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Morphometric Analysis of *Macaca nemestrina* Exposed to Ethanol During Gestation

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ABSTRACT This study was part of a multidisciplinary investigation of the effects of gestational ethanol exposure in nonhuman primates. Thirty-one pregnant *Macaca nemestrina* were exposed to weekly ethanol doses of 0.0, 0.3, 0.6, 1.2, 1.8, 2.5, 3.3, or 4.1 g/kg maternal weight. Dose cohorts 0.0 through 1.8 were exposed to the initial ethanol dose within 10 days postconception. Dose cohorts 2.5 through 4.1 received their initial dose after the fifth week of gestation. Morphometric analyses performed on cranial radiographs showed that animals exposed to high doses of gestational ethanol had, on average, slightly smaller, distorted crania than control animals. A dysmorphic, flat face characteristic of fetal alcohol syndrome was recognized in one animal of the 1.8 g/kg cohort. The animal that received the highest doses of gestational ethanol was microcephalic. Similar malformations were not seen with low ethanol exposures or in controls. These data suggest a pattern of cranial distortion that may be recognizable and characteristic of ethanol teratogenesis.

Fetal alcohol syndrome (FAS) is a pattern of malformation produced by gestational exposure to ethanol and is defined by: central nervous system dysfunction, growth deficiency, and a specific cluster of minor facial anomalies (Clarren and Smith, '78). Since growth and developmental delays are found in numerous birth defect syndromes, the specific cluster of minor facial anomalies is important in distinguishing FAS. The fact that facial anomalies have been recognized as causally related to prenatal ethanol exposure has given rise to speculation about the mechanism of teratogenesis, and the threshold and spectrum of ethanol teratogenesis at different gestational ages.

This study of facial form in nonhuman primates was part of a multidisciplinary investigation of gestational ethanol exposure. The methods of alcohol administration, handling of the pregnancies, pregnancy outcome results, overall physical condition, and cognitive and developmental performance of the infants have been detailed in previous reports (Clarren et al., '87b, '88). The purpose of the morphometric investigation was to use radiographs to characterize the crania and facial features of control animals and compare them with the features of animals exposed to ethanol during gestation.

MATERIALS AND METHODS

The 31 *Macaca nemestrina* used in this study were exposed prenatally to weekly doses of ethanol. The maternal oral ethanol dose assignments were 0.0, 0.3, 0.6, 1.2, 1.8, 2.5, 3.3, and 4.1 g/kg maternal weight. Those animals assigned to dose cohorts 1.8 g/kg and below received their initial weekly dose within 10 days after conception (referred to as "full gestational exposure" or "FGE"). Animals assigned to cohorts 2.5 through 4.1 g/kg received their initial

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TABLE 1. Distribution of the sample

	Maternal ethanol cohort								
	C[1]	C	1	2	3	4	5	6	7
Number of males	7	2	3	3	3	3	1	1	1
Number of females	8	3	3	2	2	1	1	2	0
Total subjects	15	5	6	5	5	4	2	3	1
Weekly maternal ethanol dose (g/kg)	0.0	0.0	0.3	0.6	1.2	1.8	2.5[2]	3.3[2]	4.1[2]
Cohort mean maternal peak plasma ethanol concentration (mg%)	0	0	23	61	131	215	262	427	539

[1]Control subjects from mothers who were not gavage fed glucose during gestation.
[2]Subjects received their initial ethanol exposure between 33 and 46 postconceptional days.

weekly dose between 33 and 46 days postconception (referred to as "delayed gestational exposure" or "DGE").

Standardized lateral cephalometric radiographs of their progeny were taken with the anesthetized animal seated in a cephalostat designed for nonhuman primate research. Lateral radiographs were taken at age 1 ($\bar{x}$ = 221 postconceptional days, range 195–281 postconceptional days), and at age 2 ($\bar{x}$ = 337 postconceptional days, range 285–370 postconceptional days). Frontal radiographs were made after sacrifice, with the preserved specimen heads oriented to a standard plane in the cephalostat. Age at sacrifice ranged from 330 to 381 postconceptional days, with a mean of 356 postconceptional days.

