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Prophylactic Effects of Silymarin and Vitamin E on Cyclophosphamide-Induced Skeletal Malformations in Rat Embryos

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Abstract: There are many reports that the teratogenic effects of cyclophosphamide (CP) can be prevented by application of antioxidant drugs and stimulation of the maternal immune system. Also, there is some evidence that vitamin E and silymarin are antioxidant. Therefore, in this study, the prophylactic effects of silymarin and vitamin E on teratogenic effects of CP was compared. This study was performed on 24 pregnant rats that were divided into four groups. Control group received normal saline and test groups received CP (15 mg/kg), CP (15 mg/kg) plus silymarin (100 mg/kg) and CP (15 mg/kg) plus vitamin E (100 mg/kg) intraperitonealy at 13th day of gestation, respectively. Fetuses were collected at 20th day of gestation and after determination of weight and length; they were stained by Alizarin red - Alcian blue method. Cleft palate incidence was 38.46, 9.37 and 16.66% in fetuses of rats that received only CP, CP plus silymarin and CP plus Vitamin E, respectively. In addition, skeletal anomalies incidence including limbs, vertebrae, sternum and scapula defects were decreased by silymarin. The mean of weight and length of animals' fetuses that received silymarin and vitamin E were significantly greater than those received only CP. It is concluded, silymarin has significant effect in preventing CP-induced malformations and in cases like CP-induced cleft palate better prophylactic effect than vitamin E.

Key words: Cyclophosphamide · Embryo Malformations · Silymarin · Vitamin E · Teratogenicity · Rats

INTRODUCTION

Cyclophosphamide (CP) as an alkylating agent is used for treatment of cancer and to prevent rejection of tissue transplantation. CP has several toxic effects including hemorrhagic cystitis. Metabolites of cyclophosphamide, especially acroleine modulates its toxic effects [1, 2]. Cyclophosphamide (CP) is the best known teratogenic drug in human and laboratory animals [2-4].

Several studies show that the stimulation of maternal immune system can decrease or prevent drug-induced embryonic abnormalities [5, 6]. 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 [6]. In another study, interferon gamma reduced urethane-induced cleft palate and granulocyte-colony stimulating factor decreased cyclophosphamide-induced distal limb abnormalities in mice [4].

Data from laboratory research and critical trials also suggest beneficial influences of the maternal immune system on pregnancy outcome [7, 8]. Nonspecific immune stimulation by injection of Freund's complete adjuvant (FCA) reduced early embryo loss in CBA/J mice [5]. In rodents [9] and humans [7], 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 [10]. Mechanism for these effects remains unclear; however, the possible involvement of cytokines produced by immune cells has been suggested [10].

Ivnitsky *et al.* (1998) found that CP- induced brain and craniofacial anomalies in mice were associated with increased TNF-α in fetal head and brain. Maternal immunostimulation decreased severity of CP-induced malformation in these mice and decreased TNF- α expression in fetal heads [11]. In related studies, Savion *et al.* (1999) reported that maternal dosing with

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granulocyte macrophage-colony stimulating factor (GM-CSF) significantly reduced CP-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 CP-induced fetal malformations, immune-mediated protective effects have been related to altered levels of cytokines in both the uteroplacental unit and in the fetus [12].

In the other hands, vitamin E, a natural antioxidant, is believed to help prevent diseases associated with oxidative stress [13]. Vitamin E is considered safe in pregnancy, although experiments evaluating the safety of high-doses vitamin E treatment in pregnancy have not been reported [7].

Silymarin, the mixture of flavonolignans extracted from blessed milk thistle (*Silybum marianum*) is a scavenger of radicals, such as hydroxyl, superoxide and hydrogen peroxide (H_2O_2) and increases SOD and decrease lipid peroxidation [14, 15, 16]. Silymarin has been shown to be safe in animal models and no significant adverse reactions are reported in human studies [16].

In the present study, the prophylactic effect of silymarin and vitamin E on cyclophosphamide -induced skeletal malformations in rat embryos was compared.

MATERIALS AND METHODS

Silymarin powder (Sigma, USA) and cyclophosphamide and vitamin E (Darupakhsh, Iran) were purchased.

Male and female healthy Wistar rats, 3-4 month old of age, weighing 220-240g were purchased (Razi Institute, Karadje, 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 khurakdam, Shushtar, Iran.) and tap water. A 12h light:12h dark was mentioned. Room temperature was at 23±2°C with a relative humidity of 45-55%.

Females were mated overnight with males. Pregnancy was ascertained the next morning by presence of a vaginal plug and this time was designated as gestational day (GD) 1. Pregnant females were divided into four groups (n=6) and treated as follow:

Control group received normal saline; test groups received CP (15 mg/kg) (17), CP (15 mg/kg) plus silymarin (100 mg/kg) (16) and CP (15 mg/kg) plus vitamin E (100 mg/kg) (18) intraperitonealy, respectively.

