Acta orthop. scand. *54*, 104–112, 1983

AXIAL SKELETAL MALFORMATIONS ASSOCIATED WITH CRANIOSCHISIS APERTA AND EXENCEPHALY

The Result of Experimental Intervention after the Neural Tube Closure in Rats

R. PADMANABHAN & SHAMER SINGH

Department of Anatomy, Institute of Medical Sciences, Banaras Hindu University, Varanasi, India

Maternal administration of a single dose of cyclophosphamide (20 mg/kg) after the neural tube closure (on day 12) resulted in exencephaly and cranioschisis in 100% of rat fetuses at term. Extensive hemorrhages and edema were regularly associated with these defects. Alizarin-red stained skeletal preparations revealed absence of skull vault, premature closure of basicranial synchondroses, exaggeration of the craniovertebral angle, and agenesis and hypoplasia of the vertebrae, ribs and sternum. It is suggested that failure of the neural tube to close is not the primary cause of axial skeletal malformations and even after closure, the axial skeletal anlagen remains susceptible to teratogenic insult.

Key words: axial skeletal defects; cyclophosphamide; neural tube defects; rat embryos

Accepted 7.viii.82

Central nervous system (CNS) malformations constitute a sizeable percentage of the total incidence of congenital malformations second only to cardiac malformations (Myrianthopoulos & Bergsma 1979). Because of the serious functional derangements associated with such defects, in most instances they are brought to the notice of the clinicians and hence well documented. As a result, our knowledge about CNS malformation syndromes, their genetic and environmental determination, early diagnosis, prevention, and surgical and medical treatment has advanced considerably in the last few years (Myrianthopoulos & Bergsma 1979). But the etiological factors and pathogenetic mechanisms have remained largely unexplored. Tacit approval of von Rechlinghausen's (1889) theory of nonclosure of the neural tube as the cause of several CNS malformations has led to the widespread use of the term 'dysraphia' to denote anencephaly, exencephaly with cranioschisis, craniorachischisis, rachischisis, etc. Only recently was reopening of the closed neural tube and subsequent aberration by amniotic fluid put forward as an alternative mechanism of such severe anomalies (Gardner 1966, 1973, 1980), of course without much experimental evidence. The structural complexities of these defects could perhaps explain the controversy and lack of unifying concept about these malformations (Marin-Padilla 1980). Surprisingly in almost all clinical and experimental reports while so much is written about the neurological symptoms, little attention has been paid to the less obvious and often clinically silent underlying skeletal abnormalities associated with them (Kalter 1968, Karch & Urich 1962, Marin-Padilla 1965, 1980). The present study was therefore undertaken to induce CNS malformations in the rat after the neural tube closure and document the associated axial skeletal malformations.

MATERIAL AND METHODS

Female rats of CF strain (about 225 g) were mated with males of the same strain in the evening and pregnancy was confirmed by the sperm positive vaginal smear examined on the following morning. On day 12 of gestation (sperm positive day = day 0) i.e. well after the closure of the neuropores (Nishimura & Tanimura 1976, Wilson 1973) a single dose (20 mg/kg) of cyclophosphamide (Endoxan-asta, Khandelwal – Bombay), freshly prepared in distilled water, was administered. The control animals were untreated. Hind-Lever diet and tap water were provided *ad libitum* throughout gestation. On day 20, fetuses were collected by hysterotomy under ether anesthesia and were weighed individually and observed under a dissecting microscope for external malformations. Forty fetuses from seven experimental litters and 30 from five control litters were fixed in rectified spirit and processed for Alizarin-red stain according to Hurley's technique (1965). Intact skeletal preparations were observed under a dissecting microscope for axial skeletal malformations.

RESULTS

A single dose of cyclophosphamide (CPA) on day 12 of gestation resulted in exencephaly and cranioschisis in 100 per cent of living fetuses. Besides resorptions (Table 1) significant intrauterine growth retardation ($P < 0.001$), micrognathia, exophthalmia, cataract, cleft palate, subcutaneous and deep hemorrhages, edema, low set ears, digital and tail anomalies were almost invariably found (Figure 1). Histopathological details of exencephaly and cranioschisis will be communicated separately.