Fifteen additional sex- and age-matched control *M. nemestrina* were obtained from the primate breeding colony that had been the source for the ethanol cohort sample. Criteria for selection included: no intra- or extrauterine exposures to conditions or medicaments that might adversely affect craniofacial growth and ages comparable with those of animals in the cohort sample. These additional control animals had lateral cephalometric radiographs taken that were identical to the fetal ethanol cohort sample.

Table 1 illustrates the distribution of the sample. Each animal's identification number, sex, dose cohort, maternal peak plasma ethanol concentration (MPPEC), and postconceptional age for lateral and frontal radiographs are recorded for each animal included in this project in Table 2.

Identification of landmarks used for digitization was done with a magnifying glass to inspect radiographs illuminated on a viewbox in a dark room. The coordinates of the landmarks were recorded using a digitizing tablet and microcomputer. Figures 1 and 2 illustrate and define the landmark points used in the analyses.

The digitized points were analyzed using morphometric methods developed by Bookstein ('86). The methods quantify mean differences in cranial or facial shape, which may distinguish the experimental group from a control group. Data generated in a study of facial features of humans with FAS have been similarly analyzed with these methods, as introduced by Bookstein ('86).

Landmark analysis was carried out primarily in terms of triangles defined by sets of three landmark points. One edge of each triangle was arbitrarily selected and assigned a standard length with the point coordinates (0,0) and (1,0) in a Cartesian (x,y) system. All information about the shape of the triangle was contained in the relative x,y coordinates of the third point, the shape coordinates (Fig. 3A).

After standardizing a triangle, such as Bregma-Orbitale-Lambda, in the manner described above, shapes were plotted as an x,y scatterplot of the shape coordinates (Fig. 3B). The mean shape for a group of triangles is represented by the average value of its scatter of shape coordinates. The significance of the observed difference in mean shape between ethanol-exposed and unexposed groups was assessed using Hotelling's two-sample T^2 test applied to the bivariate shape coordinate data (Clarren et al., '87a). Multiple triangles describing cranial and facial shape were examined.

Some individual comparisons were made by superimpositions of outlines of the anterior cranial base anatomy. The anterior wall of the sella turcica, anterior contours of the middle cranial fossae, contours of the cribiform plate and the frontoethmoidal crest, and the cerebral surface of the orbital roofs were aligned by eye to achieve the best possible fit. These reference structures are commonly used in orthodontic diagnosis and research, as they are located outside of the face itself (Bjork, '60; Bjork and Skieller, '83).

TABLE 2. Subject number, sex, maternal ethanol cohort, maternal mean peak plasma ethanol concentration, and age at times of radiographs

Subject number	Sex	Maternal weekly ethanol dose (g/kg)	Mean maternal peak plasma ethanol (mg%)	Postconceptional age		
				Lateral X-ray 1	Lateral X-ray 2	Frontal X-ray
1	F	0.0	0	219	354	359
2	F	0.0	0	202	322	352
3	F	0.0	0	280	338	381
4	M	0.0	0	242	341	346
5	M	0.0	0	206	332	347
6	M	0.3	18	214	319	330
7	F	0.3	23	213	327	345
8	F	0.3	24	200	354	363
9	M	0.3	24	215	348	367
10	M	0.3	25	229	348	374
11	F	0.3	25	201	370	370
13	F	0.6	51	205	350	350
14	M	0.6	51	203	301	358
15	M	0.6	67	236	326	338
16	M	0.6	67	222	360	363
17	F	0.6	70	225	360	380
19	F	1.2	115	221	342	363
20	F	1.2	117	235	359	359
21	M	1.2	124	281	360	360
22	M	1.2	140	235	353	361
23	M	1.2	161	201	318	348
24	M	1.8	189	228	326	350
25	F	1.8	208	218	352	358
26	M	1.8	214	224	340	354
27	M	1.8	248	210	352	358
28	M	2.5[1]	260	206	324	355
29	F	2.5[1]	264	202	343	350
30	F	3.3[1]	419	199	330	338
31	M	3.3[1]	431	195	328	348
32	F	3.3[1]	432	210	331	344
33	M	4.1[1]	539	254	326	362
34	M	0.0	0	249	—	—
35	M	0.0	0	251	—	—
36	F	0.0	0	214	—	—
37	F	0.0	0	227	—	—
38	F	0.0	0	244	—	—
39	M	0.0	0	—	366	—
40	M	0.0	0	—	342	—
41	M	0.0	0	—	298	—
42	M	0.0	0	—	338	—
43	M	0.0	0	—	324	—
44	F	0.0	0	—	285	—
45	F	0.0	0	—	370	—
46	F	0.0	0	—	373	—
47	F	0.0	0	—	295	—

