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Craniofacial growth of immature rats following administration of vincristine and doxorubicin

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SUMMARY The aim of the present study was to investigate the possible short-term effect of two anti-neoplastic drugs, vincristine and doxorubicin, on the craniofacial skeleton in young rats. On the basis of findings from pilot experiments, one dose of 0.0375 mg/kg vincristine or 1.0 mg/kg doxorubicin was given parenterally to inbred Long–Evans/Turku rats at 10 or 30 days of age, and followed up until 30 or 50 days, respectively. Some 30-day-old rats received two additional doses of the drugs, 3 and 6 days after the first injection. Controls were given physiological saline. A total of 310 rats were used: 40 for the pilot study, 180 medicated, and 90 control animals for the experiment itself. The weights of the rats were recorded, a number of craniofacial dimensions were measured, and the neurocranial volume determined in the case of the most severely affected rats.

The weight gain of the younger rats was retarded, as was that of the older rats that received repeated drug injections. Most dimensions of the craniofacial skeleton were significantly smaller in the vincristine-treated young animals, and following multiple injections of vincristine or doxorubicin also in the older ones when compared with the controls. Contrary to the general pattern, the measurements of the foramen magnum increased in the older rats, a feature associated with the decrease in brain cavity volume observed in those that received vincristine.

These findings indicate that anti-neoplastic agents can have a short-term adverse effect on the craniofacial growth and that the morphological changes are differential, rather than uniform.

Introduction

The anti-neoplastic drugs, vincristine and doxorubicin, are commonly used in the treatment of childhood cancers, including acute leukaemia, lymphoma, sarcoma, Wilms' tumour, and neuroblastoma. The cytotoxic effect of vincristine, which is a member of the vinca alkaloid group, is primary related to its ability to inhibit mitotic spindle formation, causing metaphase arrest during mitosis. The most important mechanism of anthracyclines, including doxorubicin, is associated with topoisomerase-mediated DNA damage (Balis *et al*., 1997). Chemotherapeutic drugs result in acute toxicities, which often are transient, but the growing child may be more vulnerable to the delayed adverse sequelae of therapy, such as effects on growth, fertility and neuropsychological function (Blatt *et al*., 1997).

Decreased linear growth is a common problem during anti-neoplastic therapy in children. Although catch-up growth may occur, short stature is permanent or even progressive in some instances (Blatt *et al*., 1997). Cranial irradiation is regarded as the most important factor in long-term retarded growth, while the role of chemotherapy is still controversial (Ogilvy-Stuart and Shalet, 1995; Ilveskoski *et al*., 1997).

Radiation to the head and neck of young cancer patients reportedly causes microcephaly, i.e. reduced head circumference (Waber *et al*., 1990) and abnormalities of the maxillofacial skeleton (Nwoku and Koch, 1975; Jaffe *et al*.,

1984; Berkowitz *et al*., 1989; Kaste and Hopkins, 1994). A detailed analysis of the craniofacial structures of children subjected to cranial or total body irradiation as a part of anti-neoplastic therapy, revealed that the constituents of the facial skeleton appear to be selectively affected, in that the reduction in length was, for instance, more pronounced in the mandible than in the maxilla (Dahllöf *et al*., 1989; Sonis *et al*., 1990). The reduction in facial height observed in such patients may be related to a disturbance in dental development incidental to radiation (Dahllöf *et al*., 1988, 1989). While irradiation at an early age as a part of anti-neoplastic therapy causes severe disturbances in dental development, dental abnormalities have also been found in children subjected to chemotherapy alone (Jaffe *et al*., 1984; Rosenberg *et al*., 1987; Pajari, 1988; Sonis *et al*., 1990; Näsman *et al*., 1997).

Cranial irradiation causes microcephaly in rats and, when combined with chemotherapy, also alters the craniofacial proportions (Schunior *et al*., 1990). No marked long-term retardation in growth of the craniofacial skeleton has been observed in the rat following administration of drugs alone without radiation (Schunior *et al*., 1994). On the other hand, the anti-neoplastic agents, methotrexate and doxorubicin, for instance, are known to have an osteotoxic effect on the rat skeleton (Friedlaender *et al*., 1984).

