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In vivo/in vitro studies on the effects of cyclophosphamide on growth and differentiation of hamster palate

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A study was undertaken to examine the effects of cyclophosphamide (CP) on growth and differentiation of palatal tissues. An *in vivo/in vitro* approach was designed to analyze (1) whether the damage caused by *in vivo* administration of CP in the developing palate can be altered *in vitro*, and (2) to determine the effects of CP on the synthesis of collagen and glycosaminoglycan (GAG), which are essential for proper palate development. In addition, effects of vitamin B_1 and/or B_6 on *in vivo* modulation of CP teratogenicity was evaluated. Pregnant hamsters were given 30 mg/kg CP or 1 ml saline on day 10 of gestation. Control and CP-treated embryonic palates were dissected on day 11 of gestation and incubated *in vitro* in the presence or absence of CP. In order to allow metabolic activation of CP *in vitro*, either a slice of hamster liver or microsomal S9 fraction of liver was added to the culture medium. To study collagen and GAG synthesis, palates were obtained between days 10 and 13 of gestation, and incubated in growth medium supplemented with [^{14}C]proline or [^{3}H]glucosamine, as appropriate. The rates of collagen and GAG synthesis were determined. The results showed that, in the controls, the presence of a liver slice or S9 fraction in the culture medium had no effects on *in vitro* closure of palate. *In vivo* CP exposed palates did not fuse *in vitro*. When drug was given *in vitro*, or both *in vivo* and *in vitro*, palatal closure did not occur. CP reduced synthesis of both collagen and GAG in the vertically developing palate. The drug-treated shelves reoriented only after the rates of collagen and GAG synthesis were restored to the levels comparable to the control counterparts. Co-administration of vitamin B_1 and B_6 did not interfere with the teratogenicity of CP. It was suggested that CP treatment affected DNA synthesis and injured growing cells, which in turn reduced the synthesis of GAG and collagen and affected the expansion of shelf volume to delay the reorientation of the palatal shelves. Furthermore, it appears that *in vivo* treatment with CP changes the programming of palatal tissues to prevent the fusion process *in vivo*, which could not be altered *in vitro*.

Key words: Cyclophosphamide, collagen, glycosaminoglycan, hamster, palate, vitamins.

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Introduction

Cyclophosphamide (CP), a polyfunctional synthetic alkylating agent, is a potent tumor- and immunosuppressor with a wide spectrum of activity.[1–3] The drug is also a powerful teratogen in both animals and humans,[4–14] and produces malformations of limb, palate, mandible, brain and tail. The mechanism by which CP exerts its effect on neoplastic or developing tissue is unclear. However, to be biologically effective, CP must be metabolically converted in the liver to hydroxycyclophosphamide.[1,15,16] The hydroxy-CP then affects DNA synthesis leading to chromosomal damage.[15,17–19] CP can also be activated *in vitro* by using either intact embryonic cells in the presence of liver microsomal proteins or hepatic cells.[20,21] In addition, it has been suggested that the teratogenic effects of CP can be modulated *in vivo* by co-administration of several agents,[19,22–24] including vitamin B_1 (Thiamin) and B_6 (Pyridoxine), which could modify both the rate and severity of CP-induced malformations.[25–27]

The developing palate of mammals provides a useful system to study the effects of antitumor agents. Starting with vertical development from the roof of the mouth, alongside the tongue, the embryonic palatal shelves reorient and unite with one another dorsal to the tongue. This sequence of events during palate formation involves spatio-temporally regulated events at cellular and biochemical levels characterized by proliferation, migration and differentiation of cells, synthesis of various extracellular matrix (ECM) molecules, and programmed cell death.[28–30] Theoretically, any of these events can be affected by chemical agents, which would change the course of growth and differentiation of the palate and induce a cleft palate.