Skull bones (Table 2)

Since every experimental fetus was exencephalic with cranioschisis, the skull vault was obviously missing and the brain with the meninges re-

Table 1. Effect of maternal administration of CPA (20 mg/kg – day 12) on rat fetuses

	No. litters	No. implants	No. resorptions	Body weight (g) Mean ± SD	No. malformed
Control	5	38	2	3.92±0.18	0/36
Experimental	7	52	5	2.91±0.12*	47/47

* $P < 0.001$ when compared with control.

Table 2. Effect of maternal administration of CPA (20 mg/kg – day 12) on axial skeletal development in rat fetuses – Skull bones

Bones	% ossified	
	Control	Treated
Mandible	100.0	100.0 (no ramus)
Maxilla	100.0	97.3 (R)
Nasal	100.0	24.3 (R)
Frontal	100.0	64.9 (only orbital plate)
Squamous Temporal	100.0	0.0
Parietal	100.0	8.1 (R)
Squamous occipital	100.0	0.0
Supraoccipital	100.0	51.4 (R)
Exooccipital	100.0	45.9 (R)
Presphenoid	100.0	83.8 (R)
Basisphenoid	100.0	97.3 (R)
Basioccipital	100.0	97.0 (R)
Hyoid	100.0	0.0

R, rudimentary.

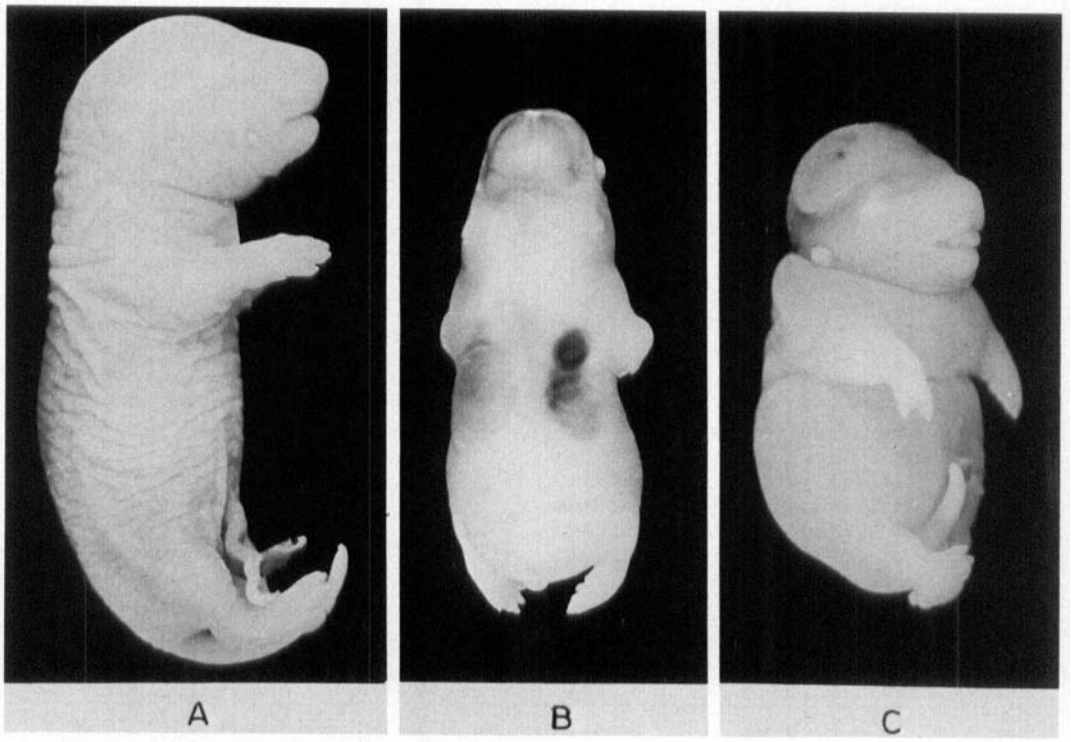


Figure 1. Gross appearance of rat fetuses on day 20 of gestation. A: Control. BC: Experimental. Note the cranioschisis, low set ears, hemorrhage (B), edema and digital malformations (C).

