

A Comparison Study of the Effects of *Echinacea purpurea* Ethanolic Extract and Mesna on Cyclophosphamide-Induced Macroscopic Fetal Defects in Rats

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Abstract

Objective(s)

There are some reports that the teratogenic effects of cyclophosphamide (CPA) can be prevented by application of antioxidant drugs and stimulation of the maternal immune system. *Echinacea purpurea* extract is antioxidative and immunomodulator drug. Mesna (Sodium 2-mercaptoethane sulfonate) is used for decreasing side effects of CPA, especially hemorrhagic cystitis. In this study, we compared the prophylactic effects of mesna and *Echinacea* extract on teratogenic effects of CPA.

Materials and Methods

This study was performed on 32 pregnant rats that were divided into 4 groups. The first group (control group) received normal saline and the other groups received CPA (15 mg/kg intraperitoneally) on 13th day of gestation. Mesna and *E. purpurea* extracts were administrated at doses of 100 and 400 mg/kg by IP injection, respectively, along with it and 12 hr later, after CPA injection. Rats were dissected on day 20 of gestation, embryos harvested and after determination of gross malformations they were stained by Alizarin red-Alcian blue method.

Results

Cleft palate incidence was 38.46, 30.77 and 14.28% in fetuses of rats that received only CPA, CPA with mesna and CPA with *Echinacea* extract, respectively. In addition, skeletal anomalies incidence including limbs, vertebra, sternum, and scapula defects were decreased by *Echinacea* extract.

Conclusion

E. purpurea has significant effect on preventing CPA-induced malformations and better prophylactic effect than mesna on cases like CPA-induced cleft palate.

Keywords: Cyclophosphamide, *Echinacea*, Macroscopic Anomalies, Mesna, Pregnancy, Rat

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Introduction

Some chemical agents and drugs can induce teratogenic effects and abortion (1). Developmental defects are a major health problem as in the USA 3-5% of fetuses have congenital abnormality (2). It is estimated that 7-10% of human anatomic anomalies result from the disruptive actions of drugs, viruses, and other environmental factors (3). De Sanntis *et al* also estimated that defects attributable to drug therapy represent about 1% of congenital defects of known etiology (4). Although, 40 agents are teratogenic for human fetuses, more agents are teratogenic in laboratory animals. Valperoic acid, cyclophosphamide (CPA), methylnitrous urea and phenytoin are the best known teratogenic drugs in human and laboratory animals (5, 6).

Several studies show that the stimulation of maternal immune system can decrease or prevent drug-induced embryonic abnormalities (7, 8). For example, in one study, macrophage activation decreases incidence of cleft palate and digital and tail anomalies in fetuses of mice that received urethane and methylnitrous urea (8). In another study, interferon gamma reduced urethane-induced cleft palate and granulocyte-colony stimulating factor decreased cyclophosphamide-induced distal limb abnormalities in mice (9).

Data from laboratory research and critical trials also suggest beneficial influences of the maternal immune system on pregnancy outcome (10, 11). Nonspecific immune stimulation by injection of Freund's complete adjuvant (FCA) reduced early embryo loss in CBA/J mice (10). In rodents (12) and humans (11), alloimmunization with paternal lymphocytes has reported efficacy for prevention of early embryo loss. In teratogen-exposed rodents, a significant decrease in morphologic defects was observed after maternal immune stimulation (13). Mechanism for these effects remains unclear; however, the possible involvement of cytokines produced by immune cells has been suggested (13).

Ivnitsky *et al* (1998) found that CPA-induced brain and craniofacial anomalies in mice were associated with increased TNF- α in fetal head and brain (14). Maternal

immunostimulation decreased severity of CPA-induced malformation in these mice, and decreased TNF- α expression in fetal heads (14). In related studies, Savion *et al* (1999) reported that maternal dosing with granulocyte macrophage-colony stimulating factor (GM-CSF) significantly reduced CPA-induced limb malformation in mice. This effect was comparable to that produced by intrauterine leukocyte administration, and resulted in increased maternal IL-2 and IL-3 production as well as increased Mac-1 positive leukocyte in the uteroplacental units of pregnant mice. Thus, for CPA-induced fetal malformations, immune-mediated protective effects have been related to altered levels of cytokines in both the uteroplacental unit and in the fetus (15).

CPA as an alkylating agent is used for treatment of cancer and to prevent rejection of tissue transplantation. CPA has several toxic effects including hemorrhagic cystitis. Metabolites of CPA, especially acroleine modulates its toxic effects (16).

Mesna (sodium 2-mercaptoethane sulfonate) is used for decreasing toxic effects of CPA in patients. In addition, mesna was effective in lowering CPA-induced malformation in rats (17).

