The influence of procarbazine and thiocyanate on embryonic bone maturation

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Summary

The substance procarbazine (Natulan®) is a teratogen known to induce cleft palates in rats on day 14 post-conception. The application of thiocyanate (SCN^-) alone to rats on day 10 and 14 of pregnancy had no effects on fetal bone maturation. However, when procarbazine was used, the maturation and growth of fetal bones was delayed. Upon additional application of thiocyanate, the effects of procarbazine (Natulan®) were increased. Thus, no antiteratogenic effect of thiocyanate occurs. We propose that charge and metabolites of teratogenic agents play a key role in developing the effects of thiocyanate.

Key words: Thiocyanate – SCN^- – Procarbazine – Embryonic skeleton – Rats

Introduction

Current models describing the malformations induced by procarbazine and the prevention of such malformations through thiocyanate are insufficiently characterized. This work provides information about bone maturation after the application of thiocyanate and procarbazine. Further morphological research exploring the induction and prevention of cleft palates has been carried out by Martens (2003). Blood samples from the mothers and amniotic fluid samples taken at the same time provided the biochemical data found. The use of thiocyanate and procarbazine is of interest insofar as procarbazine causes cleft palates on day 14 post conception. However, to date this mechanism is insufficiently explained. Thiocyanate in vivo proved to be an antimutagenic agent at induced tumors (Nagasawa et al., 1980; Grisk et al., 1981; Kramer et al., 1986, 1987). The hyperpolarizing effects of thiocyanate (SCN^-) on cell membranes and its protective characteristics against teratogens are well known (Böhland, 1982, 1986; Weuffen et al., 1990; Kramer and Böhland, 1996). The present work addresses the antiteratogenic effects of SCN^- against procarbazine in fetal rat skeletons. Procarbazine is a common substance used to induce malformations in animal experiments (Gundlach, 1986; Weingärtner et al., 2002). With procarbazine we deal with a N-methylated, and with isopropylbenzamide a substituted hydrazine. Its effects are especially exerted on DNA synthesis (Moser and Stacher, 1986; von Kreybig, 1975) and hence this substance is applied therapeutically in the treatment of tumours at a dose of 100-150 mg per square metre of the body surface.

Materials and Methods

Rats and Husbandry

The test model used female, primiparous, and inbred rats (LEW.1A). Pelleted rat chow and watering with soured water were given ad libitum. Rats were kept in K3-cages (two each). Humidity was between 50 to 60% and animals were adapted to the light regime (12h light from

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1 a.m. to 1 p.m.) for at least 14 days. During experimentation, two dams were kept together with a buck for mating from 6 p.m. to 10 p.m. They were separated on the basis of positive vaginal smears (proof of sperm) for treatment. The day after successful mating was counted as day 1 of the experiment. Pregnant rats were split into different groups: group K (Control group), group T (thiocyanate application), group N (application of procarbazine [Natulan®, Sigma-Tau Arzneimittel Comp.]) and group TN (Natulan® and thiocyanate application). The substances were applied as follows: thiocyanate (KSCN) was given at a dose of 3.2 g per 100 g body mass on days 10 and 13 of pregnancy (subcutaneous) and procarbazine at a dose of 20 mg per 100 g body mass on day 14 of pregnancy (intraperitoneal).

The number of dams was 10 in each group. On day 21 of pregnancy, the dams were anaesthetized and hysterectomized to extract the fetuses. The body mass (BM) and the crown-rump length (CR) of the fetuses were measured. A total of 37 fetuses in group K, 28 in group N, 35 in group T, and 30 in group TN were obtained. Subsequently, the fetuses were digested and the skeletons preserved following the method of Brylla and Wendler (1979).

Quantification of cartilage bone maturation and statistical test

Owing to the characteristics of the cartilage-bone staining a very fine distinction can be made between both bone and cartilage. All parts of the skeletons were rated according to their maturity. The different stages of maturity were scored as follows: 0 = not existing; 1 = cartilaginous; 2 = cartilaginous, with ossification centre; 3 = ossified. By means of a SPSS-procedure (Mann-Whitney-test), all mean values among the groups were compared with all parameters.

Results and Discussion

Body mass, head, thorax and pelvis

Concerning CR and BM, there were significant differences between the NT and N groups in comparison with the T and K groups. The animals receiving Natulan had a lower rate of growth, with lower BM and shorter CR values (Figure 1). These results could not compensated by additional application of thiocyanate (SCN^-). However, group T showed no significant differences in comparison with group K.