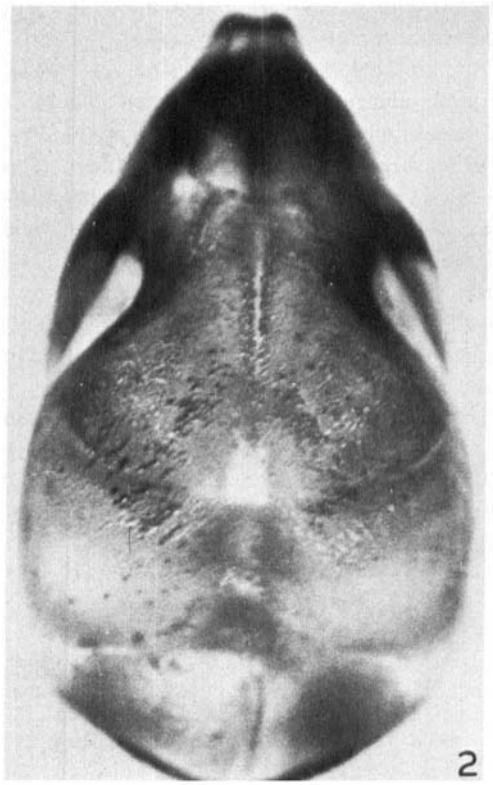
mained exposed. Except for the orbital part, the frontal bone was not identifiable (Figures 2–6). The squamous temporal and the squamous occipital were completely missing. Only in 8.1 per cent of cases could the parietal bone be identified, i.e. only its posterolateral margin. The exoccipital and supraoccipital were either totally absent or partially ossified. The presphenoid (83.8 per cent) was invariably very small. The basisphenoid was small and of varying shape. In two cases it was split. A striking feature in most of CPA fetuses was that the presphenoid-basisphenoid and the basisphenoid-basioccipital synchondroses had prematurely undergone synostosis (Figures 4–6). This had led to a gross reduction in the length of the basicranium and hence reduction in cranial volume. The ethmoid was poorly ossified in a few cases. The petrous temporal and tympanic bones had not ossified. The nasal bone was present in only 4.3 per cent of cases in a rudimentary form and the maxilla was extremely small. The hyoid bone could never be identified. The ramus of the mandible was absent in every case and the two halves of the body of mandible formed an acute angle.

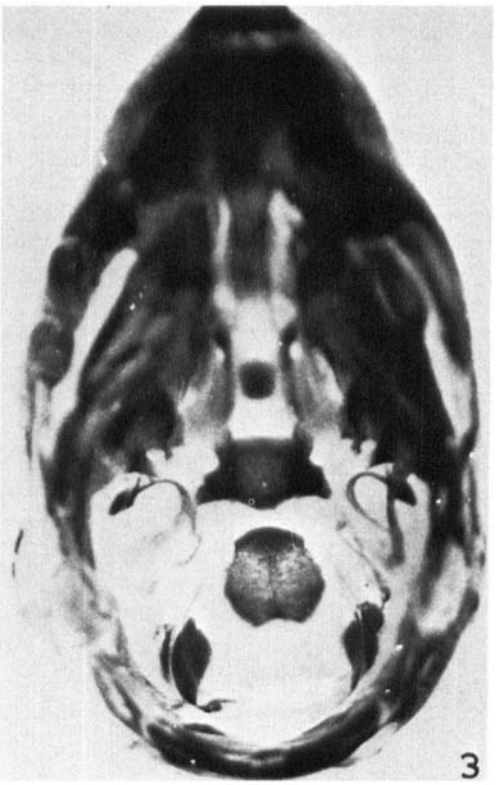
Sternebrae and ribs (Table 3)

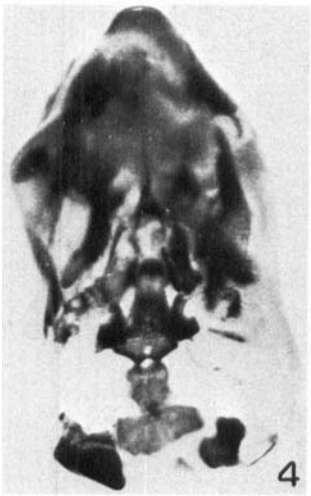
The sternebrae were the worst affected; only in 1.1 per cent of cases could a maximum of 1 sternebra be identified (Figures 7 and 8). All the control fetuses examined revealed 13 pairs of well ossified ribs (Figure 9) whereas only the upper 6–8 pairs of ribs had ossified in the experimental embryos; all of these were very rudimentary (Figures 10–14).

