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SKELETAL DEFECTS INDUCED BY CYCLOPHOSPHAMIDE (ENDOXAN-ASTA) IN CHICK EMBRYOS—PRELIMINARY REPORT

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Cyclophosphamide, a potent antitumour agent, recently synthesised and put to clinical use since 1958, belongs to the alkylating group of drugs used in cancer chemotherapy. It is not active *in vitro* as such and is believed to be transformed to an alkylating agent *in vivo* (Brock 1967). It has proved to be teratogenic to various laboratory animals, e.g. rat (Wilson 1964, Kreybig 1965, Kreybig & Schmidt 1967, Chaube et al. 1967, Singh 1970), rabbit (Gerlinger 1964), and mice (Shoji & Ohzu 1965, Gibson & Becker 1968). Chick embryos were the first to be subjected to the teratological effects of cyclophosphamide (Gerlinger et al. 1963) when this drug was injected into the albumen of the chick eggs prior to their incubation. There appears to be no subsequent report on the teratogenic effect of cyclophosphamide in chick embryos. This is a preliminary report describing the effects of cyclophosphamide when injected into the yolk sac during 1–6 days of the incubation period.

MATERIALS AND METHODS

Fertile eggs of the white leghorn chicken were obtained from a Government poultry farm. Fresh solution of the cyclophosphamide was prepared in normal saline every time prior to injection, and within 2 hours the injections were made into the yolk sac of the eggs by the technique described earlier (Tuli 1970). Whereas all the experimental eggs received 0.04 cc of the solution containing varying amounts of the drug, the control eggs received the same quantities of only saline. Some of the control eggs were also incubated without injection. Smaller doses of cyclophosphamide (0.005 mg, 0.01 mg and 0.02 mg) were injected on days 1–6 during incubation, along with corresponding controls in over 200 eggs, when days

Table 1. Lethal and teratogenic effect of cyclophosphamide in chick embryos

	Controls			
1. Day of treatment	4th		5th	
2. Amount of norm. saline in c.cs.	–	0.04	–	0.04
3. Quantity of the drug in mgs	–	–	–	–
4. No. of eggs used	20	25	25	25
5. No. of deaths	*1 (5%)*	*3 (12%)*	*1 (4%)*	*5 (20%)*
	8.8%		12%	
6. No. of abnormals	–	–	–	–

* Significant when compared with control on 5th day $P < 0.01$.
** Significant when compared with control on 4th day $P < 0.01$.
\+ Significant when compared with same dose on 4th day $P < 0.02$.
\++ Significant when compared with same dose on 4th day $P < 0.001$.
\+++ Significant when compared with 0.05 mg on 4th day $P < 0.001$.
° Not significant when compared with same dose on 4th day $P > 0.05$.

4–5 were found to be more susceptible with the higher dosage. Further injections in the experimental eggs were made on the 4th and 5th day of incubation with 0.035 mg to 0.08 mg of the drug in 500 eggs (Table 1). Control eggs were run with each experimental group. All eggs were opened on the 19th or 20th day of incubation and the chicks were killed by drowning in a tray of water. Gross malformations were carefully observed and recorded, and the specimens were weighed before preserving them in the fixatives.

OBSERVATIONS

In the first series of experiments (doses 0.005–0.02 mg) stunting of growth was more marked on the first three days of incubation, especially with the higher doses. However, moderate stunting was always noted in all cases when the drug was injected during the 5th day of incubation, even in the smallest doses. Ectopia viscerum and exophthalmos were found in a chick which received 0.002 mg of the drug on the 4th day of incubation. An interesting case of a chick with three beaks and three eyes was obtained with an injection of 0.005 mg of the drug injected on the 4th day; the case has been reported (Tuli & Singh 1970). This seemed to be an accidental finding, because this anomaly could not be reproduced when the same dose on the same day was repeated in another 150 eggs.

When higher doses of cyclophosphamide (0.035 to 0.08) were injected on the 4th and 5th day of incubation, the maximum number of

when injected into the yolk sac on the 4th and 5th day of incubation.

