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ORIGINAL ARTICLE

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Do folic acid and thiocyanate have a preventive effect on exogenously induced disturbances of embryonic cranial development?

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Abstract The preventive influence of folic acid and thiocyanate on procarbazine-induced disturbances of embryonic cranial development was investigated on experimental animals. Low dosages of folic acid or thiocyanate demonstrated no prophylactic effect. When thiocyanate was administered alone, an increased cleft rate was unexpectedly found for the secondary palate. The combined application of folic acid and thiocyanate showed a cleft-prophylactic effect in the secondary palate in addition to growth protection in the primary palate. It can be assumed that thiocyanate has a positive effect on chondral and osseous growth of the palate during the post-sensitive phase of embryogenesis, while in the sensitive phase, it can function as a carrier for teratogenous and toxic substances.

Keywords Cranial development · Folic acid · Rat · Thiocyanate

Introduction

One of the most common birth defects is cleft lip, alveolus, and palate, with a regionally differing incidence of 1:500 to 1:1,500 among newborns in Europe [18, 29]. The causal genesis is multifactorial. Besides genetic defects, poor nutrition of the periconceptional mothers-to-be apparently also plays a decisive role. A major problem seems to be deficiencies of vitamins B6, B11 (folic acid) and B12 among these women [21].

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Folic acid is an important methyl-group donor for the methylation cycle. Thus, folic acid functions as a cofactor in the biosynthesis of DNA and RNA. The essential importance for cell division and tissue growth, especially during embryogenesis, is a result of this [12, 13, 14]. Studies with pregnant women have indicated that supplemental folic acid contributes to the prevention of cheilopalatognathoschisis and other birth defects [19, 20, 31]. Because of the particular importance of the periconceptional phase for teratogenesis, the administration of folic acid is recommended at that time [11, 30, 36].

In nature, water-soluble thiocyanate (SCN^-) is ubiquitous and up to two thirds is endogenously formed in the body [33]. Thanks to its negative charge, it possesses special bonding capacities to cations [8, 9], resulting in its medical and biological importance. Its functional mechanisms have yet to be explained in detail, but the essential biological effects of thiocyanate are that it activates enzymes (collagenase, Na-, K-ATPase) [6, 16] and modulates the membrane permeability of cells [27, 28]. By influencing cAMP metabolism, thiocyanate inductively participates in hormonal regulation mechanisms. Further, it plays a crucial role in immune defense [7, 10, 32, 34]. The essential functions of thiocyanate are shown in Fig. 1, as taken from Wood [35]. A critical metabolic intermediate product in the thiocyanate cycle is SO_4^{2-}. This is considered an important building block for the synthesis of chondroitin-4 sulfate and chondroitin-6 sulfate. In this manner, SCN^- is involved in the synthesis of cartilaginous and bone substance. High concentrations of SCN^- have been found in the placenta and in the following fetal tissues: cartilage, bone, skin, and muscle [26].

The cytostatic agent procarbazine possesses a teratogenic effect [1, 24] and induces clefts in the secondary palates of fetal rats when applied on day 14 of gestation [2, 3, 5]. The purpose of this study was to examine the putative prophylactic effect of folic acid with and without added thiocyanate on embryonic rat skulls following previously induced malformation using procarbazine.