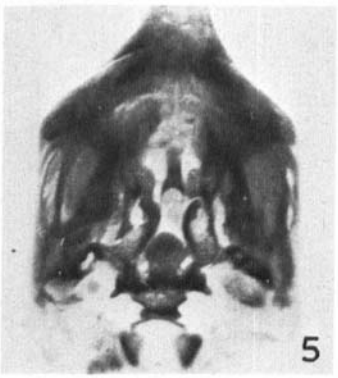
Vertebral column (Table 4)

The number of ossified vertebral arches and bodies were significantly ($P < 0.001$) reduced in CPA fetuses. The caudal arches were severely









Figures 2–4. Alizarin preparation of the skeletons of rat fetuses on day 20 of gestation.

Figure 2. The skull vault of a control fetus showing well ossified, frontal, parietal and squamous occipital bones. The nasal and zygomatic bones also have undergone ossification.

Figure 3. The base of the same skull shown in Figure 2 after removal of the vault. Note the vomer, presphenoid, basisphenoid, basioccipital, exoccipital, tympanic and petrous parts of the temporal bone, etc.

Figures 4–6. Experimental skulls. Observe that the skull vault is missing in every case. Gross distortion and underossification of the components of the basicranium premature closure of the presphenoid-basisphenoid and basisphenoid-basioccipital synchondroses and different shapes of these fused elements.

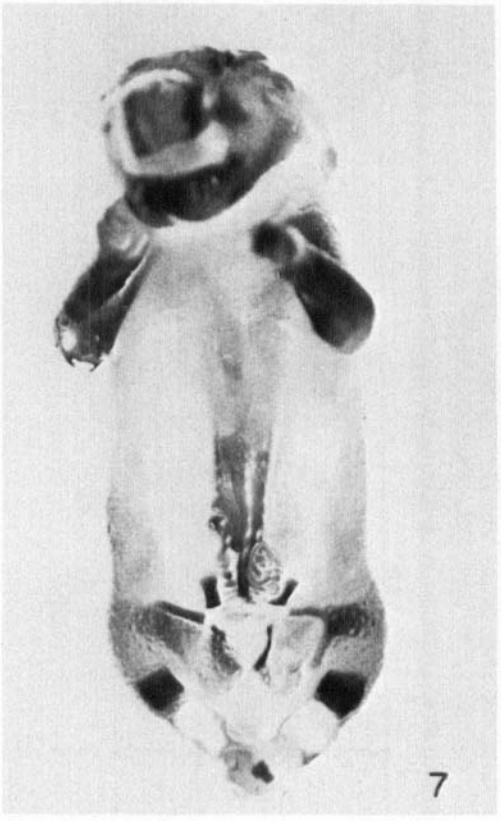
Table 3. Effect of maternal administration of CPA (20 mg/kg – day 12) on axial skeletal development in rat fetuses – Ribs and sternum

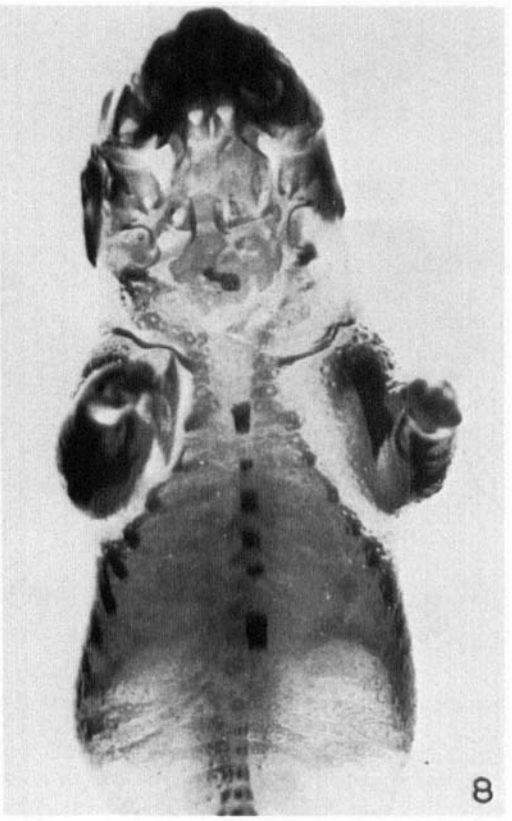
Bones	% ossified			
	Control		Treated	
	Right	Left	Right	Left
Ribs[a]	100 (13)	100 (13)	64.9 (1–8)	67.6 (1–6)
	Control		Treated	
Sternebrae[b]	100 97.5 (6)		1.1 100 (1)	

