Protection of Mice from Teratogen-Induced Cleft Palate by Exogenous Methionine (43887)

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Abstract. **A major challenge for biomedical research is the reduction and/or prevention of congenital craniofacial abnormalities which can be induced by some extrinsic toxicants such as retinoic acids (e.g. isotretinoin, Accutane®) and glucocorticoids (corticosteroid hormones) during embryonic craniofacial morphogenesis. Our present studies using a genetically susceptible mouse strain (B10.A) indicate that the teratogenic actions of exogenous retinoic acid or glucocorticoid in secondary cleft palate induction can be largely reduced or even completely rescued by subsequent administration of methionine. The greatest reduction in frequency of all-*trans* retinoic acid- or triamcinolone-induced secondary cleft palate was obtained by a single-dose IP administration of methionine at 187 mg/kg to pregnant mice on E13 21 hr. It appears that detrimental toxic effects were not observed in mice treated with this therapeutic level of methionine. Our present findings support the need for further research into the role of exogenous methionine in cleft palate reduction, that will provide a biological rationale for considering methionine as a therapeutic agent.**

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Intake of high doses of glucocorticoids during mammalian embryonic development abrogates craniofacial morphogenesis and causes cleft palate (CP) (1–8). Similarly, intake of high doses of retinoic acids (RA) during mammalian pregnancy results in CP defect (9–15). Glucocorticoid or RA binds as ligands to their specific receptors which mediate their teratogenic actions on target genes in responsive tissues. Glucocorticoid binds as a ligand to glucocorticoid receptor and effects a number of target genes. Retinoic acid binds as ligand to one or more classes of retinoic acid receptors (RAR-alpha, beta and gamma), or retinoid-X receptors (RXR-alpha, beta and gamma), and effects a number of target genes, some of which control normal embryonic morphogenesis. The major events of palate morphogenesis occur between E13 (Day 13 of gestation) and early E15. Administration of teratogenic doses of triamcinolone to genetically susceptible mouse strains (e.g. B10.A mice) on E11 21 hr, or all-*trans* RA on E13 21 hr, effectively produces CP.

Methionine (Met), choline, folate and vitamin B_{12} belong to a class of nutritionally important substance known as lipotrophs. Methionine and choline serve as methyl group (CH_3) donors, while folate and vitamin B_{12} serve as methyl transfer co-factors. Methionine is nutritionally essential for all mammals, because it serves as a structural component of proteins, and is involved in several major metabolic pathways such as the methylation of lipids, nucleic acids and proteins (16). Although it has been known that intake of vitamin B_6 and/or folate reduces the frequency of cortisone-induced CP in mice (17), it still lacks convincing data to demonstrate the protective effect of these vitamins in humans (18). Recently, folate has been used in preventive treatment of human cleft lip and CP (19–21). We attempted to use Met for ameliorating the teratogenic actions of glucocorticoid or RA in CP induction. Data in the present report demonstrate that Met administration considerably protects pregnant mice from glucocorticoid and RA-induced CP.

Materials and Methods

Mice. Mice of B10.A (B10.A/SgSnJ) strain used in this study were delivered from the Jackson Laborato-

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ries (Bar Harbor, Maine) at 6–8 weeks of age. After raising to 12 weeks of age or older the mice had body weights of approximately 29 g. At this time animals were mated during a 3-hr period. Identification of vaginal plugs was considered to be Day 0 (±3 hr) of pregnancy. All studies involving the use of mice were conducted according to protocols approved by the Institutional Animal Care and Use Committee, University of Southern California (USC), as well as approved by the U.S. National Institutes of Health. The care, handling and housing of mice were directly supervised by the USC Vivaria. For CP assays, mice were sacrificed on E18 after euthanizing by carbon dioxide asphyxiation and cervical dislocation. Fetuses were euthanized by decapitation, and assayed for CP by examining under a dissection microscope.

Glucocorticoid or Retinoic Acid Administration. Triamcinolone hexacetonide (the trademark is Aristospan, from Lederle Parenterals, Inc., Carolina, Puerto Rico) is supplied as 5 mg/ml suspension. Immediately before use triamcinolone was mixed in saline (0.9% NaCl, w/v) as 1 mg/ml suspension for injection. Time-pregnant mice with body weights of approximately 30 g on E11 21 hr received intramuscular (IM) injection of a single dose triamcinolone into the flank muscle of the hind leg. Because triamcinolone bioactivity is decreased during storage, each experimental group mice received administration of 100 ug triamcinolone provide a control for triamcinolone bioactivity. Resorption of fetuses was not observed according to the dose range and timing of our triamcinolone administration protocol. Triamcinolone produces at least 80% CP under these protocol conditions. Control mice received administration of 100 ul saline alone.

