D-chiro-inositol is more effective than myo-inositol in preventing folate-resistant mouse neural tube defects

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Abstract

BACKGROUND: Among mouse genetic mutants that develop neural tube defects (NTDs), some respond to folic acid administration during early pregnancy, whereas NTDs in other mutants are not prevented. This parallels human NTDs, in which up to 30% of cases may be resistant to folic acid. Most spina bifida cases in the folic acid-resistant 'curly tail' mouse can be prevented by treatment with inositol early in embryonic development. Here, the effectiveness and safety during pregnancy of two isomers, myo- and D-chiro-inositol, in preventing mouse NTDs was compared. METHODS AND RESULTS: Inositol was administered either directly to embryos in vitro, or to pregnant females by s.c. or oral routes. Although D-chiro- and myo-inositol both reduced the frequency of spina bifida in curly tail mice by all routes of administration, D-chiro-inositol consistently exhibited the more potent effect, reducing spina bifida by 73–86% in utero compared with a 53–56% reduction with myo-inositol. Pathological analysis revealed no association of either myo- or D-chiro-inositol with reduced litter size or fetal malformation. CONCLUSIONS: D-chiro-inositol offers a safe and effective method for preventing folic acid-resistant NTDs in the curly tail mouse. This raises the possibility of using inositol as an adjunct therapy to folic acid for prevention of NTDs in humans.

Key words: embryo culture, malformations, pregnancy, spina bifida, teratogen

Introduction

Folic acid supplementation during early pregnancy can prevent a proportion of neural tube defects (NTDs) (Wald et al., 1991; Czeizel and Dudás, 1992; Berry et al., 1999), whereas other cases of NTD appear resistant to folic acid. In the randomized controlled trial of the Medical Research Council, UK (Wald et al., 1991), 28% of NTD cases recurred despite supplementation with 4 mg folic acid per day. Moreover, the recent introduction of folic acid fortification of bread flour in the USA resulted in only a 19% decline in the prevalence of NTDs (Honein et al., 2001). While these findings may indicate the need for increased levels of folic acid supplementation, they are also consistent with a proportion of NTDs exhibiting resistance to exogenous folic acid.

Mouse genetic models also indicate the existence of folate-resistant NTDs. Mutant strains including 'splotch', 'crooked tail' and the 'Cart1' knockout exhibit NTDs that can be prevented by folic acid treatment during early pregnancy (Fleming and Copp, 1998; Zhao et al., 1996; Carter et al., 1999), whereas folic acid is ineffective in preventing NTDs in the mutant strains 'curly tail', 'axial defects' and the 'ephrin-A5' knockout (Essien and Wannberg, 1993; Seller, 1994; Holmberg et al., 2000).

Previously, it was demonstrated that a proportion of the folate-resistant NTDs in curly tail mice can be prevented by treating pregnant females, or embryos in vitro, with myo-inositol (Greene and Copp, 1997). These
myo-inositol in the culture medium of rat and mouse embryos caused a high incidence of cranial NTDs (Cockroft, 1988; Cockroft et al., 1992), and that NTDs developing in rat embryos cultured under diabetic conditions could be ameliorated by supplementation with myo-inositol (Baker et al., 1990; Hashimoto et al., 1990). Together, these findings suggest inositol as a potential therapeutic option for prevention of folate-resistant NTDs.

Here, the analysis of inositol as a primary therapeutic agent in preventing mouse NTDs has been extended. The study was intended to serve as a preliminary to a human clinical therapeutic trial. It was shown that inositol could prevent NTDs both in vitro and following administration to pregnant curly tail females, either by s.c. or oral routes. A detailed pathological analysis revealed no significant adverse effects of inositol therapy on mouse fetuses. Moreover, the effectiveness of myo-inositol was compared with that of D-chiro-inositol, a closely related enantiomer that differs in the orientation of the carbon-two hydroxyl group (C2-OH) relative to the plane of the six carbon ring.