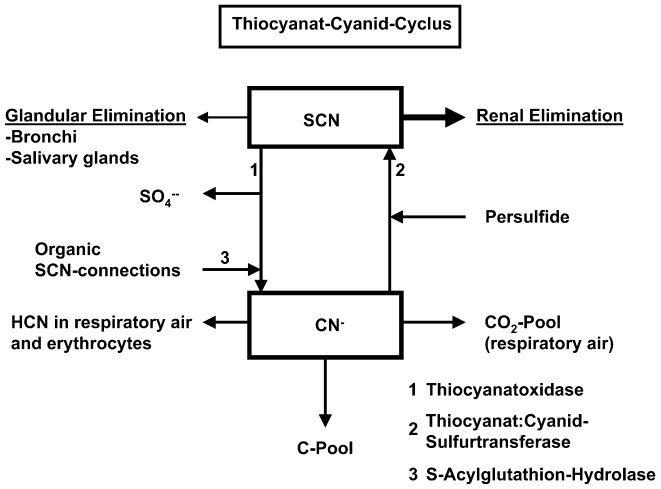

Fig. 1 Role of thiocyanate

Materials and methods

In this animal model, female primiparous inbred rats (LEW.1A) were used. Pellet food for breeding rats and acidulated water were made available ad libitum to rats, kept in pairs in K3 cages. Ambient humidity was 50–60%. The animals were adapted to the light regimen (12 h light from 1:00 to 13:00) for at least 14 days. During the experimental period, two females at a time were brought to a buck for copulation between 18:00 and 22:00, and following a subsequent positive vaginal smear (proof of sperm), were separated for treatment. The day after successful mating was considered the start of the trial (day 1). Mothers with longer times of copulation have different times of implantations. Therefore, at their 14th day the fetuses have different states of development at the time of procarbazine application. Thus, damage of the primary palate as seen by Bienengräber [4] could be ruled out.

The gravid animals were separated into treatment groups: group C (control), group P (procarbazine), group TP (thiocyanate and procarbazine), group FP (folic acid and procarbazine), and group FTP (folic acid, thiocyanate, procarbazine).

Procarbazine (Natulan, Sigma-Tau Arzneimittel) was administered to the pregnant females intraperitoneally on day 14 p.c. (postconceptionem) at a dosage of 20 mg/100 g live weight. Thiocyanate (KSCN) was administered at a dosage of 3.2 mg/100 g live weight on days 10 and 13 p.c., and folic acid was applied on days 1, 4, 7, 10, and 13 at a dosage of 0.016 mg/100 g live weight. The latter two substances were applied subcutaneously.

Each group contained ten gravid females. On day 21 p.c., the gravid females were anesthetized and hysterectomies were performed to obtain fetuses. The number of fetuses per group were: 20 in group C, 20 in P, 20 in TP, 19 in FP, and 30 in FTP. The fetuses were subsequently euthanized. The fetal heads were fixed and embedded in paraffin, then serially sectioned and hematoxylin-eosin stained for qualitative and histometric evaluation. The fixation of all heads (of both control group and treated group) was made with a phosphate buffered, 4%-formaldehyde-solution (pH 7.2) for about 48 h at 4–7°C.

Subsequently, the probes were put into an embedding machine for over 24 h. They were afterwards embedded in paraffin of 56°C. Five-micrometer thick serial cuttings were made of the heads. Deparaffinization proceeded as follows: two times xylol at 5 min each, 3 min 96% alcohol, 3 min 80% alcohol, 3 min 70% alcohol, 3 min 50% alcohol, 3 min aqua destillata. Subsequently, the hemotoxyline-eosine-staining of the cuttings was done.

The intraperitoneal procarbazine application was intended to produce temporally localized damage of the secondary palate during the sensitive phase (day 14 of gestation), which was possible due to the short half-life of procarbazine and high resorption capacity of the peritoneum. The subcutaneous application of the prophylactics was intended to incur a certain depot effect in the fetuses, which enter the fetal circulation slowly over a longer period of time due to the slow resorption of the prophylactic agents.

Statistical analysis consisted of comparisons of group averages using the t -test and the SPSS program. The significance level was defined as $p \leq 0.05$.

Results and discussion

The histological examination of the fetal heads showed that clefts were induced exclusively in the secondary palate (Table 1). This bears witness to the precision with which the trials were conducted, attained by limiting the animals' copulation time span to 4 h. Thus, during treatment of the mothers with procarbazine on day 14 p.c.—the day on which the secondary palate begins to close—all fetuses had the same developmental status. In addition, procarbazine was resorbed quickly by the maternal peritonea, so that organ-specific damage of the secondary palate occurred simultaneously.

Clefts were induced in all three prevention groups (FP, TP, FTP) and group P. Group FTP showed a tendency toward the lowest and group TP toward the highest cleft rate. The differences between the prevention groups and the damage group (P) as well as the differences among prevention groups were not statistically significant, which can be explained by the small sample size in this study.