All-*trans* RA was supplied as powder from Eastman Kodak Co. (Rochester, New York) and stored at −20°C. Immediately before use, it was dissolved in dimethyl sulfoxide (DMSO) as 1.5% (w/v) solution in subdued light. Each time-pregnant mouse of body weight approximately 32 g on E13 21 hr received intraperitoneal (IP) injection with a single dose of 1.5 mg of RA in 100 ul of DMSO. Control mice received injection of 100 ul DMSO alone. Resorption of fetuses was not observed according to our RA administration protocol.

Methionine Administration. L-Methionine (Met) was supplied as powder from Eastman Kodak Co. (Rochester, New York), and prepared in sterile water as 6% (w/v) solution for injection (solubility was hastened by heating briefly at 50°C). In order to protect mice from triamcinolone-induced CP, each pregnant mouse which had been administered 100 ug triamcinolone on E11 21 hr received a subsequent administration of Met on E13 21 hr. In order to protect mice from RA-induced CP, each pregnant mouse which had been administered 1.5 mg all-*trans* RA on E13 21 hr received a subsequent administration of Met on E13 22 hr [The 1-hr interval between two subsequent injections avoids possible pain caused by large injection volumes and possible mixing of reagents]. Control mice received Met administration on both E12 21 hr and on E13 21 hr, with 6 mg for each injection. According to the dose range and timing of our protocol, resorption of fetuses was not observed for mice treated either with triamcinolone and Met, or with RA and Met.

Data Analysis. Embryos were collected from 3–8 litters for each dose of Met and assayed for CP. Significant difference in CP frequencies between Met-treated and untreated mice was established by Chi-square analysis. Met-treated mice with a P value of 0.05 or less were considered to be significantly different from untreated mice. In addition, CP frequency was determined for each individual litter, based on which the mean value and standard deviation were calculated for various doses of Met.

Results

The CP frequency in mouse embryos was approximately 84% after administration of 100 ug triamcinolone alone on E11 21 hr to each time-pregnant mice. Whereas the palatal shelves are quite small on early E13, the major events of palate morphogenesis occur between E13 and early E15. Presumably due to a relatively slow action of triamcinolone, our previous studies indicate that lower CP frequencies are generated by triamcinolone administration either earlier or later than E11 21 hr (E. C. Lau, Z.-Q. Li and H. C. Slavkin, unpublished results). The CP frequency was significantly reduced by subsequent administration of Met at 125–250 mg/kg (4–8 mg/mouse) on E13 21 hr, at the time when palate morphogenesis was taking place (Tables I and II; Fig. 1). Triamcinolone-treated mice were almost completely rescued from CP by a single-dose injection of Met at 187 mg/kg (6 mg/mouse), and embryo-toxicity was not observed. By administering Met

Table I. Results of Treating B10.A Mice with Triamcinolone (Tc)[a], or Tc and Methionine (Met)

Treatment	No. of litters	Cleft palate	
		Observed outcomes	Mean frequencies (%)[b]
Tc alone	8	80.7% (46/57)	84.3 ± 3.5
Tc + 4 mg Met	4	33.3% (10/30)	30.1 ± 3.8
Tc + 6 mg Met	4	3.1% (1/32)	2.8 ± 5.5
Tc + 8 mg Met	4	16.1% (5/31)	15.8 ± 3.2

[a] Mice treated with Tc received 3.33 mg/kg (i.e. 100 μg each) intramuscularly on E11 21 hr.
[b] A mean frequency is calculated from the cleft palate frequencies observed for individual litters.