**Materials and methods**

**Mice**

Curly tail mice are maintained as a homozygous, random-bred stock in which NTDs develop in ~60% of individuals (Van Straaten and Copp, 2001). Experimental litters were generated by timed matings, and the day of finding a copulation plug was designated embryonic day (E) 0.5.

**In-vitro inositol treatment**

E9.5 embryos (17–19 somite stage) were cultured for 24 h at 38°C in whole rat serum, as described previously (Copp et al., 1999). At 30 min after the start of culture, myo-inositol (Sigma, UK) or D-chiro-inositol (Insmed, VA, USA) was added to the medium (62.5 µl of inositol stock per ml rat serum) to a final concentration of 5, 10, 20 or 50 µg/ml inositol. Control cultures received an equal volume of phosphate-buffered saline (PBS). Following culture, embryos were scored for: (i) posterior neuropore length (the distance from the rostral end of the posterior neuropore to the tip of the tail bud); (ii) crown–rump length; and (iii) somite number.

**In-utero inositol treatment**

For s.c. administration, osmotic mini-pumps (capacity 100 µl, delivery rate 1 µl/h; model 1003D, Alzet) were filled with solutions of 30, 75 or 150 mg/ml inositol (delivering 29, 72 and 144 µg inositol/g body weight per day respectively for a 25 g mouse), or PBS as a control. Mini-pumps were incubated in sterile PBS at 37°C for 4 h and then implanted s.c. on the back of pregnant mice at E8.5. General anaesthesia was induced by an i.p. injection of 0.01 ml/g body weight of a solution comprising 10% Hypnovel® (midazolam 5 mg/ml) and 25% Hypnorm® (fentanyl citrate 0.315 mg/ml, fluanisone 10 mg/ml) in sterile distilled water. For oral administration, pregnant mice were gavaged with 0.5 ml inositol solution in PBS twice daily intervals from E8.5 to E10.5 (six doses in total; 800 µg/g body weight per day).

**Analysis of fetuses following inositol treatment in utero**

Pregnant females were killed at E18.5, and the total number of implantations, classified as viable fetuses or resorptions, was recorded. Fetuses were dissected from the uterus and inspected immediately for the presence of open lumbo–sacral spina bifida and tail flexion defects: the primary manifestations of the curly tail genetic defect (Gruneberg, 1954; Van Straaten and Copp, 2001). A randomly selected sample of fetuses was fixed in Bouin’s fluid and subjected to detailed internal pathological analysis by free-hand serial sectioning (Wilson, 1965). Other fetuses were fixed in 95% ethanol and processed for skeletal examination (Whittaker and Dix, 1979).

**Statistical data analysis**

Continuous variables (somite number, crown–rump length, posterior neuropore length, litter size) were compared between treatment groups using analysis of variance or Kruskal–Wallis test. Ordinal regression was used to quantify further the effects of treatment and dosage on posterior neuropore length. Fisher’s exact or χ²-tests were used to compare...
phenotype frequencies between groups. To evaluate possible litter effects, multilevel ordinal logistic models were fitted to the data in Table III (MLwiN v1.10.0006) (Goldstein, 1995). Logistic regression was used to determine whether the number of resorptions in utero was linked to dose and/or treatment.

### Results

**D-chiro-inositol is more effective than myo-inositol in normalizing neural tube closure in embryo culture**

Curly tail embryos were cultured in the presence of inositol for 24 h from E9.5, the period during which the neural tube is closing at the posterior neuropore of the mouse embryo. In curly tail embryos, neuropore closure is delayed or fails to be completed, leading to the development of tail flexion defects and spina bifida, respectively (Figure 1A–C) (Copp, 1985). Posterior neuropore length at E10–10.5 is positively correlated with the likelihood that an embryo will progress to develop a spinal NTD (Copp, 1985; Van Straaten et al., 1992).