In children, commonly used multi-agent chemotherapy alone or in combination with radiotherapy does not allow the evaluation of possible effects of a single chemotherapeutic drug on the craniofacial growth. Although the size and shape of the craniofacial structures differ between humans and rats, growth mechanisms are similar. Therefore, the present investigation focused on the initial short-term effects of two anti-neoplastic agents, vincristine and doxorubicin, on the craniofacial skeleton of young rats.

Materials and methods

A total of 310 inbred Long–Evans/Turku rats were used in the investigation: 40 for the pilot study, 180 medicated, and 90 control animals for the main experiment. Animal ethical approval was given by the district administrative board (Permit no. 719/97).

Pilot experiments

The aim at this stage was to test whether the therapeutic doses used for childhood cancer diseases, vincristine (0.05 mg/kg) and doxorubicin (1.0 mg/kg), were suitable for the Long–Evans/Turku strain rats. Their tolerance was tested by giving the drugs parenterally to 40 rats at 10 or 30 days of age. In the present study, 10-day-old rats were chosen to represent prepubertal animals, while 30-day-old represented pubertal ones.

The pilot studies indicated that the 10-day-old rats tolerated only one subcutaneous injection of 0.0375 mg/kg vincristine, i.e. the mortality rate was 50 per cent or more following one injection of 0.05 mg/kg vincristine or two injections of the smaller dose with a 3-day interval. It was decided that the same dose (0.0375 mg/kg) should be used for the 30-day-old rats as well, even though they tolerated 0.05 mg/kg even in repeated injections. Intraperitoneally administered doxorubicin (1.0 mg/kg) was well tolerated by both age groups.

Experiment

On the basis of these findings, the drug administration observed the following regimen:

1. Vincristine (Vincrin®, 1.0 mg/ml, Lääkefarmos/Farmos, Turku, Finland, 0.0375 mg/kg), was diluted 1:100 with physiological saline (Natrosteril®, 9 mg/ml, Orion, Finland) and injected subcutaneously into the neck region of two groups of rats at 10 and 30 days old. A parallel group of 30-day-old rats received additional injections 3 and 6 days after the first one. Each of the three groups consisted of 30 rats, 15 females and 15 males (Table 1).
2. Doxorubicin (Adriamycin®, 2.0 mg/ml, Pharmacia & Upjohn, Nerviano, Italy, 1.0 mg/kg), was diluted 1:25 with physiological saline and injected intraperitoneally following the same regimen as above (Table 1).
3. Sham controls were given physiological saline subcutaneously, the mean volume corresponding to that of the drug injections,

Table 1 Administration regime of the agents. Each group consisted of 30 rats: 15 females and 15 males.

Age at start	Agent	Number of injections and dose (mg/kg)	Injection schedule, age in days
10 days	Vincristine	1 × 0.0375	10
	Doxorubicin	1 × 1.0	10
	NaCl	1 × 10.0	10
30 days	Vincristine	1 × 0.0375	30
	Doxorubicin	1 × 1.0	30
	NaCl	1 × 10.0	30
	Vincristine	3 × 0.0375	30, 33, 36
	Doxorubicin	3 × 1.0	30, 33, 36
	NaCl	3 × 10.0	30, 33, 36

approximately 0.2 ml for the younger rats (10–30 days) and 0.8–1.0 ml for the older ones (30–50 days).