Table II. Reduction of Triamcinolone (Tc)-Induced Cleft Palate by Exogenous Methionine (Met)

(a) Mice treated with different doses of Met:

	Yes		No		Totals
	O	E	O	E	
Tc alone	46	23.56	11	33.44	57
Tc + 4 mg Met	10	12.40	20	17.60	30
Tc + 6 mg Met	1	13.23	31	18.77	32
Tc + 8 mg Met	5	12.81	26	18.19	31
	62		88		150

(b) Compare Met-treated and untreated mice:

	Yes		No		Totals
	O	E	O	E	
Tc alone	46	23.56	11	33.44	57
Tc + Met[a]	16	38.44	77	54.56	93
	62		88		150

Ho: Assuming that there is no difference at all between Met-treated and untreated mice.
O: Observed outcomes.
E: Expected outcomes.
The χ_1^2 is 37.9, which is much greater than 3.84, and thus $P < 0.001$.
[a] The Met-treated group is obtained by combining all the mice administered with Met (4–8 mg).
The χ_1^2 is 34.47, which is much greater than 3.84, and thus $P < 0.001$.

at either higher or lower than 187 mg/kg, there was less protection of mice from triamcinolone-induced CP. By administering Met at 312 mg/kg (10 mg/mouse) to triamcinolone-treated mice, there was no significant reduction in CP.

The CP frequency was approximately 48% in the embryos after administration of 1.5 mg tRA alone on E13 21 hr to time-pregnant mice. Probably due to a relatively short half-life of retinoic acid *in vivo*, lower CP frequencies were generated by retinoic acid administration either earlier or later than E13 21 hr. The CP frequency was significantly reduced by subsequent administration of Met at 125–250 mg/kg (4–8 mg/mouse) on E13 22 hr (Tables III and IV; Fig. 2). The greatest reduction in frequency of RA-induced CP (to 9 ± 6% only) was obtained by a single-dose injection of Met at 187 mg/kg (6 mg/mouse), and embryo-toxicity was not observed. By administering Met at either higher or lower than 187 mg/kg, there was less protection of RA-treated mice from CP induction.

For control mice, it appeared that detrimental toxic effects were observed neither for pregnant mice received Met administration alone on both E12 21 hr and on E13 21 hr, with 6 mg each injection; nor for pregnant mice received consecutive administration of 100 ug triamcinolone on E11 21 hr, 10 mg of Met on E12 21 hr, and 10 mg of Met on E13 21 hr.

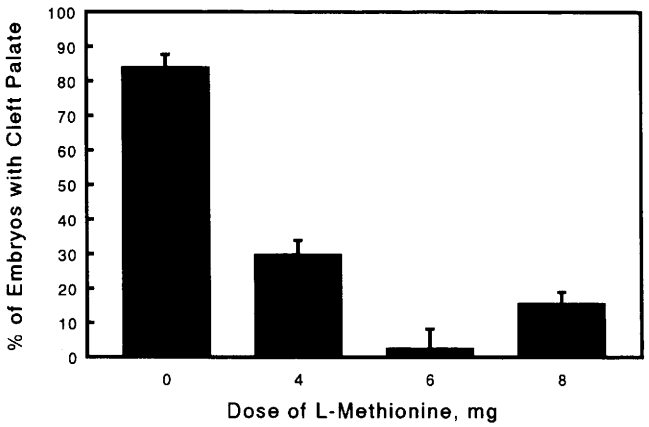

Figure 1. Bar chart for cleft palate frequency in the embryos of B10.A mouse strain after sequential administration of 100 ug triamcinolone (3.3 mg/kg) *intramuscularly* at E11 21 hr, and different doses of L-methionine (Met) between 0–8 mg *intraperitoneally* to each mouse (0–250 mg/kg) at E13 21 hr. Reduction in cleft palate frequency was observed after administration of 4–8 mg Met (125–250 mg/kg), but the maximum reduction was achieved by administering approximately 6 mg Met (187 mg/kg).

Discussion

Cleft lip with or without CP is a common human congenital malformation, and has a prevalence of approximately one in 700 live births in the United States. Little is known about its etiology or pathogenesis. It is presumed that CP is the consequence of an interplay of genetic predisposition and subsequent exposure to some extrinsic toxicants or "environmental" insults during human embryonic development between 4–10 weeks of gestation (22–27). It has been shown that intake of folate by pregnant women lowers the incidence of recurrence of cleft lip and palate (19–21). In our present study using the mouse animal model, we have demonstrated further that intake of therapeutic levels of Met effectively protects mice from CP induction by exogenous glucocorticoid or RA. Subsequent studies suggest that oral administration of these dose range of Met at E13 21h is equally effective in protecting triamcinolone-treated mice from CP (E. C. Lau and Z.-Q. Li, unpublished results). Our preliminary studies indicate that Met can also effectively protect

Table III. Results of Treating B10.A Mice with Retinoic Acid (RA)[a], or RA and Methionine (Met)

Treatment	No. of litters	Cleft palate	
		Observed outcomes	Mean frequencies (%)[b]
RA alone	4	51.9% (14/27)	47.6 ± 5.7
RA + 4 mg Met	3	23.8% (5/21)	23.3 ± 6.1
RA + 6 mg Met	4	9.7% (3/31)	9.0 ± 6.0
RA + 8 mg Met	3	38.1% (8/21)	37.6 ± 4.3

[a] Mice treated with all-*trans* RA received 47 mg/kg (i.e. 1.5 mg each) intraperitoneally on E13 21 hr.
[b] A mean frequency is calculated from the cleft palate frequencies observed for individual litters.