Earlier it was shown that injection of CP in pregnant hamsters at the time of vertical palate development seemed to affect the mesenchymal cell proliferation, i.e. DNA synthesis, and then cytodif-

ferentiation, eventually leading to a delay in the shelf reorientation, and thus a cleft palate.[13,31] In order to further elucidate the effects of CP on growth and differentiation of palatal tissues, an *in vivo/in vitro* model is described in the present study. The model evaluates two hitherto unknown aspects of CP-treated palates: (1) whether *in vivo* CP-treated palates are capable of fusing *in vitro* and (2) the effects of CP on the production of ECM (collagen and GAGs), because it is well established in the literature that a timely synthesis of ECM molecules is one of the necessary components that determines proper palatal development. The model could be of further biological significance in analyzing the mechanism of action of CP since it has been suggested that, in a developing system, the effects of CP can be altered by co-treatment with various other agents.[19,24–27] Hence, in the present study, we also evaluated *in vivo* effects of vitamin B_1 and B_6 on CP teratogenicity in hamster.

Materials and methods

The methods for environmental conditioning and mating of Golden Syrian hamsters were identical to those described earlier.[32] For the organ culture experiment, the pregnant animals were divided into four treatment groups (Table 1).

Group 1 served as a control and received 1 ml saline on day 10:00 (day 10, 0 h) of gestation.

Group 2 received 30 mg/kg CP in 1 ml saline on day 10:00 of gestation (this dose–time regimen induces cleft palate in all fetuses by delaying reorientation of the shelves from a vertical to a horizontal plane without embryotoxic effects[13]).

Group 3 received 200 mM CP *in vitro* only.

Group 4 received CP both *in vivo* and *in vitro* in the doses described for the Groups 2 and 3.

When grown *in vitro* prior to day 11:00 of gestation, the hamster embryonic palatal shelves do not achieve their full morphological potential, i.e. they do not fuse.[33] Hence, in the present study, palatal shelves were obtained from embryos on day 11 of gestation and placed on millipore filters resting on stainless steel grids as described before.[33] The assembly was then incubated in Dulbecco's minimum essential medium (DMEM) containing 10% fetal calf serum (FCS), with or without CP, as appropriate (Groups 1–4), at 37°C in 5% CO_2 and 95% air. In order to allow metabolic activation of CP, a 1–3 mm thick slice of hamster liver (homologous mother) was placed in culture plates, in a manner similar to cell inoculation[20] or microsomal S9 fractions were added to the culture medium as described by Wiger *et al.*[21] to allow metabolic activation of CP. In some *in vitro* experiments, neither liver slice nor microsomal S9 fractions were added to the media.

The palates were removed from cultures at 24 or 48 h after incubation and fixed in Bouin's solution for 48 h. They were then processed for histological examination (6 μm serial sections, hematoxylin & eosin stain) as described before.[13]

The details for the measurements of collagen synthesis are described earlier.[34] The control and treated palates (Groups 1 and 2) were obtained at various times between days 10:06 and 13:00 of gestation (Figure 3), because hamster palate morphogenesis is accomplished during this period. The dissected palates were incubated in growth medium (DMEM + 10% FCS) supplemented with 0.1 ml ascorbic acid (5 mg/100 ml) and 0.1 ml [^{14}C]proline (specific activity 250 μCi/mmol; New England Nuclear, Boston, MA) for 1 h at 37°C in an atmosphere of 5% CO_2/95% air. Following incubation, the palates were washed twice with [^{14}C]proline-free DMEM to remove excess radioactive proline from the surface of the tissue. The washed palates were sonicated in 2 ml 0.5 N acetic acid containing 10 mM EDTA at 0–4°C and left in the refrigerator for 12–18 h for protein digestion. The samples were then used for collagen digestion assay and total protein determination.