The rats were weighed every third day until killed by carbon dioxide asphyxia 20 days after the start of the experiment, i.e. at 30 or 50 days of age. The heads were freed from soft tissues, bleached with hydrogen peroxide and stored in glycerol. A number of measurements were made to the nearest 0.1 mm, using a digital sliding caliper (Figure 1):

(1) *neurocranial length*, distance from the frontonasal suture to the superior margin of the supraoccipital bone;
(2) *calvarial width*, longest distance between the temporal crests;
(3) *neurocranial width*, intertemporal width immediately superior to the zygomatic process;

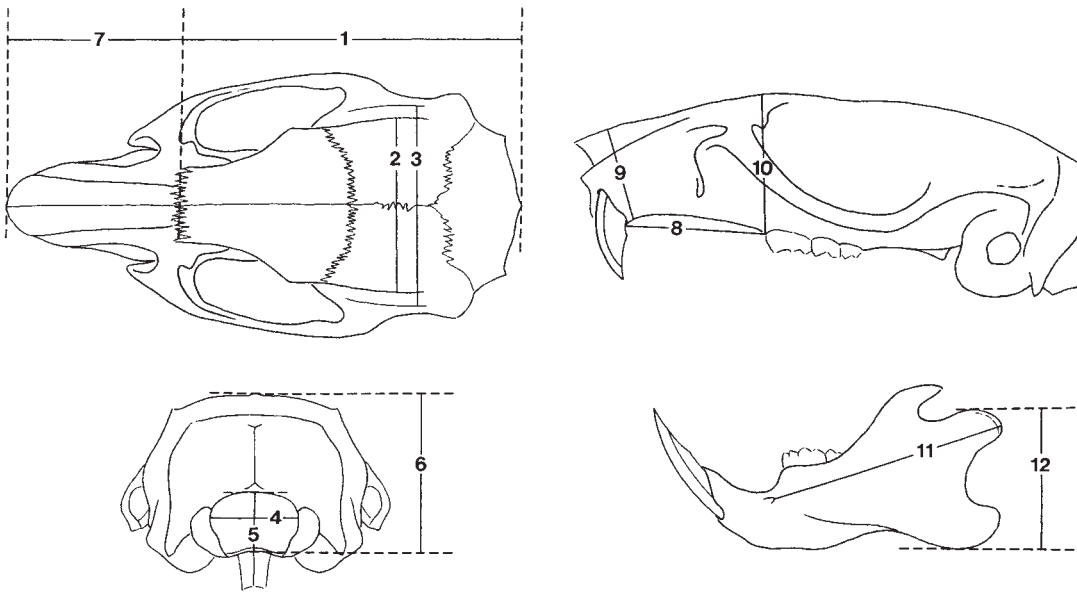

Figure 1 Linear measurements performed on the craniofacial skeleton of the rat.

(4) *width of the foramen magnum*, longest transversal diameter of the foramen magnum;
(5) *height of the foramen magnum*, vertical diameter of the foramen magnum;
(6) *neurocranial height*, distance from the inferior surface of the basioccipital bone to the vertex;
(7) *nasal length*, distance from the anterior margin of the nasal bone to the frontonasal suture;
(8) *sagittal diastema*, distance from the posterior junction of the premaxilla and incisors to the anterior cemento-enamel junction of the first maxillary molars;
(9) *anterior height of the snout*, distance from the inferior surface of the premaxilla to the superior surface of the nasal bone;
(10) *posterior height of the snout*, distance from the frontomaxillary suture to the alveolar bone anterior to the first maxillary molar;
(11) *mandibular length*, distance between the mental foramen and posterior aspect of the condylar process;
(12) *mandibular height*, distance between the inferior border of the mandible and superior aspect of the condylar process.

Statistical analysis

Statistical differences between the experimental and control animals were first tested with a one-way analysis of variance (ANOVA). Only those variables that showed significant differences between the groups in ANOVA were processed further by comparing the group means with Tukey's multiple range test. As the test revealed only one significant sex difference (between the measurements of calvarial length in the older rats), the dimensions recorded for the females and males were pooled.