Echinacea stimulates the immune system and is applied in the prevention or treatment of some diseases including influenza and common cold. *Echinacea* extract activates macrophage, polymorphonuclear leukocytes and natural killer cells (18). The effect of *Echinacea purpurea* extract on decreasing phenytoin-induced cleft palate in mice was reported (19).

In the present study, the prophylactic effects of mesna and *E. purpurea* extract on CPA-induced macroscopic fetal defects in rats were compared.

Materials and methods

Dried aerial parts of *E. Purpurea* were from Goldaruo Co. Isfahan, Iran. The plant was taxonomically identified at the Department of Botany, School of Agriculture, Shahid Chamran University, Ahwaz, Iran. The plant was powdered, using a grinder (MSE, England). One hundred grams of this powder

was placed in a beaker and 1000 ml of 70% ethanol added. The mixture was left at room temperature for 3 days. The extract separated and the remaining plant extracted with more ethanol after 2 days. The extract was filtered by Whatman (No. 1) filter paper and concentrated under vacuum evaporation and then the product dried in oven at low temperature.

Male and female healthy Wistar rats, 10-12 weeks of age, weighing 180-200 g were purchased (Razi Institute, Karaj, Iran) and housed individually (males) or at 10 per polycarbonate cage (female) for a 2-week acclimation period. Rats were fed *ad libitum* by standard laboratory pellet (Pars khorakdam, Shoshtar, Iran.) and tap water. A 12-hr light: 12-hr dark cycle was maintained. Room temperature was at 23 ± 2 °C with a relative humidity of 45-55%.

Male and female rats were housed together. Pregnant females were divided into four groups (n=8) and treated as follow:

First group received normal saline (10 ml/kg), the second group received CPA (15 mg/kg) (Slott and Hales, 1986), the third group received CPA (15 mg/kg) and along with it and 12 hr later mesna (100 mg/kg) (17), and the fourth group received CPA (15 mg/kg) and along with it and 12 hours later extract of *E. purpurea* (400 mg/kg) (19). All drugs were diluted in distilled water then administrated intraperitoneally.

The animals were sacrificed by cervical dislocation on 20th day of gestation and fetuses collected and numbered; then weight and length of them measured and gross malformations determined. Fetuses were stained by Alizarin red-Alcian blue method (20) and investigated by stereomicroscope (Nikon, Japan) for skeletal defects. The incidence of macroscopic defects was determined and was compared in the groups. Statistical significance between groups determined using SPSS program. The minimum level of significance was $P<0.05$.

Results

There were not any aborted or absorbed fetuses from normal saline group. Total

number of collected fetuses from groups 1, 2, 3 and 4 were 26, 49, 27 and 47, respectively.

No macroscopic anomalies were observed in the control animals. In the control group palatal closures of fetuses were normal on gestational day 20 (i.e., palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Figure 1A). CPA induced cleft palate at 38.46% incidence (Figure 1B). Mesna reduced incidence of CPA-induced cleft palate to 30.77%, but *E. purpurea* extract reduced it to 14.28 %. In group 2, percentage of intrauterine death of fetuses was 32.6%, but not in other groups. Percentages of absorbed fetuses were 40.8, 51.85 and 55.3 in groups 2 to 4, respectively, so mesna and *E. purpurea* increased the resorption rate.

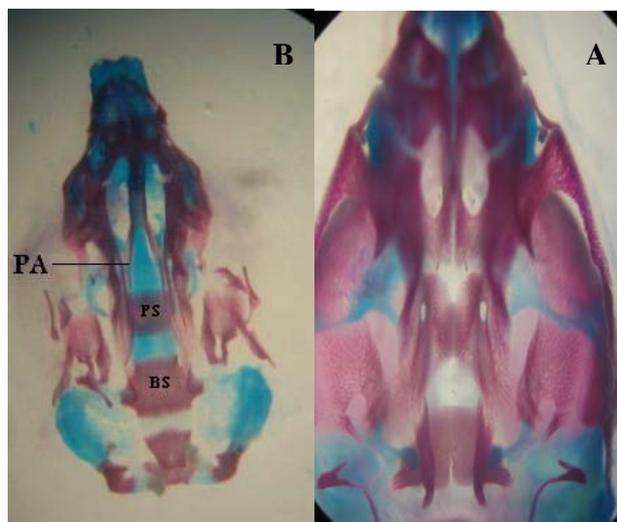


Figure 1. Ventral view of skull of GD 20 fetal rats. A) Normal palatine bone B) Cleft palate induced by cyclophosphamide (arrow) which stained with Alizarin red- Alcian blue. PS: palatine; BS: sphenoid.