[1]Subjects received their initial ethanol exposure between 33 and 46 days postconception.
—, Data not collected.

The amount of facial and cranial growth for an individual may be evaluated by superimposing cephalometric tracings made from radiographs taken at different points in time (Ghafari et al., '87). Tracings of two age- and sex-matched subjects superimposed on the anterior cranial base have been used to qualitatively describe facial and calvarial shape differences between the subjects (Inouye et al., '85).

RESULTS

One FGE animal (no. 25) in the 1.8-g/kg cohort (MPPEC = 208 mg%) was dysmorphic, with a flat philtrum and a flattened midface (Clarren et al., '88). The facial shape coordinates of this animal did not differ statistically from the control group. However, superimposition of the radiographic tracing of animal 25 with an age- which facial characteristics contributed to

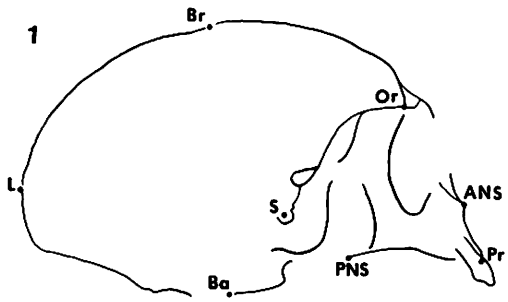

Fig. 1. Landmarks on lateral radiographs. Br, bregma, point at junction of coronal and sagittal sutures; Or, orbitale, junction of orbital roof and inner table of the frontal bone; S, sella, center of the sella turcica; ANS, anterior nasal spine; Pr, prosthion, most anterior, inferior point on premaxilla; PNS, posterior nasal spine; Ba, basion, most anterior point of foramen magnum; L, lambda, most superior point on lambdoid suture.

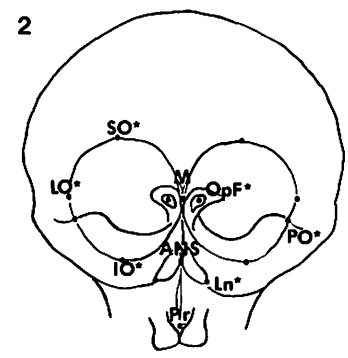

Fig. 2. Landmarks on frontal radiographs. *Bilateral points. SO*, most superior portions of orbital rim; LO*, most lateral portion of orbital rim; PO*, intersection of petrous portion of temporal bone and lateral orbital rim; OpF, center of optic foramen; M, intersection of medial portion of orbits with cribiform plate; IO*, most inferior portion of orbital rim; ANS, anterior nasal spine; LN*, most lateral portion of nasal cavity; Pr, most anterior, inferior point on premaxilla.

the clinical impression of dysmorphic features (Fig. 4). This animal had a dose schedule unique in the cohort; the dosing began on the third postconceptional day, with subsequent weekly doses.

Another animal exhibited microcephaly. This animal (no. 33) was a member of the delayed gestational exposure cohort and was exposed to the highest levels of ethanol in this study (MPPEC = 539 mg%). The head circumference was below the 99th percentile for the species at birth and at 6 months (Sackett et al., '75). The facial structure did not appear abnormal on examination of superimposed radiographic outlines or in terms of facial shape coordinates (Fig. 5).