Based on the control animals, the width of the Canalis incisivus was measured under normal conditions, yielding a value of 200 μm (see Fig. 1). Broadened Canales incisivi were classified as partial (subtotal) clefts which did not involve the entire secondary palate. The group TP showed the highest percentage of complete (total) clefts of the secondary palate and the highest rate of partial clefts (including broadened Canales incisivi). The results of this group in terms of partial clefts differed statistically significantly from those of groups FTP and C.

The lengths of the primary and secondary palates are given in Table 2. Of the prevention groups, FTP displayed the longest primary palates and the groups FP and TP the shortest. The lengths of the primary palates in the remaining groups did not differ significantly from one another. It can be assumed that the combination of folic acid and thiocyanate exerts a positive influence on palatal

Table 1 Number of clefts in secondary palate

Clefts	Group K	Group P	group FP	Group TP	Group FTP
Whole (%)	0	65	57	85	40
Total clefts (%)	0	60^{K}	47^{K}	50^{K}	40^{K}
Subtotal clefts (%)	0	5^{TP}	10^{TP}	$35^{K,FTP}$	$0^{FP,TP}$

Significant differences between groups ($p \leq 0.05$) are indicated by superscripts (group signification)

Table 2 Length of primary and secondary palate (in micrometers)

	Group K	Group P	Group FP	Group TP	Group FTP
Primary palate	2,055	2,155	$2,057^{FTP}$	2,125	$2,315^{K}$
Secondary palate	$1,880^{FP,TP,FTP}$	$1,600^{FP,TP}$	$1,080^{K}$	$1,040^{K}$	$1,500^{FP,TP}$

Significant differences between groups ($p \leq 0.05$) are indicated by superscripts (group signification)

growth. However, this influence is limited to those organs and tissues that have already gone through the sensitive phase of embryogenesis. The result is compensatory growth of the nonsensitive palatal regions after procarbazine damage in group FTP.

In the present study, the highest cleft rates and shortest lengths of the secondary palate were observed when thiocyanate alone had been administered. This means that retarded growth of the secondary palate was associated with cleft induction. This result contradicts results of other studies, which found thiocyanate to have a protective effect in experimental mutagenesis and carcinogenesis [17, 22, 23, 25]. In contrast, the application of thiocyanate combined with folic acid exhibits a preventive effect, which is expressed as the low cleft rate in the secondary palate and growth protection in the primary palate in group FTP. Thiocyanate has a developmentally specific effect, i.e., its effect is dependent on the respective maturity of each organ. Apparently, the thiocyanate anion is able to induce accelerated growth of the primary palate. This is demonstrated in group FTP, where presumably, the membrane modulating properties of SCN^- allow more folic acid to enter the cells of this organ. In contrast, this effect is absent in the secondary palate, since this organ is in its sensitive phase on days 14 and 15 p.c. and its development is heavily impaired by procarbazine.

The reason that folic acid in group FP of this study did not show any prophylactic effect may be that the amount applied was insufficient. Other studies with experimental animals applied greater amounts [3]. Therefore, the amount of applied folic acid should be increased in further experiments.

In medical and nutritional terms, it must be recommended that mothers-to-be ingest sufficient thiocyanate, since this substance accelerates the growth and development of the fetus. A daily allowance of 4–6 mg SCN^-/day on average is considered adequate [33]. Vegetables of the genus Brassica (broccoli, cabbage, etc.) are recommended due to their high thiocyanate content. However, given the current state of knowledge, it is unadvisable to medically subscribe additional thiocyanate during pregnancy for two reasons: 1) nothing is known about thiocyanate deficiencies in pregnant women, and 2) based on the present results, this cannot be regarded as completely harmless. Since the anion character of SCN^- facilitates the preferential transmembranal movement of cations into cells, an amplifying effect of certain medications or their—potentially toxic—metabolic intermediate products is conceivable, all of which must be taken into account when administering additional pharmaceuticals during pregnancy.

Folic acid still occupies a key position in the prevention of birth defects (cheilopalatognathoschisis, neural tube defects). Care should be taken during pregnancy that sufficient folic acid is ingested, since latent deficiencies are common [15]. If a folic acid deficiency is confirmed, the mother-to-be should receive a folic acid supplement of 5 mg/day up to week 14 of pregnancy, and where pregnancy is planned, starting 1 to 2 months prior to conception because of the greater benefit [31].

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