For the collagen digestion assay, acid-digested samples were dialyzed exhaustively over a 48 h period, first against 0.5 N acetic acid, followed by 0.5 M Tris–HCl buffer (pH 7.4; 0–4°C). In each case, the appropriate solutions were changed at 6 h intervals. The radiolabeled samples were then digested at 37°C for 4 h with bacterial collagenase (0.2 mg/ml; Sigma, St Louis, MO, catalogue C-0773) in the presence of 0.1 mM *N*-ethylmalemide. The undigested proteins from each sample were precipitated overnight at 0–4°C with 10 μl of 10% FCS and 125 μl of a mixture of 10% trichloroacetic acid (TCA) and 1% tannic acid (TA). The samples were then centrifuged at 13 000 r.p.m. for 40 min. The pellets were discarded and 200 μl of the supernatant from each sample were counted in a liquid scintillation counter (Phillips, Model PW 4700). The measurements were corrected for counting efficiency and the disintegration per minute (d.p.m.) was determined. The total protein was assessed by the Biuret method.[35] Each experiment was repeated three to five times. Means and standard deviations were calculated. The data on the rate of collagen synthesis

were evaluated by ANOVA method or Student's *t*-test, as appropriate. The comparison between treated and control groups were evaluated by Student's *t*-test at a significant level of 0.05.

The detail procedures for measurement of GAG synthesis were described earlier.[36] Briefly, the embryos from three litters (mean letter size 13 ± 2.2) were obtained at 6 h intervals between days 10:00 and 12:00 and then at 2 h intervals until day 12:06 of gestation. CP-treated embryos were similarly obtained until day 13:00 of gestation (Figure 5). An average of six palates were incubated for each experiment in a growth medium containing 1.8 ml DMEM, 0.2 ml fetal calf serum (10%) and 0.04 ml [^{3}H]glucosamine–HCl to a final concentration of 1.0 mCi/ml (specific activity 30 Ci/mmol; NEN Research) for 6–8 h at 37°C in an atmosphere of 5% CO_2/95% air as described by Derby and Pintar.[37] The palates were then washed in [^{3}H]glucosamine-free medium, homogenized and digested in 1.0 ml of 0.2 M Tris–HCl, containing sodium azide (0.02%) and pepsin (1.0 mg/ml) at pH 8.0 for 24 h at 56°C to liberate GAGs and proteins.[38] The pepsin digestion was terminated by heating samples at 100°C for 5 min. Protein was precipitated by 0.5 ml of 50% TCA at 0–4°C for 30 min. The samples were then centrifuged at 2000 *g* for 10 min at 0–4°C. The 100 μl supernatant from each sample was used for liquid scintillation counting as described above. The precipitate was dissolved in 1.5 ml of 1.0 N NaOH for total protein determination by the Biuret method.[35] Each experiment was repeated five or six times. The data on the rate of GAG synthesis were evaluated by ANOVA or Student's *t*-test as described for collagen.

In a separate experiment, the effects of co-administration of vitamins on CP teratogenicity were evaluated. The pregnant animals received either CP (day 10, i.p., 20 or 30 mg/kg), vitamin B_1 (day 10, 10 mg/kg, i.m.), vitamin B_6 (days 9 and 10, 10 mg/kg, i.m.), or their combinations (Table 2). The control received 1 ml saline. The fetuses were obtained on day 15 of pregnancy, weighed and fixed in Bouin's fluid for 48 h. They were then examined for external malformations of limb, palate, eye, brain, ear, mandible, tail and gut. The Student's *t*-test was used for statistical analysis of the data.

Results

In vitro development

The data on *in vitro* growth of control and CP-treated palates are outlined in Table 1. In the absence of *in vivo* or *in vitro* CP-treatment (Group 1), the palatal shelves show an epithelial seam followed by a mesenchymal union within 48 h of explantation (Figures 1 and 2). Presence of a liver slice or S9 fraction did not appear to affect the fusion process. When CP was administered *in vivo*, and palatal shelves subsequently explanted *in vitro* (Group 2), the palate closure did not occur (Figure 3). When the drug was given either *in vitro* (Group 3), or both *in vivo* and *in vitro* (Group 4), palatal closure was not achieved (Table 1).

Collagen synthesis

In control palates, the rate of collagen synthesis remained steady between days 10:00 and 12:00 of gestation, when the shelves were growing vertically (Figure 4). The rate then increased two-fold ($p < 0.01$) between days 12:00 and 12:04 of gestation, i.e. when the shelves reorient and fuse.[39] Following a transient decline during the next 4 h, the rate of collagen synthesis increased rapidly and was approximately 5-fold higher on day 12:12 than that seen on day 12:08 of gestation (Figure 4).