Regarding bone maturation (cf. Table 1), in principle bone maturity decreased both from cranial to caudal and from proximal to distal. Only a few significant differences were discernible at the head and rump. Thus, inhibititory malformations could be traced to the nasal bone, 1st cervical vertebra, and 13th rib.

Upper and lower limbs

The most prominent differences between the groups were observed in the appendicular bones (cf. Table 2). These gross observations do not provide any evidence as to which bones were in their sensitive phase on day 14 of pregnancy, when procarbazine was applied, and which bones were left unaffected. Those not affected by the application of Natulan had their sensitive phase at least before day 14 of pregnancy or after that day, respectively. Concerning the head, the values for nasal bones and palates are of interest. Since on day 14, among others, closure of the secondary palate takes place and limb buds begin to appear, these areas are damaged most (Abou Tara, 1975; Bienengräber et al., 1994, 1999, 2001; Malek et al., 1996). Upon comparing distinctly damaged lower limbs with lesser damaged upper limbs, it is clear that both limb buds are developed on and around day 14. However, the upper limbs are developed somewhat earlier.

On comparing the malformations observed in groups N and TN, the latter shows the most distinct malformations (Table 2). These results were unexpected. Regarding the TN group, thiocyanate had no positive influence on the

Table 1.– Maturity of head and rump bones

Group	TN (n=30)		T (n=35)		N (n=28)		K (n=37)	
Cleft	a		b		c		d	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Body mass (BM) in g	1,83 ^{b,d}	0,4	3,17 ^{c}	0,37	1,83^{d}	0,26	3,35	0,35
CR in cm	26,53 ^{b,d}	2,3	35,89 ^{c}	1,92	26,79 ^{d}	1,64	36,3	1,81
Fontal bone	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Temporal bone	1,00	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Parietal bone	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Nasal bone	2,13 ^{b,d}	0,40	2,74 ^{c,d}	0,44	2,00 ^{d}	0,54	2,95	0,23
Zygomatic bone	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Maxilla/Mandible	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Incisive bone	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Palate	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Interparietal bone	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Sphenoid	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Praesphenoid	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Occipital	2,00 ^{b,d}	0,50	2,71 ^{c}	0,46	2,00 ^{d}	0,00	2,57	0,05
Hyoid	1,90	0,00	2,00	0,00	2,00	0,00	2,00	0,00
Cervical vertebrae 1.-2.	2,00 ^{b}	0,20	2,11	0,32	2,00	0,00	2,03	0,16
Cervical vertebrae 3.-7.	2,00	0,00	2,00	0,00	2,00	0,00	2,00	0,00
Thoracic, lumbar, sacral vertebrae	2,00	0,00	2,00	0,00	2,00	0,00	2,00	0,00
Caudal vertebrae	1,00	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Sternum	1,97	0,00	2,00	0,00	2,00	0,00	2,00	0,00
Clavicle	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Scapula	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Ribs 1.-12.	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Rib 13.	2,90 ^{b,c,d}	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Pelvic bones	2,00	0,00	2,00	0,00	2,00	0,00	2,03	0,16

SD = standard deviation, n= number of animals in each group.
Significant differences ($p<0.05$) are marked with small superior letters after the mean values. These letters correspond to the mean value of the respective groups (e.g. group TN = column a; group K = column d).