Experimental						
4th			5th			
0.04	0.04	0.04	0.04	0.04	0.04	0.04
0.035	0.04	0.05	0.035	0.04	0.05	0.08
90	130	30	30	120	50	50
33 (36.4%)	*31 (23.8%)*	*12 (40%)*	*3 (10 %)*+	*31 (25.8%)*°	*9 (18%)*°	*39 (78%)*+++
	30.4%**			32.8%*		
4 (4.4%)	7 (5.3%)	- (0%)	5 (16.6%)°	28 (23.3%)++	21 (42%)++	1 (2%)°

malformed embryos were observed in the latter group (Table 1). The higher dose of 0.05 mg on the 4th day of incubation was rather more lethal (40 per cent) than teratogenic (0 per cent). However, the same dose was found to be most teratogenic when given on the 5th day of incubation and produced malformation in the 21 surviving chick embryos out of the 50 eggs injected, i.e. 42 per cent (Figure 1). When the dose was increased to 0.08 mg, it proved to be most lethal for the 5-day-old embryo resulting in 39 immediate deaths out of 50 eggs injected (78 per cent). Malformations produced by cyclophosphamide injections (Figures 2 and 3) included defects in the eyes (e.g. exophthalmos, bleb formation over the eyes and absence of eye-lids), beak defects especially of the lower beak (shortening of the beaks, crossing of the beaks, parrot beak), limb deformities (shortening, curving and dislocation), toe defects (varying degrees of ectrodactylae, bending, and misdirection), exencephaly due to crania bifida, ectopia viscerum and stunting of growth (Table 2). The number of anomalies per malformed embryo was observed to be more in the group treated on the 4th day than in the one treated on the 5th day, i.e. 3/chick vs. 2.4/chick (Tables 1 and 2). Stunting was most marked with 0.05 mg doses injected on the 5th day of incubation resulting in an average wet weight of 15 g (average wet weight in controls was 25 g). Ectopia viscerum was the commonest malformation observed with practically all doses. In a severe type it presented liver and heart also. Some

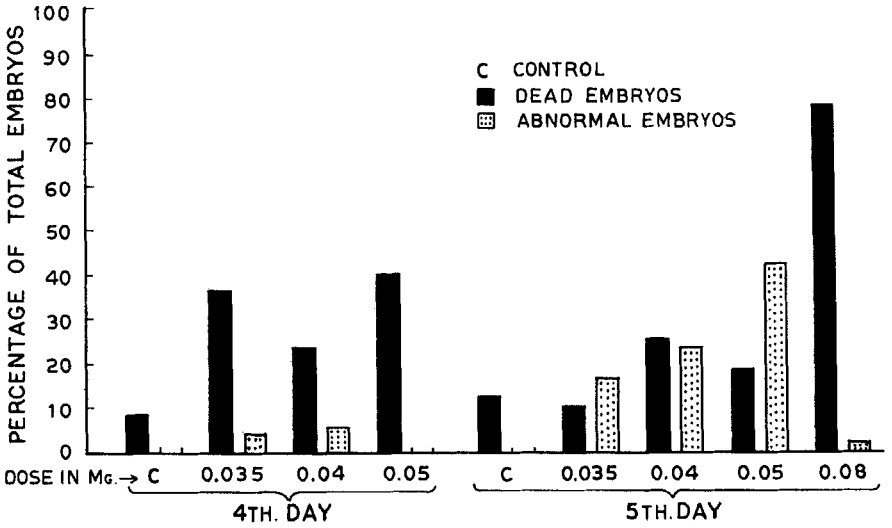

Figure 1. Lethality and teratogenicity induced by cyclophosphamide in chick embryos.

Table 2. Frequency of malformations observed during 19–20 days in chick embryos after cyclophosphamide injection into the yolk sac on the 4th and 5th days of incubation.

Day	4th day			5th day			
Doses	0.035 mg	0.04 mg	0.05 mg	0.035 mg	0.04 mg	0.05 mg	0.08 mg
Malformations							
1. Eye	1	2	–	–	5	3	–
2. Beak	1	3	–	–	6	9	–
3. Limbs	1	1	–	–	5	–	–
4. Toes	2	5	–	–	6	7	–
5. Exencephaly	1	2	–	–	4	2	–
6. Ectopia viscerum	3	7	–	3	12	8	1
7. Stunting (moderate)	2	1	–	5	17	8	–
8. Stunting (severe)	–	2	–	–	14	16	–
	11	23	–	8	69	53	1
Average wet weight in (Normal = 25 g)	23.3	21.3	–	24	18	15	–

N.B.: Multiple anomalies of the individual chicks have been counted separately.