During the initial 12 h in CP-treated palates the rate of collagen synthesis was comparable to the control ($p < 0.05$). Between days 10:12 and 12:04

Table 1. Effects of CP on hamster palate grown on millipore filter *in vitro*

Treatment group[a]	24 h				48 h			
	No. of Explants	Open	Seam	Mesenchymal union	No. of Explants	Open	Seam	Mesenchymal union
1	12	8	3	1	10	1	—	9
2	11	11	—	—	8	8	—	—
3	12	11	1	–	10	9	1	—
4	8	8	—	—	8	8	—	—

[a]See Materials and methods for details.

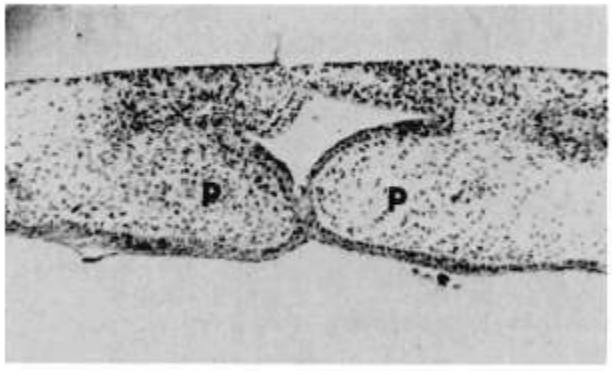

Figure 1. Hamster palatal shelves (P) cultured on day 11:00 of gestation showing epithelial seam after 24 h *in vitro*. ×50.

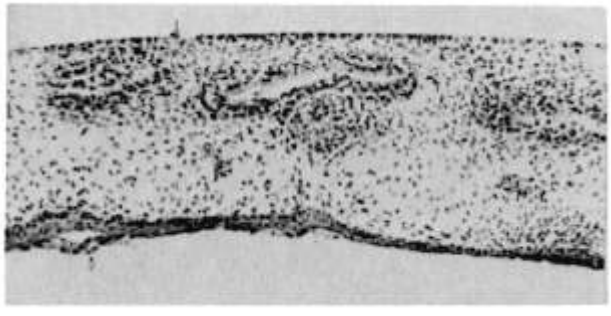

Figure 2. Hamster palatal shelves cultured on day 11:00 of gestation showing mesenchymal union after 48 h *in vitro*. ×50.

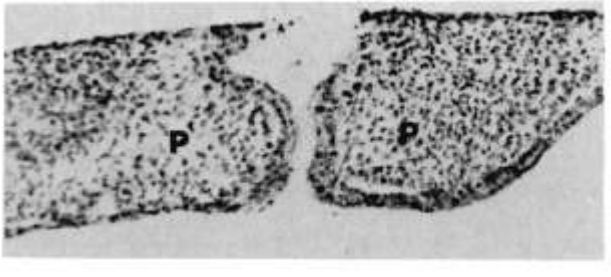

Figure 3. *In vivo* CP-treated hamster palatal shelves (P) cultured on day 11:00 of gestation showing unfused palatal shelves after 48 h *in vitro*. ×31.5.

of gestation, although the rate remained unchanged (Figure 4), it was reduced overall by 2- to 5-fold in comparison to controls ($p < 0.01$). In the ensuing 16 h, the rate of collagen synthesis increased approximately 3-fold in CP-treated palates, and between days 12:20 and 13:00 of gestation (i.e. the time at which reorientation occurs in CP-treated palates) it was comparable to the rate seen during shelf reorientation in controls (i.e. day 12:02 of gestation).