Comparison of all ethanol-exposed specimens with the controls revealed no statistically significant differences in shape between the groups. Comparison of controls with individual FGE cohorts and with a cohort composed of all DGE animals yielded no significant findings. The small number of subjects in each cohort restricted the power of statistical tests. In subsequent analyses, the four highest FGE (1.8 g/kg) and the six DGE (2.5, 3.3, 4.1 g/kg) subjects were compared with a group combining the 16 low-ethanol exposure subjects with the 20 control animals.

Lateral analysis

There were no clearly significant relationships between facial shape coordinates and classification by ethanol exposure at either age. There was a difference in cranial shape, however, defined by the triangles within the quadrilateral Br-Or-S-L (Table 3). The mean cross-sectional area of Br-Or-S-L was approximately 3.5% smaller in high-dose animals (Table 4). The mean difference was statistically significant ($P \leq 0.04$, one-sided test) on the basis of a random effects regression analysis of log (area) appropriate for longitudinal data, which took into account each subject's sex, age at the times of radiography, degree of ethanol exposure, and the fact that most subjects had data collected at two ages while others were observed only once (Laird and Ware, '82). Figure 6 interprets this mean distortion in cranial shape with respect to approximate registration on Or-S. Mean two-dimensional cranial area in the high-dose animals was reduced by 1.7% compared with controls.

Frontal analysis

Morphometric analysis of multiple triangles describing facial shape and features in the frontal plane revealed no significant alteration of frontal facial features in the high-ethanol exposure group.

Digitization accuracy

Four radiographs were redigitized. The coordinates for homologous points were compared statistically using least squares

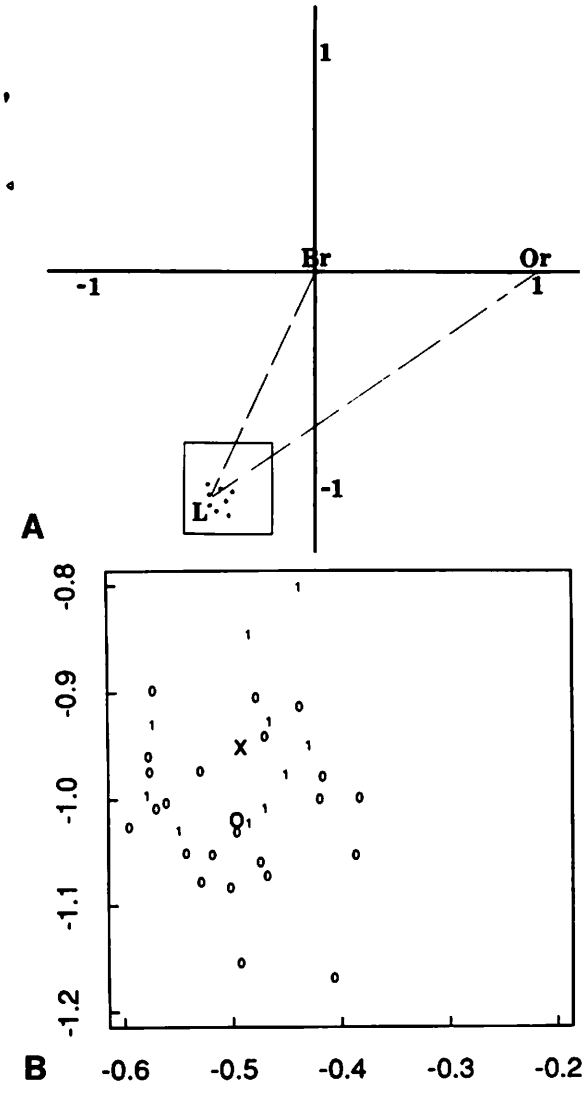

Fig. 3. A: Cartesian coordinates of scatterplot of shape coordinates for triangle Br-Or-L. B: Scatterplot of shape coordinates for triangle Br-Or-L. x,y coordinates for point L. O, control subjects; 1, high-dose ethanol subjects; 0, mean value of shape coordinates for control subjects; X, mean value of shape coordinates for high-dose ethanol subjects.