Table 2.– Maturity of limb bones

Group	TN (n=30)		T (n=35)		N(n=28)		K (n=37)	
Cleft	a		b		C		D	
Parameters	Mean	SD	Mean	SD	Mean	SD	Mean	SD
Humerus, Radius, Ulna	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Carpals	1,00	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Metacarpus 1	0,03 ^{b,d}	0,00	1,00 ^{c}	0,00	0,07 ^{d}	0,26	1,00	0,00
Metacarpus 2	0,63 ^{b,d}	0,00	2,00 ^{c}	0,00	0,57 ^{d}	0,50	2,00	0,00
Metacarpus 3	1,20 ^{c,d}	0,00	2,00 ^{c}	0,00	1,71 ^{d}	0,46	2,00	0,00
Metacarpus 4	1,37 ^{b,d}	0,00	2,00 ^{c}	0,00	1,39 ^{d}	0,88	2,00	0,00
Metacarpus 5	0,53 ^{b,d}	0,00	1,09 ^{c}	0,28	0,64 ^{d}	0,49	1,00	0,00
Finger 1	0,03 ^{b,d}	0,00	1,00 ^{c}	0,00	0,04 ^{d}	0,19	1,00	0,00
Finger 2	0,03 ^{b,d}	0,00	1,00 ^{c}	0,00	0,04 ^{d}	0,19	1,00	0,00
Finger 3	0,07 ^{b,c,d}	0,2	1,00 ^{c}	0,00	0,46 ^{d}	0,51	1,00	0,00
Finger 4	0,60 ^{b,d}	0,2	1,00 ^{c}	0,00	0,68 ^{d}	0,48	1,00	0,00
Finger 5	0,17 ^{b,c,d}	0,00	1,00 ^{c}	0,00	0,46 ^{d}	0,51	1,00	0,00
Femur, Tibia	3,00	0,00	3,00	0,00	3,00	0,00	3,00	0,00
Patella	1,00	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Fibula	1,43 ^{b,c,d}	0,00	3,00 ^{c}	0,00	2,07 ^{d}	0,47	3,00	0,00
Tarsals	1,00	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Metatarsus 1	0,00 ^{b,d}	0,00	1,00 ^{c}	0,00	0,00 ^{d}	0,00	1,00	0,00
Metatarsus 2	0,27 ^{b,c,d}	0,00	2,00 ^{c}	0,00	0,07 ^{d}	0,26	2,00	0,00
Metatarsus 3	1,03 ^{b,d}	0,00	2,00 ^{c}	0,00	1,04 ^{d}	0,19	2,00	0,00
Metatarsus 4	1,30 ^{b,d}	0,00	2,00 ^{c}	0,00	1,43 ^{d}	0,63	2,00	0,00
Metatarsus 5	0,03 ^{b,d}	0,00	2,00 ^{c}	0,00	0,00 ^{d}	0,00	2,00	0,00
Toe 1	0,00 ^{b,d}	0,00	1,00 ^{c}	0,00	0,04 ^{d}	0,19	1,00	0,00
Toe 2	0,27 ^{b,d}	0,00	1,00 ^{c}	0,00	0,11 ^{d}	0,31	1,00	0,00
Toe 3	0,97	0,00	1,00	0,00	1,00	0,00	1,00	0,00
Toe 4	0,90 ^{b,d}	0,00	1,00	0,00	0,93	0,26	1,00	0,00
Toe 5	0,07 ^{b,d}	0,00	1,00 ^{c}	0,00	0,04 ^{d}	0,19	1,00	0,00

maturation of bones that were in their sensitive phase on day 14 of pregnancy. It can therefore be concluded that thiocyanate increases the teratological effects of procarbazine. This mechanism suggests that the cyclophosphamide model (Kramer et al., 1983) cannot be readily transferred to procarbazine. Both cytostatics have alkylating characteristics and develop their teratogenic or rather anticarcinogenic characteristics after activation into carbocations, and cause linkings as well as fissions of DNA strings (Robbiano et al., 1994; Rutishauser and Bollag, 1963; Dold et al., 1993; Dorr and Fritz, 1980). Evidently, the biotransformed metabolites of these agents have a different affinity for SCN^--anions.

Accordingly, in contrast with the findings of Wattenberg (1979) thiocyanate has no antiteratogenic effect. Presumably, the antiteratogenic effect depends on the binding characteristics of agents with protective effects. The present results also show that the bones of the skeleton undergo their sensitive phases against teratogenic agents at different stages of development.