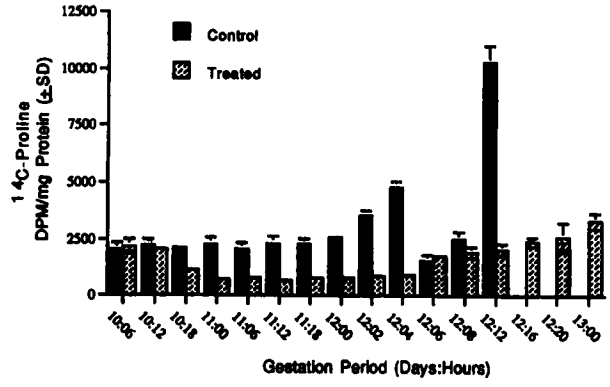

Figure 4. Collagen synthesis in normal a CP-trated developing hamster palate.

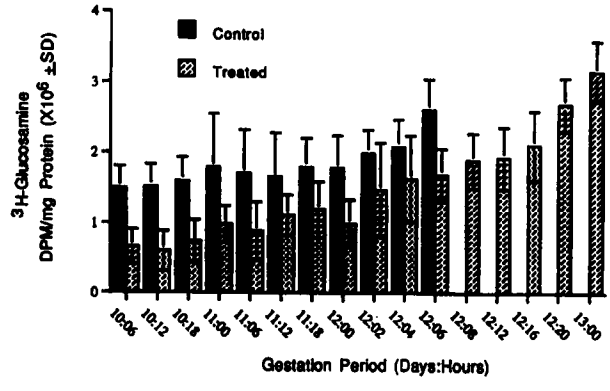

Figure 5. GAG synthesis in normal and CP-treated developing hamster palate.

GAG synthesis

In the control palates, the rate of GAG synthesis remained constant between days 10:00 and 12:06 of gestation (Figure 5). In CP-treated palates, the rate of GAG synthesis was unchanged between days 10:06 and 12:00 of gestation. During this period, however, in comparison to the controls, it was reduced by 50% ($p < 0.01$). The rate then increased gradually during the next 12 h and on day 12:12 the rate of GAG synthesis in CP-treated palates was comparable to that seen in the control reorienting palate on day 12:02 of gestation ($p < 0.05$). In the ensuing 12 h, i.e. the period during which the CP-treated palate reorient, the rate increased further by 50% ($p < 0.05$).

Co-administration of vitamin B_1, B_6 and CP (Table 2)

Effects of co-administration of CP, vitamin B_1 and B_6 are summarized in Table 2. When given alone, vitamin B_1 or B_6 did not show any embryotoxic or ter-

atogenic effects. CP treatment (20 or 30 mg/kg) showed teratological response similar to that described earlier.[13] Co-administration of 20–30 mg/kg CP and vitamin B_1 did not affect the rate of fetal resorption or malformation.

Simultaneous treatment of vitamin B_6 and 30 mg/kg CP increased the resorption rate ($p < 0.01$) but not the malformation rate. However, when 20 mg/kg was injected with vitamin B_6 both the malformation and resorption rates were unchanged.

When vitamin B_1 and B_6 were co-administered with CP, the malformation rates was low, but not significantly different from 30 mg/kg. All litters had several malformed fetuses.

When overall frequencies of individual external malformations, such as cleft palate and limb defects, were compared there were no differences between

Table 2. Effects of vitamins B_1 and B_6 on teratogenicity of CP

Drug dose[a] (mg/kg)	No. of litters	No. of live fetuses	Resorptions[b] (%)	Malformed fetuses (%)	Mean fetal weight[c] (g ± SD)
CP (30)	3	37	5	97	1.82 ± 0.21
CP (30) B_1 (10)	3	40	5	100	1.85 ± 0.23
CP (30) B_6 (10)	3	32	16	94	2.03 ± 0.35
CP (30) B_1 (10) B_6 (10)	3	44	0	84	1.68 ± 0.20
CP (20)	5	60	3	15	2.24 ± 0.27
CP (20) B_1 (10)	5	58	3	12	2.00 ± 0.49
CP (20) B_6 (10)	3	37	3	32	1.98 ± 0.22
CP (20) B_1 (10) B_6 (10)	5	59	5	15	2.07 ± 0.40

[a]All fetuses were normal following injection of vitamin B_1 and B_6 alone or in combination.
[b]Resorption rate in saline-treated controls 4–10%.
[c]Mean fetal weight in saline-treated control 2.2 ± 0.2 g.