a, b, figures in parentheses indicate the range of ossified ribs (a) and sternebrae (b).

affected. The arches were longitudinally fused in most cases; the fusion involved mostly the cervical and thoracolumbar segments (Figures 10–13). Besides reduced ossification, the two halves of the vertebral bodies also showed longitudinal fusion. As a result, there was a median fissure. In 10 cases the lumbar bodies had fused longitudinally, obliterating the intervertebral articulation (Figures 10–12). In the absence of fusion, the vertebral arches were loosely held, leading to a considerable degree of scoliosis. Such loose articulation and scoliosis were more pronounced in fetuses with extreme edema (Figure 14).

The angle between the cervical spine and the basicranium (the craniovertebral angle) in CPA fetuses was considerably increased compared to the controls. There was extreme lordosis in severe cases of exencephaly.





Figures 7–8. Ventral chest walls of rat fetuses. No single sternebra is ossified in the experimental fetus (7) in contrast to the six sternebrae in the control (8).

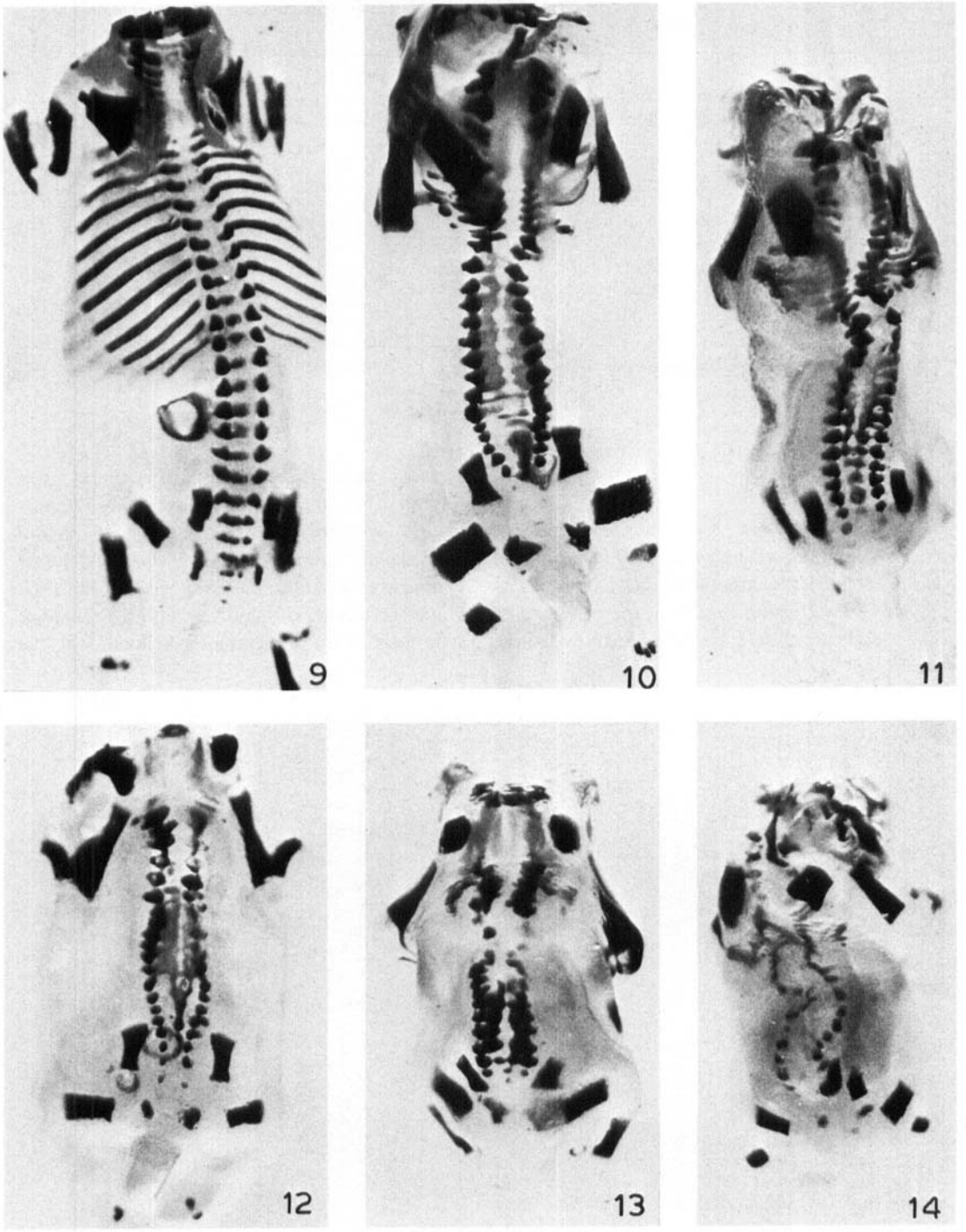