![Figure 1.](https://example.com) **Figure 1.** (A) Curly tail embryo after 24 h culture from E9.5 to E10.5. The box indicates the posterior neuropore region. (B,C) Higher magnification of the caudal region of cultured curly tail embryos. The posterior neuropore, a region of open neural folds, occupies the dorsal part of the caudal region, between the arrows in (B) and (C). Embryos with a large neuropore (C) develop spina bifida and/or a tail flexion defect, whereas embryos with a small neuropore (B) complete neural tube closure normally. (D–G) Skeletal preparations of E18.5 curly tail fetuses in dorsal view (D,E: rostral to the top) or right lateral view (F,G: rostral to the right). Compared with the normal fetus in (D), the fetus with spina bifida (E) exhibits vertebral pedicles widely spaced apart and absent neural arches in the low lumbar/sacral region (long arrow in E). The tail (short arrow in E) is enclosed in a skin sac and appears reduced in length owing to deformation and fusion of caudal vertebrae. The tail flexion defect (G) comprises a 360° curl of the tail, compared with the normal fetus which exhibits a straight tail (F). Scale bars: A = 0.5 mm; B,C = 0.2 mm; D–G = 2 mm.

Both myo- and D-chiro-inositol exhibited a dose-dependent normalization of posterior neuropore length in embryo culture, as judged by the reduction in neuropore length observed in embryos treated with higher inositol doses (Table I; Figure 2A). Strikingly, D-chiro-inositol significantly reduced neuropore length at dose levels of both 20 and 50 µg/ml, whereas a comparable effect was seen with myo-inositol only at 50 µg/ml. Embryos exposed to 20 µg/ml myo-inositol (or 5–10 µg/ml D-chiro-inositol) exhibited neuropore lengths that were not different from PBS-treated controls.

![Table 1.](https://example.com) **Table 1.** Comparison of growth and developmental parameters in curly tail embryos cultured in the presence of myo- and D-chiro-inositol

**Figure 2.** (A) Exposure of curly tail embryos in culture to myo- (dark grey bars) or D-chiro-inositol (black bars) causes a dose–dependent
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in the frequency of spina bifida among the offspring of mice treated with either myo- or D-<i>d</i>-<i>chiro</i>-inositol (Figure 2B), and a significant shift in the distribution of fetuses between the three phenotype categories (Table II). D-<i>d</i>-<i>chiro</i>-inositol was most effective, causing a 86% reduction in the frequency of spina bifida, while a 53% reduction was observed for myo-inositol.

An ordinal multilevel regression analysis confirmed that fetuses treated s.c. with D-<i>d</i>-<i>chiro</i>-inositol (<i>P</i> < 0.0005) and myo-inositol (<i>P</i> < 0.002) were significantly less likely to have spina bifida than those treated with PBS. In the oral dosing study, fetuses treated with D-<i>d</i>-<i>chiro</i>-inositol were less likely to have spina bifida than PBS-treated controls (<i>P</i> < 0.01), whereas the trend towards reduced spina bifida frequency in litters treated with myo-inositol did not reach statistical significance.

A further investigation was made to determine whether clustering of fetuses of particular phenotypes within litters may have affected the outcome of the comparison between myo-inositol, D-<i>d</i>-<i>chiro</i>-inositol and PBS. The multilevel analysis (which took into account the potential non-independence of fetuses within litters) showed that the difference between treatment groups is unaffected when possible litter effects are taken into account.

No evidence of an adverse effect of inositol on pregnancy success or fetal outcome

One possible explanation for a decrease in spina bifida frequency following maternal inositol administration could be an increase in loss of affected fetuses during pregnancy. When both resorption rate and litter size were examined in pregnancies receiving either s.c. or oral inositol, no significant difference was found between pregnancies treated with inositol and those receiving PBS alone (Table III).

To identify any adverse effects of inositol treatment on fetal outcome, fetal crown–rump length was measured, but no significant differences were found between treatment groups (Table III). An extensive pathological analysis of treated fetuses was performed using both free-hand serial sectioning and skeletal preparation. This analysis confirmed the occurrence of spina bifida and tail defects in a proportion of fetuses (Figure 1D–G), as also scored by external fetal inspection. Additionally, exencephaly, a failure of cranial neural tube closure, was observed in a small proportion of fetuses (Table IV). Exencephaly is a recognized, infrequent defect in curly tail homozygotes (<i>Embury et al</i>., 1979). No overall increase or decrease in this defect was observed in inositol-treated fetuses compared with PBS controls, although the low frequency of affected fetuses may have obscured any treatment effect.