The animals were sacrificed by cervical dislocation at 20th day of gestation. Following laparatomy, the uterus was exteriorized and the number and location of fetuses and resorption were noted, then their weight and length (crown- rump length) were measured. Individual fetuses were examined carefully for external anomalies then fetuses were stained by Alizarin red-Alcian blue method [19] and investigated by stereomicroscope (Nikon, MTZ 200, Japan) for skeletal malformations. The incidence of skeletal malformations was determined and was compared in the groups.

Statistical significance between groups was determined using SPSS program and compared by one way analysis of variance (ANOVA). Binomial data were examined using the Chi-square test. The minimum level of significance was $p< 0.05$.

RESULTS

No maternal death or abortion occurred in any experimental groups. There were not any aborted or absorbed fetuses from normal saline group. There were not observed macroscopic anomalies in the control animals. In the control group palatal closures of fetuses were normal at gestational day 20 (i.e., palatal shelves had grown vertically on the sides of the tongue, then horizontally to meet and fuse) (Fig. 1 A). CP induced cleft palate at 38.46% incidence (Fig. 1 B). Vitamin E reduced incidence of CP-induced cleft palate to 16.66 %, but silymarin reduced it to 9.37 %. In group that received CP, percentage of intrauterine death of fetuses were 32.6% but not in other groups. Percentages of absorbed fetuses were 40.8, 14.28 and 27.43 in groups that received CP, CP plus silymarin and CP plus vitamin E, respectively, so silymarin and vitamin E decreased resorption rate (Table 1).

Table 1: Incidence of anomalies in fetuses of groups. Group1 (control) without macroscopic anomalies; Group2 received CP; group3 received CP + silymarin; group 4 received CP + Vitamin E

	Incidence (%)		
Anomaly	Group 2	Group 3	Group 4
Cleft palate	38.46	9.37	16.66
Exencephaly	100.00	43.75	76.66
Omphalocele	11.11	0.00	0.00
Open eye	23.07	21.87	30.00
Brachygnathia	23.07	12.50	16.66
Sternum defects	37.93	6.25	10.00
Vertebral defects	65.50	6.25	3.33
Limbs defects	100.00	0.00	10.00

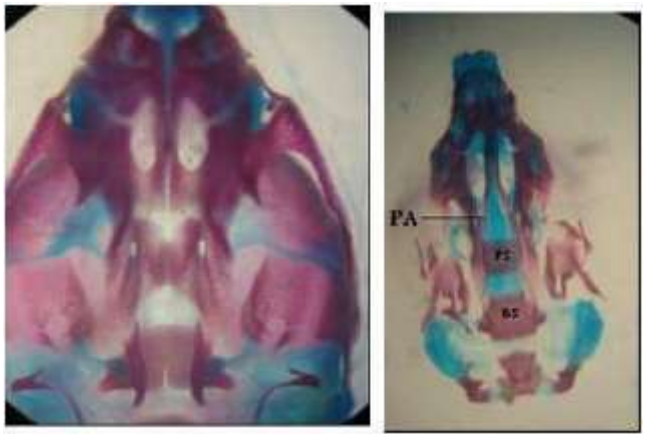

Fig. 1: Ventral view of skull of GD 20 fetal rats. A) Normal palatine bone B) Cleft palate induced by cyclophosphamide (arrow) which stained with Alizarine red- Alcian blue. PA: Palatine; PS: Presphenoid; BS: Basisphenoid

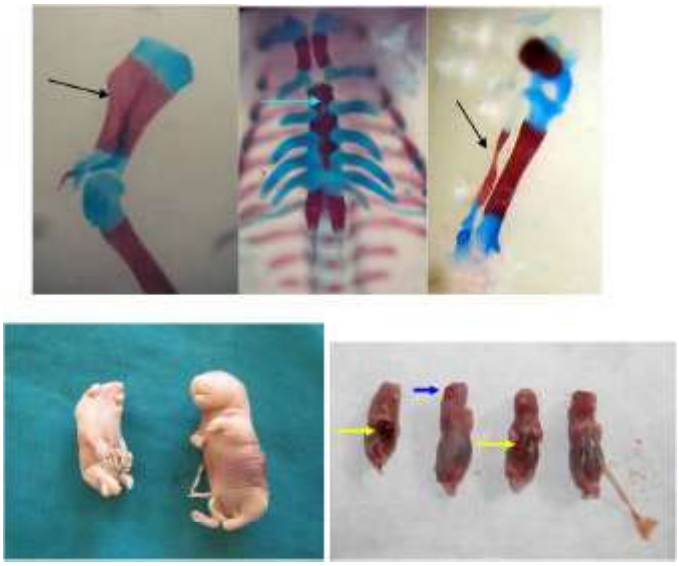

Fig. 2: Some skeletal defects in fetuses of rats. Curved scapula and absence deltoid tuberosity (up-left); fused sternebrae and split xiphoid process (up-middle); curved fibula (up-right); fetus deformity (left-down); exencephaly and omphalocele (right- down)