Table 3. Effects of vitamins B_1 and B_6 on CP-induced malformations

Drug dose (mg/kg)	No. of malformed fetuses	No. of fetuses with malformations[a]		
		Complete palatal cleft	Incomplete palatal cleft	Limb defects
CP (30)	36	28	8	2
CP (30) B_1 (10)	40	38	2	22
CP (30) B_6 (10)	30	24	9	13
CP (30) B_1 (10) B_6 (10)	37	33	4	8
CP (20)	9	0	9	0
CP (20) B_1 (10)	7	2	5	0
CP (20) B_6 (10)	12	12	0	0
CP (20) B_1 (10) B_6 (10)	10	4	5	0

[a]Frequencies of malformations of eye, ear, gut and mandible were between 2 and 5%.

various CP-treated and/or vitamin-treated groups (Table 3). However, increased frequency of severe forms of cleft palate was seen following the co-treatment of 20 mg/kg CP and vitamin B_6. Since the affected fetuses were seen in all litters, it appears that simultaneous treatment of CP and vitamin B_6 may have deleterious effect on the developing palate.

Discussion

It is well recognized in the literature that metabolic activation of CP is essential for exerting its biological effect. Under *in vivo* circumstances, CP is metabolically activated in liver.[1,15,16] Under *in vitro* conditions, CP can be activated by addition to the culture of either liver microsomal fraction (S9), or proteins obtained following stimulation of liver by phenobarbital, polychlorinated biphenyl and methylcholanthrene, or 4-hydro-peroxy-CP, or by using drug metabolizing cells.[18,20,21,40–46] In the present study, a thin slice of liver was placed at the bottom of the culture plate to activate CP in a manner similar to that described for cells (see Figure 1 in Manson and Simons[20]). The results showed that even in the presence of a liver slice or S9 fraction in the culture media, the control palates underwent epithelial seam formation and mesenchymal union in a temporal manner similar to that observed in the absence of liver,[33] indicating that the presence of liver or S9 fraction has no adverse effect on *in vitro* palatal closure. Furthermore, when CP was administered *in vitro* palatal closure was prevented in the presence, but not in the absence of a liver slice or microsomal S9 fraction (data not shown).

Studies on *in vitro* behavior of developing palate following an *in vivo* drug treatment are rare. Such studies, however, could be potentially useful in developing experimental systems to study the mechanism of drug action.[47] When *in vivo* steroid-treated palates were grown *in vitro* some of them fused.[48] In the present study, *in vivo* CP-treated palates, when grown in cultures in the presence or absence of CP, failed to fuse. It appears that during the first 24 h following *in vivo* drug treatment, i.e. prior to the beginning of culture period, CP may have caused irreversible changes in the programming of the developing palatal tissues, which cannot be altered by *in vitro* explantation at least under the conditions defined in the present study. Earlier, it was indicated that CP affects DNA synthesis and injures cells in the developing palate within 6–8 h after drug administration.[13,31] Although the rate of DNA synthesis in the palate was restored immediately, the cellular injury persisted for 48 h in CP-treated palates.[31] These changes, coupled with an altered extracellular environment reflected by reduced synthesis of both GAG and collagen, prevented *in vivo* drug-treated palatal shelves from fusing *in vitro*, even in the absence of CP in the media. A reduction in cell proliferation in bone marrow, limb bud and developing neural tissue, and changes in the microenvironment of grafts to restore hemopoetic tissues have been observed following both *in vivo* and *in vitro* CP exposure.[49–54] Also, it has been shown that CP arrests cells in S and G_2 phases of cell cycle before causing cellular damage.[52,53,55] CP-induced cellular damage is also observed in other tissues such as lung and various embryonic structures.[53,56–60] Depending on the types of tissues and the experimental circumstances, the cellular damage, although observed only in a few cells within 1 h, peaks between 10 and 18 h after the treatment. The CP-damaged cells could, in turn, affect the production of ECM molecules (discussed below). Thus, the observations of the present study, along with those reported previously,[13,31] on the effects of CP on developing palate are consistent with the data from the literature (references cited above) and further suggest that an *in vivo/in vitro* developing system could be potentially useful in studying mechanisms of action of anti-cancer drugs.