The measurements were repeated with an interval of at least 2 weeks on 40 randomly selected control animals. The repeatability of the measurements was calculated from the formula:

$$\sqrt{\sum d^2/2n}$$

where d is the difference between the duplicate measurements in each case and n is the number of duplicates. The error varied between 0.03 and 0.17 mm in different measurements whose repeatability was estimated applying the intra-class correlation coefficient (British Standards Institution, 1987). One measurement was discarded due to poor repeatability (error 0.17 mm and intra-class correlation coefficient 0.33), whereas the error in those remaining was regarded as having an insignificant effect on the results. In most cases the intra-class correlation coefficient was >0.90 (measurements 1, 3, 4, 7–12), while measurements 2, 5, and 6 were less readily repeatable (correlation coefficient 0.75–0.77).

Neurocranial volume was measured in the rats that showed the most conspicuous reduction in external neurocranial dimensions, the vincristine group, and in the corresponding controls. The measurement was made by pouring glass beads 1.0–1.2 mm in diameter (weight 168.4 g/100 cm^3), in through the foramen magnum and weighing the beads. The mean values of two successive weighings were used in the statistical comparison of the two groups with a Student's *t*-test.

Results

The rats treated with the drugs differed somewhat from the controls on visual inspection. The site of the subcutaneous or intraperitoneal injection became temporarily bald, i.e. the hair was gradually lost over about 1 week from the start of treatment, particularly in the young rats that received vincristine, but a tendency toward recovery was evident before the termination of the experiment. The young vincristine animals did not move about in the same way as the controls, they seemed disinclined to eat and appeared to become thin and dehydrated following the injections, but subsequently showed signs of recovery.

Young rats (10–30 days)

Vincristine. Most craniofacial measurements, with the exception of the dimensions of the foramen magnum and the height of the neurocranium (measurements 4, 5, 6), were significantly smaller

Table 2 Measurements (mm) of skulls and weights (g) of rats that were given one injection of vincristine (0.0375 mg/kg), or doxorubicin (1.0 mg/kg) at 10 days of age and killed at 30 days. Control rats received physiological saline.

Variable	A Vincristine		B Doxorubicin		C Control		Significance		
	Mean	SE	Mean	SE	Mean	SE	A–C	B–C	A–B
1. Neurocranial length	**22.77**	0.13	**23.65**	0.12	**23.65**	0.07	***	NS	***
2. Calvarial width	**13.18**	0.03	**13.36**	0.03	**13.33**	0.02	***	NS	***
3. Neurocranial width	**14.79**	0.04	**14.92**	0.05	**15.05**	0.03	***	*	*
4. Width of foramen magnum	6.24	0.03	6.21	0.04	6.31	0.02			
5. Height of foramen magnum	5.32	0.02	5.34	0.02	5.38	0.02			
6. Neurocranial height	10.14	0.04	10.21	0.03	10.24	0.03			
7. Nasal length	**11.24**	0.10	**11.45**	0.07	**11.61**	0.05	**	NS	NS
8. Sagittal diastema	**8.33**	0.06	**8.65**	0.04	**8.64**	0.04	***	NS	**
9. Anterior height of the snout	**5.97**	0.03	**6.10**	0.02	**6.07**	0.03	**	NS	***
10. Posterior height of the snout	**8.20**	0.05	**8.41**	0.04	**8.38**	0.03		NS	***
11. Mandibular length	**14.74**	0.10	**15.20**	0.06	**15.22**	0.06	***	NS	***
12. Mandibular height	**8.07**	0.05	**8.29**	0.04	**8.27**	0.04	**	NS	**
13. Weight at start	17.0	0.37	17.71	0.34	17.51	0.32			
14. Final weight	**66.23**	1.74	**67.48**	1.47	**71.53**	1.25	*	NS	NS

The bold figures represent data that have been subjected to Tukey's test. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.
The regular type figures represent statistically non-significant differences between animal groups, as determined with the ANOVA test.

in the treated animals than in the controls, as was the tendency regarding final weight (14; Table 2). The measurements of the neurocranial volume did not differ significantly between the medicated rats and the controls (1.28 versus 1.31 cm^3; $P > 0.05$).