Exencephaly, omphalocele, open eye, brachygnathia, and several different anomalies in vertebrae, sternum, scapula and limbs were observed (Figure 2) which their incidences are shown in Table 1. Their incidences (except exencephaly) were decreased by *Echinacea* extracts. Open eye and brachygnathia were not observed in animals treated with mesna and *E. purpurea*. Mean weight ($P<0.043$) and length ($P<0.02$) were significantly decreased in the group which received only CPA. The means of weight ($P<0.044$) in the group that received mesna were significantly greater than the group received only CPA.

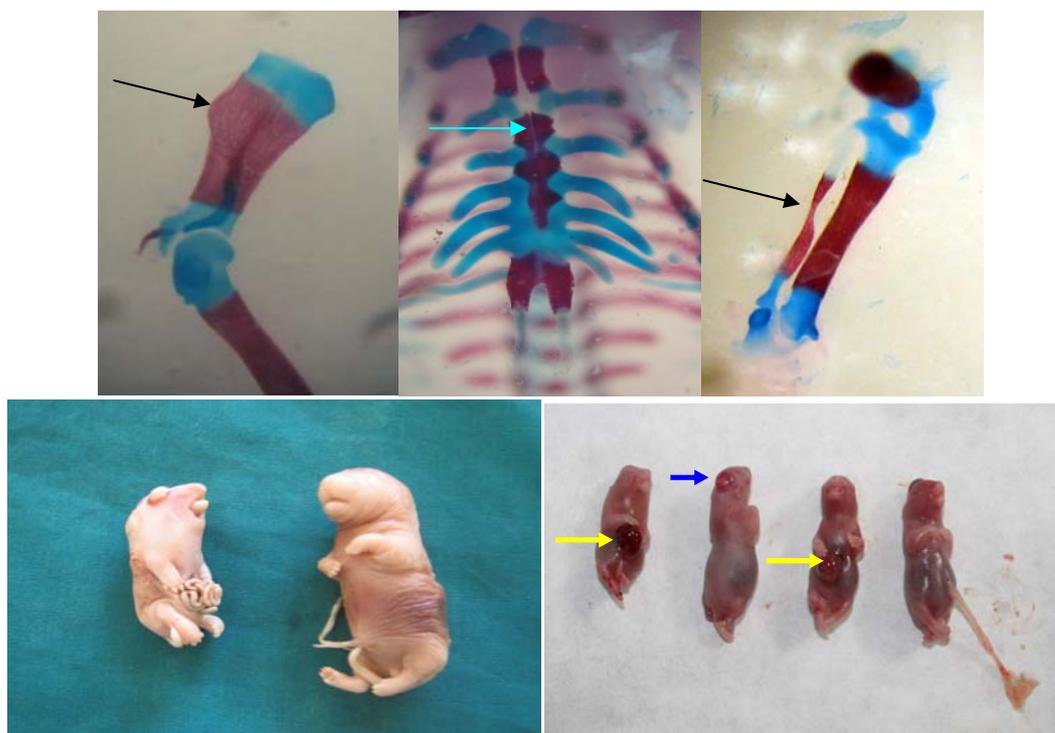


Figure 2. Some skeletal defects in fetuses of rats. Curved spine of scapula and absence deltoid tuberosity (up-left); fused sternbrae and split xiphoid process (up-middle); curved fibula (up-right); fetus deformity (left-down); exencephaly and omphalocele.

The mean weight and length in the group that received mesna did not differ significantly with the control group. The mean weight ($P<0.049$) and length ($P<0.001$) were significantly decreased in the group which received *Echinacea* extract and differed significantly in comparison to the control group (Figures 3, 4).

Table 1. Incidence of anomalies in fetuses of groups. Group1 (control) without macroscopic anomalies; Group 2 received cyclophosphamide; group 3 received CPA+mesna; group 4 received CPA+*Echinacea* extract.

Anomaly	Incidence (%)		
	Group 2	Group 3	Group 4
Cleft palate	38.46	30.77	14.28
Exencephaly	100	92.3	100
Omphalocele	23.07	7.69	4.76
Open eye	23.07	84.6	0
Brachygnathia	23.07	53.8	0
Sternum defects	37.93	53.8	33.3
Vertebral defects	65.5	100	61.9
Limbs defects	100	100	76.19

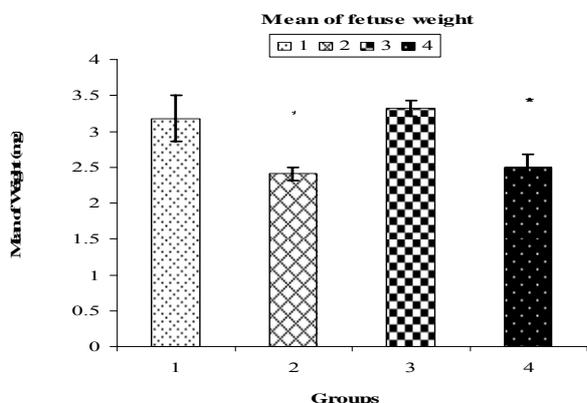


Figure 3. Weight (mean±SEM) of fetuses in normal saline and test groups: 1: normal saline (1 ml/100g IP); 2: CPA (15 mg/kg IP); 3: CPA+mesna (100 mg/kg IP); 4: CPA+*Echinacea* (400 mg/kg IP). n=8;* Significant difference with normal saline group ($P<0.05$).