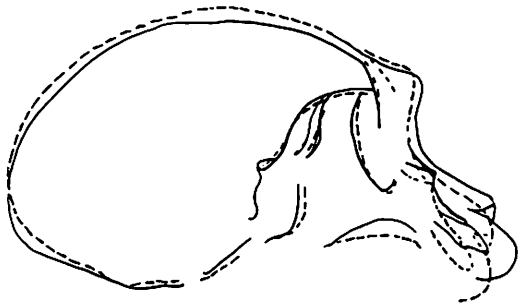

Fig. 4. Superimposition of radiographic tracings using "best fit" on anterior cranial base. Dysmorphic subject compared with age- and sex-matched control. Solid line, control (no. 1) female, 354 postconceptional days; dashed line, dysmorphic subject (no. 25) female, 352 postconceptional days.

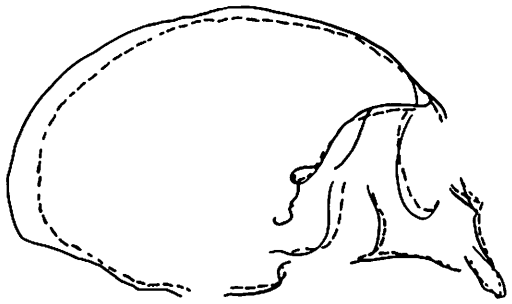

Fig. 5. Superimposition of radiographic tracings using "best fit" on anterior cranial base. Microcephalic subject compared with age- and sex-matched control. Solid line, control (no. 5) male, 332 postconceptional days; dashed line, microcephalic subject (no. 33) male, 326 postconceptional days.

TABLE 3. Statistical results of morphometric analysis of cranial shape

Age	No.	Landmarks	T-square	*P* value
1	33	Br-Or-L	6.11	.07
2	38	Br-Or-L	4.01	.16
1	33	Br-Or-S	3.88	.17
2	38	Br-Or-S	4.86	.11
1	33	Br-Or-L-S	6.39	.06[1]
2	38	Br-Or-L-S	5.23	.10[1]

[1]Computed from the average of the shape coordinates for triangles Br-Or-L and Br-Or-S.

analysis; digitization accuracy was determined to be high, with no shape coordinate varying by more than 2.6%.

DISCUSSION

It was hypothesized that a direct relationship would be found between increasing exposures to ethanol and the presence and degree of craniofacial malformations. The most severe craniofacial alterations were expected to occur in the group of animals that had been exposed to the higher weekly dose of ethanol from the beginning of gestation. The animal that was clinically dysmorphic was a member of the 1.8-g/kg cohort, the highest full-gestational dose exposure. The decreased protrusion of the midface, flattened philtrum, and long upper lip of

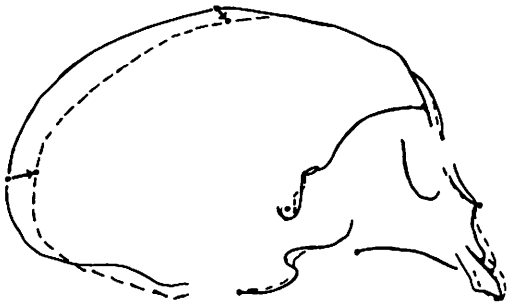

Fig. 6. Superimposition of radiographic tracings using "best fit" on anterior cranial base registered on points S and Or. High-dose ethanol subject compared with age- and sex-matched control. Solid line, control (no. 5) male, 206 postconceptional days. Coordinates of this subject were nearly identical to mean coordinates of the group combining the 16 low-ethanol exposure subjects with the 20 control animals. Dashed line, high-dose ethanol subject (no. 28) male, 206 postconceptional days. Coordinates of this subject were virtually coincident with mean coordinates of the group comprised of the 1.8-, 2.5-, 3.3-, and 4.1-g/kg cohorts. Arrows indicate the direction of distortion for points L and Br in the high-dose ethanol subjects, relative to the registration line S-Or.