Figure 2. Normal chick embryo 20 days old (control).

defects, e.g. scoliosis, meningocele, swelling in the neck region and rudimentary wings, were scattered in their occurrence.

DISCUSSION

This preliminary study has demonstrated that the cyclophosphamide when injected in the yolk sac of developing chick embryos produces deformities of the limbs, beak, skull, spine, and eyes, besides causing ectopia viscerum and stunting of growth in most of the cases. Some of the malformations, e.g. of the beak, limbs, eyes and inhibition of growth, have been described by Gerlinger et al. (1963) in their report where this drug was injected into the albumen of the chick eggs prior to incubation. However, the ectopia viscerum frequently observed with every dose of cyclophosphamide in the present experiments has not been mentioned by them. The exencephaly and meningocele have not been found either in their experiments, whereas the reduction in the tail described by them has not been seen in the present cases. These differences can be due to different times of injections (prior to incubation or during incubation) or due to the injection of the drug into different media (albumen or the yolk sac). All major organogenesis in the chick has begun by 48 hours of incubation, and as the chick embryo develops in a cleidoic system, any drug or dye injected before or during the first few hours of incubation will be present when the

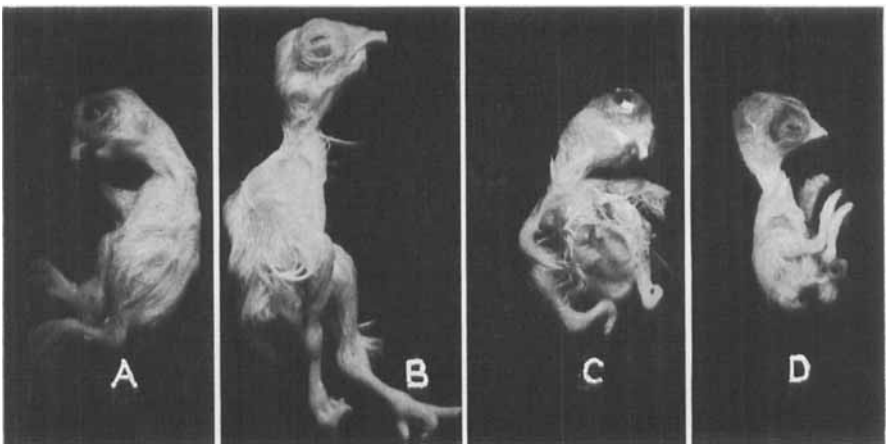

Figure 3. All embryos 20 days old.

A. Embryo showing parrot beak deformity, absence of eyelids and ectrodactylae induced by 0.04 mg of cyclophosphamide injected into the yolk sac on 5th day of incubation.

B. Embryo showing micrognathia of lower beak, rotation of right limb and ectrodactylae induced by 0.04 mg of the drug on 5th day.

C. Embryo showing advanced deformity of the limbs with a single toe in each, rudimentary wings, shortened lower beak, absent eyelids, marked ectopia viscerum showing liver and part of the heart and severe stunting of growth caused by 0.04 mg of cyclophosphamide injected into the yolk sac on 5th day of incubation.

D. Embryo showing multiple anomalies, e.g. rudimentary wings, short limbs with ectrodactylae, meningocele, shortened lower beak, bleb over the eyes, and ectopia viscerum and severe stunting of growth caused by 0.04 mg of cyclophosphamide injected in the yolk sac on 5th day of incubation.