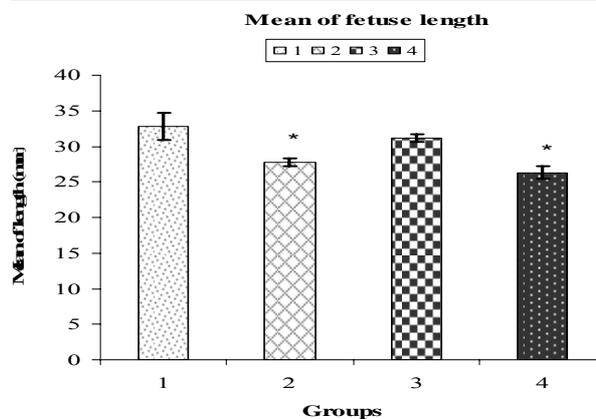


Figure 4. Length (mean± SE) of fetuses in normal saline and test groups. 1: normal saline (1ml/100g IP); 2: CPA (15 mg/kg IP); 3: CPA+mesna (100 mg/kg IP); 4: CPA+*Echinacea* (400 mg/kg IP). n=8;* Significant difference with normal saline group ($P<0.05$).

Discussion

Several studies have verified that the maternal immune stimulation can reduce teratogenic anomalies (21). Mechanisms of this effect remain unclear, but it is thought the fetal gene expression has been modulated (8).

The enhancing antioxidative effects can protect fetuses against phenytoin teratogenicity (9, 22). Sharova *et al* showed that interferon-gamma and Freund's complete adjuvant reduced severity of the urethane-induced cleft palate in mice (23). In the present study, the prophylactic effects of mesna and *Echinacea* on CPA-induced macroscopic fetal defects were compared in rats. Both mesna and *Echinacea* reduced the severity of incidence of cleft palate. *Echinacea* was greater in decreasing the incidence of cleft palate than mesna. Mesna had better protective effect than *Echinacea* on weight and length of fetuses.

Sloth and Hales (1986) evaluated effect of mesna on CPA-induced teratogenicity. They used CPA at dose 10 and 15 mg/kg in rats in 13th day of gestation. They observed the CPA can produce teratogenicity in 50 and 100% of fetuses with 10 and 15 mg/kg, respectively (17). They determined fetal defects similar with our study including hydrocephaly, omphalocele, open eye, brachygnathia, and limb defects. These anomalies were decreased by 30 mg/kg mesna.

Cytokines have been reported to mediate CPA-induced neurotoxicity (7). Granulocyte-macrophage colony stimulating factor (GM-CSF) as cytokine and injection of leukocytes decreased CPA-induced teratogenicity including limb defects (7, 8).

E. purpurea stimulates various immune cells including macrophages and natural killer cells. It has anti-inflammatory effects (18). In one study, *E. purpurea* root increased levels of interleukine 1 and 6, tumor necrosing factor and antibody production more than the extracts

of *E. angustifolia* and *E. pallida* (24). Bukovsky *et al* (1995) reported that ethanolic extract of *E. purpurea* increased activity of mouse peritoneal macrophage following 5 days exposure (25). In one double-blinded study in 24 men, oral administration of *E. purpurea* extract increased polymorphonuclear (PMN) phagocytic activity for 5 days that reached its peak levels on the 5th day (18). Moreover, *Echinacea* has anti-oxidative and free-radical scavenging activity (26). *Echinacea* is used for treatment of acute upper respiratory infections including common cold and influenza (18, 27). In other study, *Echinacea* appears to attenuate the response of macrophages to an immune stimulus and its combination of phytochemicals exhibits different pharmacological properties to one or more of the isolated major individual components (28). *Echinacea* alkylamides modulate induced immune responses in T-cells (29).

Conclusion

In the present study, *E. purpurea* extract had prophylactic effect on incidence of CPA-induced skeletal anomalies. Mesna chelates metabolites of CPA including acrolein and reduces its side effects. Effect of CPA on teratogenicity is mediated indirectly by inducing oxidative stress. Thus, *E. purpurea* can decrease anomalies from CPA by antioxidative or immunostimulant effect, which we believe deserves further investigation.

Acknowledgment

The authors wish to express their gratitude to the research council of Shahid Chamran University for their financial supports. The authors declare that they have no conflict of interests.

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