Table IV. Reduction of Retinoic Acid (RA)-Induced Cleft Palate by Exogenous Methionine (Met)

(a) Mice treated with different doses of Met:

	Yes		No		Totals
	O	E	O	E	
RA alone	14	8.10	13	18.90	27
RA + 4 mg Met	5	6.30	16	14.70	21
RA + 6 mg Met	3	9.30	28	21.70	31
RA + 8 mg Met	8	6.30	13	14.70	21
	30		70		100

(b) Compare Met-treated and untreated mice:

	Yes		No		Totals
	O	E	O	E	
RA alone	14	8.10	13	18.90	27
RA + Met[a]	16	21.90	57	51.10	73
	30		70		100

Ho: Assuming that there is no difference at all between Met-treated and untreated mice.
O: Observed outcomes.
E: Expected outcomes.
The χ_1^2 is 9.30, which is greater than 3.84, and thus $P < 0.05$.
[a] The Met-treated group represents a combination of all the mice administered with Met (4–8 mg).
The χ_1^2 is 5.89, which is much greater than 3.84, and thus $P < 0.05$.

mice from RA-induced cranial neural crest defects and CP. It will be anticipated that administration of exogenous Met is capable of protecting mice from CP and other craniofacial abnormalities induced by a wide spectrum of teratogens.

Although it has been shown that arachidonate administration is also capable of protecting mice from glucocorticoid-induced CP (26, 28), arachidonate is of relatively high cost and high toxicity. On the contrary, we have shown that pregnant mice were tolerant to a relatively wide dose range of Met, and detrimental toxic effects were not observed at Met levels of 187 mg/kg required for CP rescue. Furthermore, deleterious effects on growth rate or appearance for rats fed with toxic levels of Met can be alleviated by feeding them with MGS diet containing supplements of glycine and serine (29).

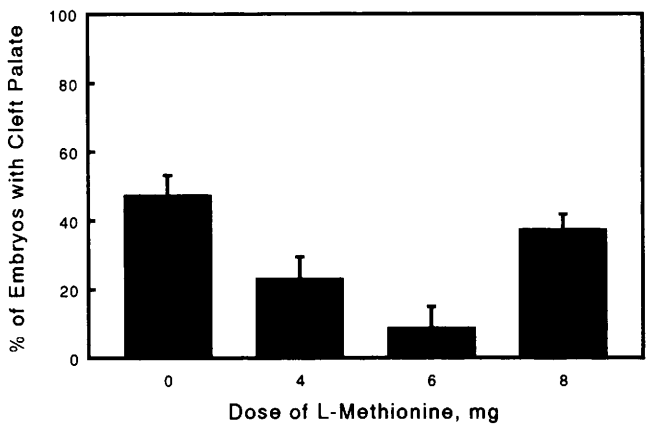

Figure 2. Bar chart for cleft palate frequency in the embryos of B10.A mouse strain after sequential administration of 1.5 mg all-*trans* retinoic acid *intraperitoneally* to each mouse (47 mg/kg) at E13 21 hr, and different doses between 0–8 mg of L-methionine (Met) *intraperitoneally* (0–250 mg/kg) at E13 22 hr. Reduction in cleft palate frequency was observed after administration of 4–8 mg Met (125–250 mg/kg), but the maximum reduction was achieved by administering approximately 6 mg Met (187 mg/kg).

In conclusion, the main finding of the present report is that exogenous Met protects mice from RA- and glucocorticoid-induced CP. Although we have demonstrated the effectiveness of exogenous Met (in the range of 125–250 mg/kg) in protecting B10.A mice from glucocorticoid- or RA-induced CP, the mechanism for amelioration of teratogenic actions by Met has not yet been known. Although DL-Met has been applied for treatment of human liver disorders, we would not extrapolate our present findings in mice to humans, and have not suggested Met for human CP prevention. Further studies with primates and other animal models will be required to assess the effectiveness and safe application of Met in human CP prevention.

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