Doxorubicin. This was well tolerated by the young animals, in that only the neurocranial width (3) tended to be smaller than in the controls (Table 2).

A comparison of the effect of vincristine and doxorubicin on craniofacial morphology showed that most measurements (1–3, 8–12) were more retarded in the vincristine-treated rats at 30 days.

Weanling rats (30–50 days)

Vincristine. The length of the neurocranium (1), and the widths of the calvarium and neurocranium (2 and 3) were smaller than in the controls, whereas the foramen magnum (5) showed a tendency to be larger (Table 3).

The changes were more pronounced in the group that had received three injections of vincristine, as most measurements of the neurocranium (1, 2, 3, and 6) were smaller than in the controls, while not only the height (5) of the foramen magnum, but also its width (4) was greater than in the controls. The height of the snout (9 and 10) and the mandible (12) was reduced, and the weight gain (14) was clearly retarded (Table 4). The brain cavity volume of the medicated rats was significantly smaller than that of the controls (1.40 versus 1.46 cm^3; $P < 0.001$).

Doxorubicin. The rats that had received one injection differed from the controls with regard to the neurocranial length (1) and the width of the calvarium (2), both of which were smaller, whereas the height of the foramen magnum (5) and the anterior height of the nose (9) were larger than in the controls (Table 3).

The changes were more pronounced in the rats that had received three injections of doxorubicin, in that in addition to the altered dimensions (1, 2, 5, and 9) caused by one injection, the width (3) and height (6) of the neurocranium, the posterior height of the snout (10), the height

Table 3 Measurements (mm) of skulls and weights (g) of rats that were given one injection of vincristine (0.0375 mg/kg) or doxorubicin (1.0 mg/kg), at 30 days of age and killed at 50 days. Control rats received physiological saline.

Variable	A Vincristine		B Doxorubicin		C Control		Significance		
	Mean	SE	Mean	SE	Mean	SE	A–C	B–C	A–B
1. Neurocranial length	**25.91**	0.13	**25.92**	0.10	**26.20**	0.11	*	*	NS
2. Calvarial width	**13.10**	0.03	**13.19**	0.03	**13.33**	0.03	***	**	NS
3. Neurocranial width	**15.36**	0.04	**15.48**	0.03	**15.48**	0.03	**	NS	*
4. Width of foramen magnum	**6.46**	0.03	**6.37**	0.03	**6.38**	0.02	NS	NS	*
5. Height of foramen magnum	**5.25**	0.02	**5.26**	0.02	**5.18**	0.02	*	**	NS
6. Neurocranial height	11.07	0.04	11.0	0.04	11.02	0.04			
7. Nasal length	**14.16**	0.06	**14.13**	0.06	**13.94**	0.09	NS	NS	NS
8. Sagittal diastema	10.33	0.04	10.35	0.04	10.30	0.04			
9. Anterior height of the snout	**7.04**	0.03	**7.16**	0.03	**7.07**	0.03	NS	**	***
10. Posterior height of the snout	9.85	0.04	9.82	0.03	9.79	0.03			
11. Mandibular length	17.82	0.05	17.71	0.06	17.70	0.07			
12. Mandibular height	10.08	0.03	10.05	0.04	10.12	0.04			
13. Weight at start	68.50	0.82	70.37	0.97	69.03	0.80			
14. Final weight	153.17	3.00	150.50	2.89	151.90	2.85			

The bold figures represent data that have been compared with the controls by using Tukey's test. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
The regular type figures represent statistically non-significant differences between animal groups, as determined with the ANOVA test.

Table 4 Measurements (mm) of skulls and weights (g) of rats that were given three injections of vincristine (0.0375 mg/kg) or doxorubicin (1.0 mg/kg) at 30 days of age and killed at 50 days. Control rats received physiological saline.