Figure 9. Dorsal view of the axial skeleton of a control fetus (the skull has been removed). All ribs, the vertebral arches and bodies have been distinctly ossified. Note also the caudal vertebral centra.

Figures 10–14. The skeleton of experimental fetuses showing scanty (10, 11) or no ossification of ribs, longitudinal fusion of vertebral arches and centra (10–13), absence of caudal vertebral centra, absence of centra and arches in midthoracic and cervical regions (13) and loose vertebral articulation and scoliosis (11 and 12). (In Figure 12, the skull has been removed).

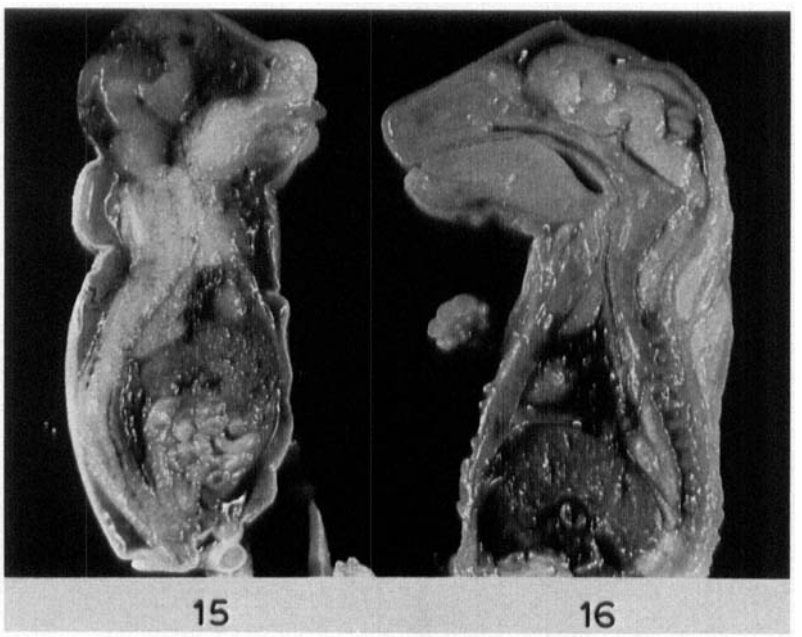
Table 4. Effect of maternal administration of CPA (20 mg/kg – day 12) on axial skeletal development in rat fetuses – Vertebrae

	% ossified									
	Cervical		Thoracic		Lumbar		Sacral		Coccygeal	
	C	T	C	T	C	T	C	T	C	T
Vertebral arches[a]	100.0 (7)	94.6 70.3 (2–7)	100.0 (13)	91.9 (5–13)	100.0 (6)	56.8 (4–6)	100.0 (5)	62.2 (2–5)	45.0 (1)	0
Vertebral bodies[b]	–	–	100.0 (13)	5.4 (1–7)	100.0 (6)	56.8 (1–6)	100.0 (5)	75.7 (1–5)	73.0 (3)	0

a, b, figures in parentheses indicate range of ossified arches (a) and bodies (b). C, control. T, treated.