Internal and skeletal examination revealed almost no major structural defects, apart from NTDs (Table IV). For instance, no major malformations of the heart, lungs, kidney, gut, limbs or skeleton were identified. Of the morphological changes observed in the analysis, most were minor (e.g. small additional liver lobe) and the great majority occurred as frequently in PBS controls as in fetuses treated with inositol. D-<i>d</i>-<i>chiro</i>-inositol-treated fetuses (144 µg/g body weight per day) showed a somewhat elevated frequency of enlargement of the renal pelvis/ureter, rudimentary ribs on the seventh cervical vertebra and occurrence of anomalous cervical
significant. Anomalous cervical vertebrae was associated with exencephaly in several litters, suggesting that cervical anomalies may form part of the spectrum of vertebral column defects present in the curly tail mouse.

Discussion

In the present study, the ability of exogenous inositol to prevent spinal NTDs in the folate-resistant curly tail mouse genetic model was evaluated. Maternal inositol administration significantly reduced the frequency of spina bifida in curly tail mice and normalized closure of the posterior neuropore in whole cultured embryos. A striking finding was the increased potency of D-chiro-inositol compared with myo-inositol—two closely related enantiomers that differ only in the orientation of the carbon–two hydroxyl group (C2–OH) relative to the plane of the six carbon ring. At identical dosage levels, s.c. administered D-chiro-inositol caused a consistently greater reduction in frequency of spina bifida than myo-inositol. Moreover, in vitro, D-chiro-inositol was effective in normalizing neural tube closure at a concentration at which myo-inositol had no effect.

Reasons for the differing potency of myo- and D-chiro-inositol in preventing NTD

It was shown previously that the protective effect of myo-inositol is mediated via the phosphoinositide cycle (Greene and Copp, 1997), which generates the second messengers inositol triphosphate and diacylglycerol (DAG) (Majerus, 1992). Among the downstream events of this signalling pathway, activation of protein kinase C (PKC) by DAG appears critical for normalization of neuropore closure in curly tail embryos. For instance, activation of PKC by phorbol esters mimics the effect of inositol supplementation, whereas PKC inhibitors abrogate the protective effect (Greene and Copp, 1997).

It is possible that D-chiro-inositol also acts through a PKC-dependent pathway, in which case the greater preventive effect of D-chiro-inositol may result from its differential incorporation and metabolism within the phosphoinositide cycle. Insulin stimulation of rat fibroblasts expressing the human insulin receptor leads to a significant increase in the incorporation of D-chiro-inositol into phospholipids, whereas the effect on myo-inositol incorporation is only marginal (Pak et al., 1993). Moreover, D-chiro-inositol induces a much larger reduction in plasma glucose level in rats rendered diabetic by streptozotocin administration compared with exogenous myo-inositol (Ortmeyer et al., 1993). In humans, D-chiro-inositol can increase the action of insulin in patients with polycystic ovarian syndrome, improving ovulatory function, reducing blood pressure, and decreasing blood androgen and triglyceride concentrations (Nestler et al., 1999). These findings suggest an inherently greater potency or bioactivity of D-chiro-inositol than myo-inositol, perhaps as a result of incorporation into different phosphatidylinositol species (Pak and Larner, 1992). It is striking, however, that these differences of in-vivo potency are maintained in the face of the demonstrated interconversion of the two inositol isomers (Pak et al., 1992). Perhaps the rate of interconversion is too low to obscure the inherently greater potency of D-chiro-inositol in short-term effects on embryonic development.

How does inositol normalize neural tube closure in curly tail mice?