embryo passes through later susceptible periods. The amount of the cyclophosphamide injected by Gerlinger et al. (1963) prior to incubation has been much more (0.1 mg) than that used by us during the 4th and 5th day of incubation (0.035 mg–0.08 mg), and it was probably available in a fair amount during a later period to cause some of the similar anomalies in their cases, or it has affected the primordium of these organs earlier at the stage of chemodifferentiation. The yellow yolk is about 8 times as viscous as the white, and the difference in viscosity of the media may account for the different rate of diffusion or migration of the drug to reach the developing embryo. Schlesinger (1958) stated that the migration of substances through the yolk depended upon the relative density of the yolk and that of the sub-

stance injected. Beaudoin (1961) found that the higher lethal and teratogenic effect of saline in one of his two groups of control eggs was due to mechanical factors involved in the technique of injection, i.e. in the white or the yellow yolk. These differences in malformations may also be due to other factors, since the lethal and teratogenic effects induced by cyclophosphamide in rat foetuses by different workers have also shown variations. Although Kreybig (1965) did not find any effect of the cyclophosphamide when injected into pregnant rats on the 11th day of gestation, Chaube et al. (1967) found that the same drug given on the 11th day of gestation produced brain and facial malformations in the litters. However, in experiments conducted by Singh (1970), cyclophosphamide caused resorption of all the embryos when injected in the mother rat on the 11th day of gestation.

Although the results of teratogenicity depend not only on the species but even on the strains of experimental animals (Cahen 1964), the malformations produced by cyclophosphamide in the chick and rat embryos have shown striking similarities. Anomalies of the head region, e.g. exencephaly, brachygnathia, meningocele, and opened eyes reported in rats and mice by different workers, have been seen in the chick embryos in the present preliminary study. Even the ram's head, i.e. thickening of the neck region described in rat foetuses (Kreybig & Schmidt 1967, Singh 1970) has also been found in these chick embryos. Limb deformities and anomalies of the toes induced by cyclophosphamide in rats (Chaube et al. 1967) and in mice (Gibson & Becker 1968) have also been produced in the chick embryos in the present investigation. The ectopia viscerum commonly observed in the chick embryos in this study has not been reported to be caused by cyclophosphamide in the rat, mice or chick embryos. These malformations are, however, known to be produced in chick embryos by other teratogenic agents as well. It is now established that apparently similar defects may be produced by agents of dissimilar nature (Landauer 1953, Duraiswami 1955). These substances may have acted at different times on the same series of developmental events, or entirely different events, all of which were necessary for the natural development of an organ, any part of the embryo, or the embryos as a whole. It seems obvious that such diverse substances which act as teratogenic agents must work primarily by interfering with some normal biochemical or metabolic activities of the developing embryonic cells and tissues, thus resulting in the abnormal development. Teratogenic specificity of any agent is reflected by the production of a syndrome of malforma-

tions characteristic of the particular agent, the nature of malformations being determined by the pathways of the action of the agent and how that relates to developmental events at the time of the treatment and afterwards.

Smaller doses of 0.035 mg have produced anomalies in 4.4 per cent of embryos when injected on the 4th day of incubation, as compared with 16.6 per cent produced by injection of the same dose on the 5th day (Table 2); the difference, however, is not statistically significant ($P < 0.1$). With doses of 0.04 mg the embryos affected on the 4th day were 7 out of the 130 eggs injected, i.e. 5.3 per cent as compared with the 23.3 per cent embryos affected on the 5th day by the same dose, the difference being highly significant ($P < 0.001$). Similarly the number of embryos affected by a 0.05 mg dose on the 5th day is much more (42 per cent), whereas none survived as a malformed embryo with the same dose injected on the 4th day ($P < 0.001$). The doses of 0.04 mg and 0.05 mg have thus proved most teratogenic in affecting the chick embryos on the 5th day of incubation (Figure 1). Although the embryos affected by the treatment on the 4th day have been significantly fewer as compared with the 5th day group, more embryos of the former group have shown multiple anomalies, e.g. 11 malformed embryos in the 4th day group have exhibited 34 anomalies (Tables 1 and 2), i.e. 3 per embryo as compared with the 5th day group ($131/55 = 2.4$ per embryo).

Lethal effects of the cyclophosphamide (Table 1) in the two experimental groups have not been much different, i.e. 30.4 per cent in the 4th day group and 32.8 per cent in the 5th day group ($P > 0.05$), although mortality with similar doses on the 5th day has been less (Figure 1). However, the mortality in each experimental group as compared with the corresponding control group has been significantly more ($P < 0.01$). The mortality rate in the control eggs themselves has been far greater in the group where normal saline was injected than in the noninjected eggs.