Variable	A Vincristine		B Doxorubicin		C Control		Significance		
	Mean	SE	Mean	SE	Mean	SE	A–C	B–C	A–B
1. Neurocranial length	**25.63**	0.13	**26.65**	0.11	**26.33**	0.13	***	***	NS
2. Calvarial width	**13.13**	0.03	**13.13**	0.03	**13.28**	0.03	***	***	NS
3. Neurocranial width	**15.37**	0.05	**15.36**	0.04	**15.51**	0.04	***	***	NS
4. Width of foramen magnum	**6.5**	0.03	**6.44**	0.03	**6.39**	0.03	**	NS	NS
5. Height of foramen magnum	**5.27**	0.02	**5.24**	0.02	**5.17**	0.02	**	*	NS
6. Neurocranial height	**10.92**	0.04	**10.92**	0.04	**11.05**	0.04	***	***	NS
7. Nasal length	14.13	0.07	14.02	0.08	14.0	0.08			
8. Sagittal diastema	10.30	0.04	10.31	0.05	10.36	0.05			
9. Anterior height of the snout	**6.98**	0.03	**7.07**	0.03	**7.10**	0.04	***	*	NS
10. Posterior height of the snout	**9.73**	0.05	**9.72**	0.05	**9.83**	0.04	*	*	NS
11. Mandibular length	17.63	0.08	17.73	0.07	17.80	0.07			
12. Mandibular height	**9.93**	0.04	**9.93**	0.04	**10.18**	0.05	***	***	NS
13. Weight at start	69.37	1.33	70.13	0.94	69.90	1.12			
14. Final weight	**147.50**	3.65	**143.83**	3.17	**156.83**	4.01	***	***	NS

The bold figures represent data that have been compared with the controls by using Tukey's test. *$P < 0.05$; **$P < 0.01$, ***$P < 0.001$.
The regular type figures represent statistically non-significant differences between animal groups, as determined with the ANOVA test.

of the mandible (12), and the final weight (14) were smaller than in the controls (Table 4).

A comparison of the rats that had received one drug injection of either doxorubicin or vincristine at 30 days, and were followed up to 50 days revealed a difference regarding the width of the neurocranium (3), the foramen magnum (4), and the anterior height of the snout (9), the effect being more pronounced after vincristine treatment. No difference was recorded between the groups that had received repeated drug injections.

Discussion

Administration of vincristine and doxorubicin to the rats resulted in definite changes in the morphogensis of the craniofacial skeleton. The dimensional deviations in the vincristine group following one injection were more obvious in the younger animals (10–30 days) than in the older animals (30–50 days), whereas no such tendency was evident for the doxorubicin group.

The retardation in dimensional growth following the administration of the two drugs differed only in the younger group of rats, in that vincristine caused more marked changes than doxorubicin. The difference between the drugs was insignificant in most of the measurements in older rats, especially in the animals that received three injections. The different response may be explained by the different dose of the drugs. In the group of younger rats, the doxorubicin dose was probably submaximal as compared with that of vincristine, i.e. with a higher dose of doxorubicin the effect could have approached that of vincristine.

It could be argued that the lag in craniofacial growth of the animals resulted from malnutrition as the weight of some treated animals was significantly retarded at the end of the experiment. However, in contrast to the trend in the present findings, the neurocranium is less affected than the splanchocranium in rats under conditions of nutritional stress (Pucciarelli, 1980). Importantly, the reduction in craniofacial growth of the medicated rats was not uniform, but rather indicative of a differential effect with both treatment regimens, i.e. some dimensions were affected more than others, while many remained virtually unaffected. In other words, recorded measurements do not just represent dimensions of younger and smaller animals, but are rather indicative of an area-specific differential growth lag.

The retardation in the length of growth of the mandible was associated with a parallel retardation in maxillary growth, thus corroborating the hypothesis of Kantomaa (1984) that the mandible is passively brought into a more anterior position with the growth of the maxilla. The growth and tissue-separating capacity of the mandibular condyle (Rönning, 1966; Peltomäki *et al*., 1997), which forces the condylar process downwards, thus contributing to the vertical growth of the mandible (Kantomaa, 1984), is evidently directly affected by these cytotoxic agents. The reduced vertical dimensions of the snout (measurements 9 and 10) in the young vincristine-treated rats would also be explained by a direct effect of the cytotoxic agent on the nasal cartilage that reportedly contributes to the vertical growth of the snout of the rat (Rönning, 1971). No explanation can be offered at present for the increased nasal height in the older doxorubicin-treated animals.