DISCUSSION

The present study has clearly demonstrated that cranioschisis and exencephaly could be induced in the rat fetuses by a single injection of CPA (20 mg/kg) well after the closure of the neural tube. The spinal part of the tube was not exposed to the surface although spina-bifida occulta was observed in a number of fetuses. These abnormalities were certainly associated with serious axial skeletal anomalies such as agenesis and/or hypoplasia and retarded ossification of the skull bones, vertebrae, ribs and sternebrae. Premature synostosis of the basicranial synchondroses, the



Figures 15–16. Razor blade sections through mid sagittal planes of the experimental (15) and control (16) fetuses. Extensive hemorrhage in the brain, absent skull vault, exaggerated vertebrobasicranial angle, short skull base and edema are quite evident in the experimental fetus.

consequent shortening of skull base and craniovertebral lordosis possibly led to a reduction in the cranial volume. The resulting overcrowding in the cranium and the pressure offered by the distended brain contained therein probably caused cranioschisis and exencephaly. The extreme distension of the hindbrain vesicle (Figures 15 and 16) could explain how the overlying supraoccipital and exoccipital rudiments were disturbed in their growth and ossification. Unlike the brain, the spinal part of the neural tube was grossly undisturbed. But the vertebral elements had extensively fused, the fusion involving certain segments of the column. In the non-fused segments the intervertebral articulation was loose, resulting in scoliosis. These fusions and scoliotic changes and their consistent association with the general edema and hemorrhage of the fetus as a whole lead one to speculate about their early origin, i.e. the precartilaginous mesenchymal masses which are the precursors of the vertebral primordia were not allowed by the fluid filled (edematous) environment to assemble properly to form the anlagens. Such disorganized primordia should either fail to develop totally (agenesis) or develop partly (hypoplasia); the latter effect being intensified by the growth inhibiting action of CPA (Connors 1975). However, the exact mechanism by which CPA initiates hemorrhage and edema cannot be adduced from an investigation of this nature. Edema can result from altered osmoregulatory mechanisms (Grabowsky 1970), vascular lesions (Poswillo 1976) or infiltration of fluid from an overdistended neural tube (Grabowsky 1970, Gardner 1980). The extensive hemorrhages and hydrocephalic brains observed in this study point to such a possibility. In 1961 Gardner hypothesized that the presence of increased extracellular fluid can lead to disruption of the developing anlagens. Although his hypothesis was based solely on his clinical observations in which neural tube anomalies were consistently associated with several non-neural malformations (Gardner 1980), this is probably the first experimental report in support of such a theory. Primary failure of neural tube closure is believed to result from notochordal abnormalities (Feller & Sternberg 1929, Saunders 1943, Willis 1964), anomalies of somites (Gallera 1951), localised overgrowth of neural tissue (Patten 1953), abnormal equilibrium of cerebrospinal fluid (Strover & van der Zwan 1939), abnormal induction of neural plate by the underlying chordamesoderm (Hamilton & Mossman 1975) and defective mitotic activity in the neuroepithelium (Langman & Welch 1966). What most investigators have overlooked is the possibility that with the closure of the neural tube, the surrounding mesodermal precursor of the axial skeleton may not become invulnerable to teratogenic insults. That it is not really invulnerable receives ample experimental evidence from the present work in which the insult was initiated well after the neural tube closure.

Cyclophosphamide is a strong inhibitor of DNA synthesis (Connors 1975). So the mesodermal deficiency resulting from its antiproliferative activity upon the undifferentiated axial mesoderm, and the concurrent occurrence of hemorrhage and edema could have contributed to the disruption of precursor cells and displacement of anlagens leading to axial skeletal malformations. But the exact mechanism by which CPA triggers edema or hemorrhage awaits further clarification.