There are numerous reviews in the literature demonstrating the significance of collagen and GAG synthesis in palate development.[34,36,61–64] The quantitative data of the present study on ECM synthesis in control palates are similar to those described for the normal palate development in literature. Specifically, a spurt in the rate of collagen synthesis at the time of shelf reorientation[34,65–68] and a steady rate of GAG synthesis through the period of palate development were also seen in the present study.

The putative roles of collagen and GAG synthesis during palate development were further evaluated by using CP because CP delays palatal shelf reorientation in mammals.[13,75,76] The results showed that between days 12:00 and 12:04 of gestation, which is the time of normal shelf reorientation in hamster, collagen synthesis in CP-treated palate was reduced. During the next 16 h, although the rate of collagen synthesis recovered, the shelf reorientation occurred between days 12:20 and 13:00 of gestation, i.e. only after the rate reached a level comparable to that seen during normal reorientation. The synthesis of GAG was also depressed initially until

day 12:00, but it recovered on day 12:12 of gestation, i.e. just prior to reorientation of CP-treated palate, to the rate comparable to normal reorienting palate. Earlier, it was shown that reduced DNA synthesis and cell injury occurred within 6 h of CP treatment, followed by a delayed expansion of shelf volume of the developing palate.[13,31] Although the rate of DNA synthesis recovered quickly, the reduced rate of GAG synthesis (within 6 h) and collagen synthesis (within 12 h) followed cell injury, and subsequently seemed to affect the shelf volume. The shelf volume in CP-treated palates was restored at the time of reorientation, but only after the resolution of cellular injury[13,31] and recovery of GAG and collagen synthesis (present study). Additionally, it has been shown that following CP-induced cell injury there were alterations at the epithelial–mesenchymal interface in the basal lamina, which were repaired before shelf reorientation.[31] Clearly, in the vertically developing palate there was a CP-induced damage phase, and a delayed but overlapping recovery phase, both of which extended over a period of approximately 64 h. These sequence of changes in CP-treated developing palate ultimately allowed shelf reorientation, but they also seem to have altered the programming of the differentiating cells to prevent fusion and cause cleft palate. To the best of our knowledge, there are no studies on the effects of CP on collagen and GAG synthesis in any developing system. Thus, findings of CP affected ECM synthesis are difficult to compare. Following administration of fluorouracil, both GAG and collagen were affected.[34,36,64] However, only after restoration of the rates of synthesis of these ECM molecules was the shelf reorientation achieved in fluorouracil-treated palates. Thus, the data from CP-induced perturbation in the developing palate, along with those from literature, suggest that a certain minimum amount of both GAG and collagen accumulation may be essential for advancing growth and differentiation of palate.

Previously, Schubert[26] observed that administration of vitamin B_1 or B_6 to mice reduced the frequency of CP-induced cleft palate. Dostal and Schubert,[27] using a different strain of mice, showed that co-administration of CP and vitamin B_1 and B_6 decreased, albeit non-significantly, the overall proportion of malformed fetuses, and changed the spectrum of malformations. In the present study on hamster, it was not possible to achieve a significant reduction in the malformation rate following simultaneous treatment of three agents. It is possible that differences in observations between Schubert's work[26] and that of others, including the one reported in the present study, may relate to the differences in animal species.

In summary, an analysis of the results of the present study show that when *in vivo* CP-treated palates are grown *in vitro*, with or without CP, they do not achieve their full morphological potential to unite. Although CP treatment reduced the synthesis of both GAG and collagen and affected the expansion of shelf volume to delay the reorientation of palatal shelf, the present study suggests that CP also affect the terminal differentiation of the palatal tissue to prevent fusion of the opposing shelves. Finally, vitamin B_1 and B_6 did not alter the teratogenicity of CP.

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