timson J. (1977). Caffeine. Mutation Res. 47, 1-52.

Weathersbee P.S. and Lodge J.R. (1979). Alcohol, caffeine and nicotine as factors in pregnancy. Post Grad. Med. 66, 165-171.

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