A reduction in some external dimensions of the neurocranium was observed in all the animals, but it was especially evident in all the vincristine-treated and in the older rat groups that had received three injections of doxorubicin. A tentative and somewhat simplistic explanation for this would be that diminished bone formation, resulting from a probable toxic effect of the chemotherapeutic agents on the osteoblasts (Norido *et al*., 1988; Friedlaender *et al*., 1984), caused the reduction in the growth of the membranous bones in the skull.

The greater than normal size of the foramen magnum in the medicated rats followed from 30 to 50 days deviates from the general pattern of reduced growth of the craniofacial structures in the present animals. A similar change, a disproportionately large foramen magnum, was observed in rats in which the growth of the neurocranium (cranial base) was experimentally disturbed. Moreover, herniation of the medulla and a portion of the cerebellum through the foramen magnum was evident, and the animals

showed behaviour related to neural damage. It should be noted that compensatory growth in the height and width of the neurocranium occurred in rats with experimentally disturbed growth of the basicranial synchondroses (Asling *et al*., 1952), associated with a virtually unaffected neurocanial volume (Rönning, 1971). However, no such compensatory growth was evident in the present measurements. Instead, the volumetric measurements of the neurocranium indicated that, in association with the increased dimensions of the foramen magnum, the brain cavity volume was smaller in the medicated rats, significantly so in the older group (30–50 days). The reduction in the volume of the older rats could possibly lead to a space insufficiency and herniation as mentioned above. On the other hand, chemotherapy may affect the central nervous system (Prassapoulos *et al*., 1997) and, consequently, the possible brain atrophy when combined with microcephaly may not necessarily lead to space insufficiency. This could be verified histologically. Clinically, the size of the foramen magnum is difficult to determine in children, for example, from a lateral head film. It is not known whether the reduced head circumference is associated with an enlarged foramen magnum in children subjected to anti-neoplastic therapy.

The visually observed features of the medicated rats, such as local alopecia, loss of appetite, and slow movements are generally known acute side-effects of chemotherapeutic drug medication. The weight gain of the younger rats was reduced after a single injection of vincristine and that of the older ones after three injections. This finding corroborates earlier data on rats (Rebert *et al*., 1984) and rabbits (Noriddo *et al*., 1988). Non-significant changes in weight gain following the administration of doxorubicin doses similar to those used in this investigation have been noted earlier in experiments on rats (Nilsson *et al*., 1990). The maximally tolerated doses of anti-cancer drugs probably differ among the strains of rats. For example, the lethal dose of the environmental poison agent TCDD (2,3,7,8-tetrachlorodibenzo-*p*-dioxin) for the Long–Evans strain is just a fraction of that needed for Han/Wistar rats (Pohjanvirta *et al*., 1993). Thus, on analogy, the doses of the drugs used in the present experiment may not as such be applicable to similar experiments with other strains of rats. This suggests that it is necessary to find specific drug doses for experiments with different strains of rats.

Conclusions

The administration of vincristine or doxorubicin to immature rats led to reduced craniofacial growth. With the doses used in this study, vincristine seemed to have a more marked effect than doxorubicin in younger rats (experimental period from 10–30 days), while in the two groups of older rats (30–50 days) the difference between the drugs was insignificant. Repeated injections, which were given only to the older rats, led to an accentuation of the changes. Medication with the drugs vincristine and doxorubicin causes differential, rather than uniform morphogenetic changes in the craniofacial skeleton of the rat.

The findings of the present experiments constitute a baseline for selective and more detailed investigations regarding the effect of anti-neoplastic drugs on the craniofacial skeleton.

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