TABLE 4. Longitudinal regression analysis of log of cranial area defined by Br-Or-S-L. (Seventy-two observations on a total of 45 subjects, 27 with area measured at two ages, 18 with area measured once)

	Coefficient[1]	Standard error
Age[2]		
Males	.00119	.00010
Females	.00101	.00012
Ethanol		
(1.8 g/kg)	.0353	.0207

[1]100 × (coefficient) reflects percentage changes in cranial area (because analysis is of log area).
[2]Postconceptional age in days.

animal 25 are all consistent with alcohol teratogenesis (Clarren and Smith, '78; Sulik et al., '81; Vtiz et al., '84). Other animals in the 1.8-g/kg cohort (nos. 24, 26, and 27) had been exposed to weekly plasma ethanol concentrations ranging from 189 mg% to 248 mg% but did not display perceptibly altered facial structures. Their calvaria were on average small and distorted when combined with the other high-exposure groups and compared with controls.

Apparently, early weekly gestational exposure to ethanol with peak plasma ethanol concentrations approximating 200 mg% can have an impact on facial form. Facial anomalies may relate to the specific dose schedule but could relate to other factors in teratogenesis, such as genetic susceptibility, maternal weight gain, or maternal alcohol dehydrogenase activity. Sulik suggested that a critical time period was likely to exist for the associated defects and malformations found in FAS (Sulik, '84). C57BL/6J mice exposed to ethanol doses ranging near 200 mg% on the seventh day of gestation had a 12% incidence of abnormalities of the nasal and upper lip regions that were characteristic of FAS (Sulik et al., '81; Sulik, '84). Newell-Morris and coauthors have demonstrated the craniofacial complex of *M. nemestrina* to be vulnerable to teratogenic influence by retinoic acid between days 20 and 44 of gestation (Newell-Morris et al., '80). Animal no. 25 was uniquely exposed to ethanol on days 24, 31, and 38, within this apparent period of embryonic vulnerability for facial anomalies. The DGE cohort animals received their initial ethanol doses (ranging from 260 mg% to 539 mg%) between days 33 and 46 postconception; no dysmorphic facial features were apparent in the DGE group. Further testing is needed to identify the critical period for the production of facial anomalies.

In this study, animals were sacrificed at age 6 months, which may explain the lack of significant findings in the analysis of facial features. A report detailing facial analysis of children exposed to varying gestational alcohol doses supported the contention that some children difficult to identify with FAS in infancy become more obviously affected later in childhood (Clarren et al., '87a). This will be investigated in a follow-up study in which animals exposed to gestational ethanol will be allowed to live to maturity.

The sole microcephalic animal in this study had been exposed to the highest dose of ethanol. This animal was part of the DGE cohort and had no ethanol exposure until the major period of organogenesis had ended. Microcephaly and scaphocephalic head shape have been reported in *M. nemestrina* exposed to an identical ethanol dose and schedule (Inouye et al., '85). Microcephaly, which is secondary to a diminished rate of brain growth, can be induced by weekly gestational exposure to extremely high doses of ethanol even if it is not administered until the major period of embryogenesis is completed.

In conclusion, a modest cohort ethanol effect was identified in this project, although the finding must be viewed as exploratory, since the analysis of animals

dosed at 1.8 g/kg or more was suggested by examination of the data rather than made as an a priori hypothesis. On average, animals exposed to high doses of prenatal ethanol had slightly smaller, distorted crania than control animals. Weekly exposure to gestational ethanol is therefore adequate to produce calvarial and facial alterations, but a dose-response threshold for dysmorphic effects and the time period critical for the effects have not been clarified. Further investigation using doses up to 1.8 g/kg administered early in gestation is planned to address these questions.

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