The mechanism by which cyclophosphamide produces these varied malformations cannot be adduced from the present findings. However, damage to the mesenchymal tissue is obvious. As the drug is inactive *in vitro* and becomes active only in the tissues of the host (mother/foetus) the transformation of the cyclophosphamide to an active alkylating agent in the chick embryo is positively occurring in the tissues of the embryo itself.

SUMMARY

Cyclophosphamide (endoxan-asta) when injected into the yolk sac of chick eggs has produced malformations of the beak, skull, spine, limbs and toes besides causing defects in the eyes, ectopia viscerum and stunting of growth.

Dosage of 0.04 mg and 0.05 mg on the 5th day of incubation has proved to be most teratogenic.

Malformations thus induced in chick embryos show a striking resemblance to the malformations produced by the same drug in rats and mice.

The brunt of the teratological effects of cyclophosphamide is mainly borne by the mesenchymal tissue.

REFERENCES

Beaudoin, A. R. (1961) Teratogenic activity of several closely related disazo dyes on the developing chick embryo. *J. Embryol. exp. Morph.* **9**, 14–21.

Brock, N. (1967) Pharmacologic characterization of cyclophosphamide (NSC-26271) and cyclophosphamide metabolites. *Cancer Chemother. Abstr.* **51**, 315–325.

Cahen, R. L. (1964) Evaluation of the teratogenicity of the drugs. *Clin. Pharmacol. Ther.* **5**, 480–514.

Chaube, S., Kury, G. & Murphy, M. L. (1967) Teratogenic effects of cyclophosphamide (NSC-26271) in the rat. *Cancer Chemother. Abstr.* **51**, 363–376.

Duraiswami, P. K. (1965) Comparison of congenital defects inducted in developing chickens by certain teratogenic agents with those caused by insulin. *J. Bone Jt Surg.* **37**-A, 277–294.

Gerlinger, P. (1964) Action du cyclophosphamide injecté à la mère sur la realisation de la forme du corps des embryons de lapin. *C. R. Soc. Biol. (Paris)* **158**, 2154–2157.

Gerlinger, P., Ruch, J. V. & Clavert, J. (1963) Action du cyclophosphamide sur la formation de l'embryon. *C. R. Soc. Biol. (Paris)* **157**, 173–176.

Gibson, J. E. & Becker, B. A. (1968) The teratogenicity of cyclophosphamide in mice. *Cancer Res.* **28**, 475–480.

Kreybig, T. von (1965) Die teratogene Wirkung Von Cyclophosphamide wahrend der embryonales Entwicklungsphase bei der Ratte. *Naunyn-Schmiedebergs Arch. exp. Path. Pharmak.* **252**, 173–195.

Kreybig, T. von & Schmidt, W. (1967) Chemisch induzierte Fetopathien bei der Ratte—Experimentelle untersuchungen uber die Wirkung Von cyclophosphamide und N-Methyl-N-nitroso—Harnstoff nach der Gade am 15 order 16. Tag der Gestation. *Arzneimittel-Forsch.* **17**, 1093–1100.

Landauer, W. (1953) Genetics and environmental factors in the teratogenic effects on chicken embryos. *Genetics* **38**, 216–220.

Schlesinger, A. B. (1958) The structural significance of the avian yolk in embryogenesis. *J. exp. Zool.* **138**, 233–258.

Shoji, R. & Ohzu, E. (1965) Effect of endoxan on developing mouse embryos. *J. Fac. Sci. Hokkaido Univ.* Ser. VI. **15**, 662–665.

Singh, S. (1970) The teratogenicity of cyclophosphamide (endoxan-asta) in rats. In press.

Tuli, S. M. (1970) Experimental methods of production of congenital skeletal malformations. In *Methods of surgical research,* ed. Udupa, K. N.& Singh, L. M., pp. 247–258. B. H. U. Press, Varanasi.

Tuli, S. M. & Singh, S. (1970) Partial anterior duplication (Anadidymus) of a chick. In press.

Wilson, J. G. (1964) Teratogenic interaction of chemical agents in the rat. *J. Pharmacol. exp. Ther.* **144**, 429–436.