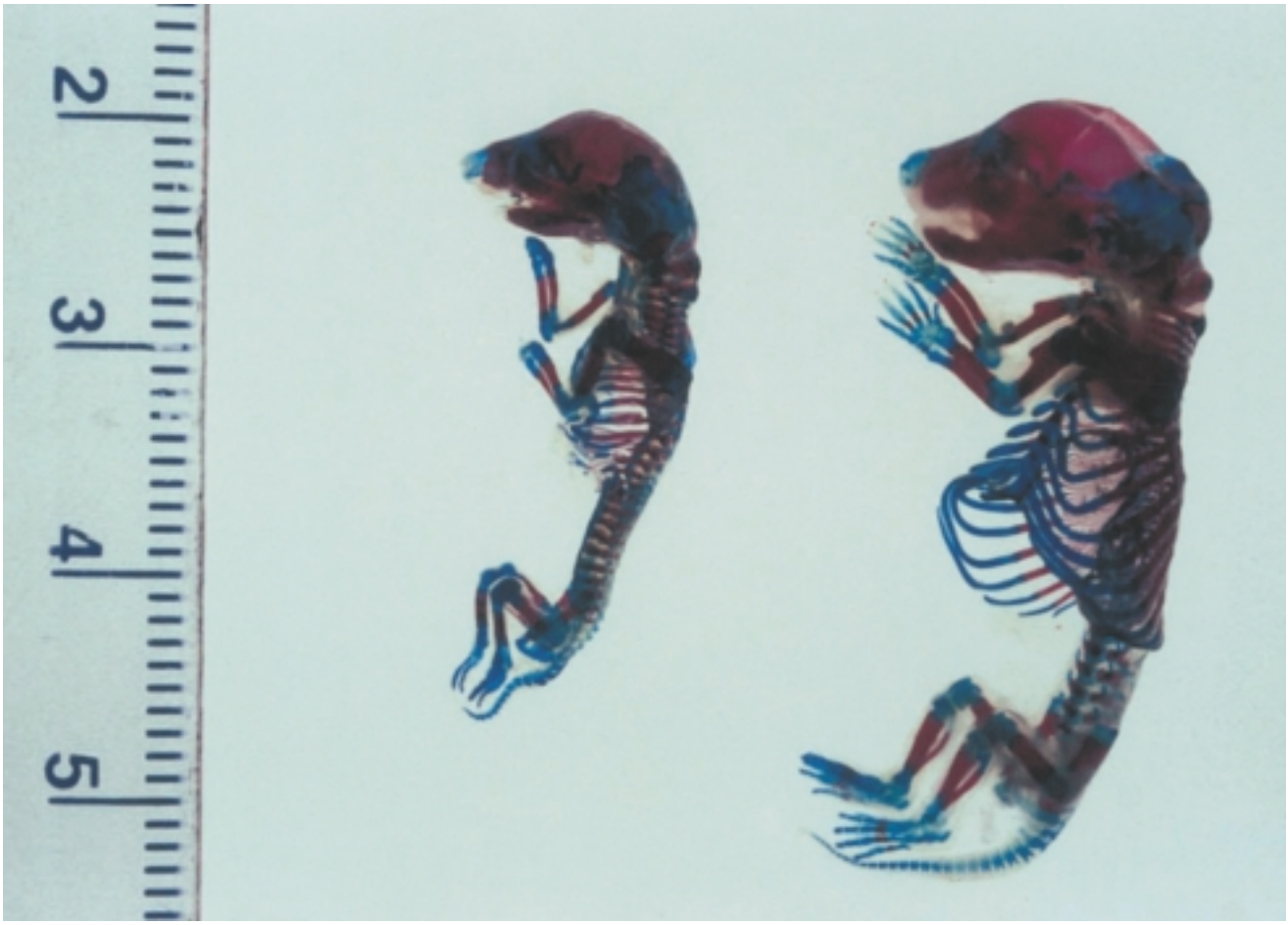

Fig. 1. Bone and cartilage staining according to Brylla and Wendler (1979), left skeleton of TN group and right skeleton of control group K.

Conclusions

Originally, this animal model was developed to prevent malformations on skeletal systems by thiocyanate after procarbazine application on embryonic day 14 in rats. The results showed no protective or antiteratogenic effects of thiocyanate.

Normal embryonic development of the rat skeleton shows different stages of bone maturity on day 21 after conception. Maturation develops from cranial to caudal and from proximal to distal. The upper extremities are more developed than the lower limbs. During the application of procarbazine, not all bones are affected in their development. Only bones in a sensitive developmental stage are influenced by procarbazine, especially the upper limbs. The sensitive phase is not identical to the degree of maturity or the state of development, respectively, and is specific to each bone on any given embryonic day.

The additional application of thiocyanate accelerates the effects of procarbazine. These effects are unknown and recommend caution in human medicine. Procarbazine is a successful drug in the treatment of patients with Morbus Hodgkin disease. Finally, we recommend that patients undergoing cancer therapy with procarbazine should not receive thiocyanate-rich foods.

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