Although the causative gene responsible for the curly tail defect is unknown (Van Straaten and Copp, 2001), the primary cellular abnormality leading to the development of spina bifida has been identified as a reduced rate of cell proliferation in the hindgut endoderm and notochord (Copp et al., 1988). The resultant growth imbalance between dorsal and ventral tissues causes excessive ventral curvature of the caudal embryonic region, which mechanically opposes closure of the posterior neuropore (Brook et al., 1991). Exogenous inositol treatment may correct the underlying cell proliferation defect in curly tail embryos. In support of this idea, inositol metabolism is known to be intimately involved with cell cycle progression in a variety of cell types. For instance, a nuclear polyphosphoinositide cycle exists in which phosphatidylinositol-specific phospholipase C generates elevated levels of nuclear DAG specifically...
required for the G2/M transition, perhaps by attracting specific activated PKC isozymes to the nucleus, where they are stabilized by binding to nuclear proteins including lamins A, B and C. The elevation of DAG levels is transient, as DAG is rapidly converted to phosphatidic acid by nuclear DAG kinases, enzymes that are also induced by growth–promoting agents (Martelli et al., 2000). It is possible that inositol treatment of curly tail embryos stimulates this nuclear DAG cycle leading, via the activation of specific PKC isozymes, to the increased proliferation of hindgut and notochordal cells, so normalizing neural tube closure.

Relevance of the findings for clinical application of inositol therapy
In order for these experimental findings to be translated into a clinical application, inositol supplementation must be not only effective in preventing NTDs, but also safe for use during human pregnancy. The current demonstration of a protective effect of inositol by s.c. and oral administration is supported by the results of previous studies in which myo-inositol was injected i.p. (Greene and Copp, 1997). Further support for the effectiveness of oral supplementation with myo-inositol comes from the finding of a reduction in the incidence of diabetes–induced abnormalities in rats by oral administration of myo-inositol (Khandelwal et al., 1998). The efficacy of inositol in preventing human NTDs has not yet been tested in a clinical trial. Nevertheless, a recent case study has documented inositol supplementation in association with a normal outcome in the third pregnancy of a woman who had two previous consecutive NTD pregnancies despite taking 4 mg folic acid throughout the periconceptional period (Cavalli and Copp, 2002). The empirical recurrence risk of NTD following two previous affected fetuses is ~1 in 9 (Seller, 1981), so the association of inositol therapy with normal pregnancy outcome in this case may have been a chance association. A larger study of pregnancies at risk of ‘folate–resistant’ NTDs is needed, to test the idea that inositol may be as effective in humans as in mice.

With regard to the safety of inositol therapy during pregnancy, the present pathological study revealed no major defects, apart from NTDs, and no increase in the frequency of embryonic or fetal loss in utero in inositol–treated mice. The present study was limited, however, and in particular the effects of inositol administration during the periconceptional period—when inositol would be taken during a clinical trial—were not assessed. The reproductive toxicology of D–chiro-inositol has been the subject of studies in rats and rabbits, with more extensive administration at doses up to 2000 mg/kg per day, and no adverse effects on mating, fertility or embryo/fetal development have been found (Insmed Inc., data on file). There is much less information available on the safety of inositol therapy in human pregnancy. In the case of the mother who took inositol in her third pregnancy following two apparently ‘folate–resistant’ NTD pregnancies, a dose of 0.5 g inositol per day was used, with no known side–effects for either mother or baby (Cavalli and Copp, 2002). In particular, there was no evidence of abnormal uterine contractions, such as have been suggested as a possible adverse effect of inositol therapy (Colodny and Hoffman, 1998). Myo-inositol has also been tested in adults for prevention of depression, panic disorder and obsessive compulsive disorder (Benjamin et al., 1995; Levine et al., 1995; Fux et al., 1996) and in children for treatment of autism (Levine et al., 1997). No significant side–effects were reported in these studies, which employed relatively high inositol doses of up to 18 g per day in adults and 200 mg/kg in children.

In conclusion, D–chiro–inositol has been shown to be highly effective in preventing folate–resistant mouse NTDs. A clinical trial could next evaluate the effectiveness of peri–conceptional inositol supplementation, as an adjunct to folic acid therapy, in preventing human NTDs. If folate–resistant NTDs can be prevented, in addition to those cases already prevented by folic acid, this could lead to a significant further reduction in the frequency of this birth defect.

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