REFERENCES

Connors, T. A. (1975) Cytotoxic agents in teratogenic research. In: *Teratology: Trends and Applications* (Eds. Berry, C. L. & Poswillo, D. E.) pp. 49–79. Springer, New York.

Feller, A. & Sternberg, H. (1929) Zur Kenntnis der Fehlbildungen der Wirbelsäule. I. Die Wirbelkörperspalte und ihre formale Genese. *Virchows Arch.* **272**, 613–640.

Gallera, G. (1951) Influence de l'atmosphere artificillement modifiee sur le development embryonnaire due poulet. *Acta Anat.* **11**, 549–585.

Gardner, W. J. (1961) Rupture of the neural tube – the cause of myelomeningocele. *Arch. Neurol.* **4**, 1–7.

Gardner, W. J. (1966) Embryonic origin of spinal malformations. *Acta Radiol. Diagnosis* **5**, 1013–1023.

Gardner, W. J. (1973) *The dysraphic states from syringomyelia to anencephaly.* p. 201. Excerpta Medica, Amsterdam.

Gardner, W. J. (1980) Hypothesis: Overdistension of the neural tube may cause anomalies of nonneural organs. *Teratology* **22**, 229–238.

Grabowsky, C. T. (1970) Embryonic oxygen deficiency. A physiological approach to analysis of teratological mechanisms. In: *Advances in Teratology* (Ed. Woollam, D. H. M.) Vol. 4, pp. 125–167. Lagos Press, London.

Hamilton, W. J. & Mossman, M. H. (1975) *Human Embryology*, 5th edn. pp. 487–489. Macmillan Press, London.

Hurley, L. S. (1965) Demonstration "A". Alizarin staining of bone. Supplement to *Teratology Workshop Manual* Second Workshop in Teratology, Berkeley, California, pp. 121–122.

Kalter, H. (1968) *Teratology of the central nervous system.* The University of Chicago Press, Chicago.

Karchi, S. B. & Urich, H. (1972) Occipitoencephalocele. A morphological study. *J. Neurol. Sci.* **15**, 89–112.

Langman, J. & Welch, G. W. (1966) Effect of vitamin A on the central nervous system. *J. Comp. Neurol.* **128**, 1–16.

Marin-Padilla, M. (1965) Study of the skull in human cranioschisis. *Acta Anat.* **62**, 1–20.

Marin-Padilla, M. (1980) Morphogenesis of experimental encephalocele (carnioschisis occulta). *J. Neurol. Sci.* **46**, 83–99.

Myrianthopoulos, N. C. & Bergsma, D. (1979) Recent advances in the developmental biology of the central nervous system malformation. *Birth Defects – Original Series.* XV: 3. Alan R. Liss, New York.

Nishimura, H. & Tanimura, T. (1976) Survey of embryology and teratology. In: *Clinical Aspects of the Teratogenicity of drugs.* p. 4. Excerpta Medica, American Elsevier, New York.

Patten, B. M. (1953) Embryological stages in the establishing of myeloschisis with spina bifida. *Am. J. Anat.* **93**, 345–365.

Poswillo, D. E. (1975) Hemorrhage in development of the face. In: *Morphogenesis and Malformation of Face and Brain* (Ed. Bergsma, D.): *Birth Defects. Original Article Series* XI: 7, pp. 61–81. Alan R. Liss, New York.

Saunders, R. L. (1943) Combined anterior and posterior spina bifida in a living neonatal human female. *Anat. Rec.* **87**, 255–278.

Strover, W. F. H. & van der Zwan, A. (1939) Anencephaly and rachischisis posterior with the description of a human hemicephalus of 18 mm. *J. Anat.* **73**, 441–450.

Von Recklinghausen, E. (1886) Untersuchungen über die spina bifida. *Arch. Path. Anat.* **105**, 243–373.

Willis, A. (1964) *Borderland of Embryology and Pathology.* Butterworth & Co., London.

Wilson, J. G. (1973) *Environment and Birth Defect.* Academic Press, New York.

Correspondence to: Professor Shamer Singh, M.S., Banaras Hindu University, Faculty of Medicine, Department of Anatomy, Institute of Medical Sciences, Varanasi-221005, India.