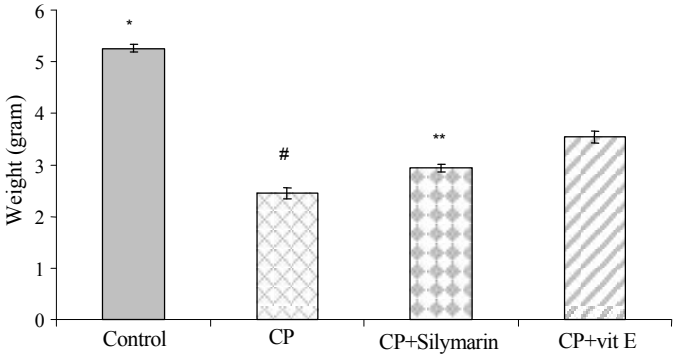

Fig. 3: Weight (mean±SE) of fetuses in normal saline and test groups: Control: normal saline; CP: Cyclophosphamide (15 mg/kg IP); CP + silymarin (100 mg/kg IP); CP + Vitamin E (100 mg/kg IP). n=6;* Significant difference when compared with other groups; ** Significant difference when compared with CP and CP + vitamin E; # significant difference when compared with CP + vitamin E ($p<0.05$)

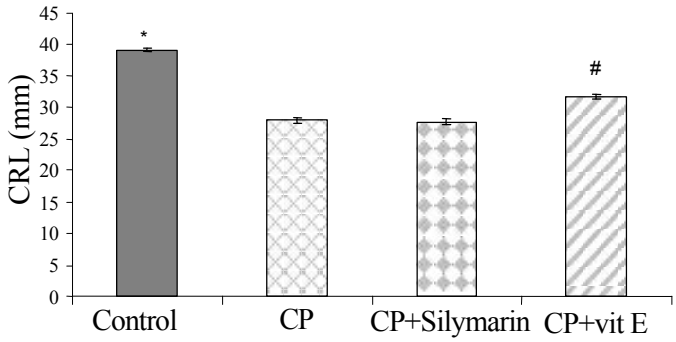

Fig. 4: Length (mean±SE) of fetuses in normal saline and test groups. Control: normal Saline; CP: Cyclophosphamide (15 mg/kg IP); CP + silymarin (100 mg/kg IP); CP + Vitamin E (100 mg/kg IP). n=6;* Significant difference when compared with other groups; # Significant difference when compared with CP and CP + silymarin groups; ($p<0.05$)

Exencephaly, omphalocele, open eye and different and several anomalies in vertebrae, sternum, scapula and limbs were observed (Fig. 2) which their incidence is shown in Table 1. Their incidences were decreased by silymarin and vitamin E. Omphalocele was not observed in animals treated with silymarin and vitamin E. Mean weight and length ($P<0.0001$) were significantly decreased in the group which received only CP. The means of weight in the groups that received silymarin ($P<0.005$) and vitamin E ($P<0.0001$) were significantly greater than the group received only CP. The mean weight ($P<0.0001$) and length ($P<0.0001$) were significantly decreased in groups which received silymarin and Vitamin E and significantly differed in comparison to control group (Fig. 3, 4). The mean weight and length in the group that received silymarin were significantly decreased in comparison with vitamin E group.

DISCUSSION

Several studies have reported that the maternal immune stimulation can reduce teratogenic anomalies [20]. Mechanisms of this effect remain unclear, but it is thought the fetal gene expression has been modulated [5].

The enhancing antioxidative effects can protect fetuses against drugs teratogenicity [21]. Sharova L. *et al.* showed that interferon-gamma and Freund's complete adjuvant reduced severity of the urethane-induced cleft palate in mice [22].

In the present study for first time, the prophylactic effects of silymarin and Vitamin E on cyclophosphamide-induced macroscopic fetal defects were compared in rat embryos. Both silymarin and Vitamin E reduced the frequency of incidence of cleft palate. Silymarin was greater in decreasing the incidence of cleft palate than vitamin E, but, vitamin E had better protective effect than Silymarin on weight and length of fetuses.

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

Cytokines have been reported to mediate CP–induced neurotoxicity [6]. Granulocyte-macrophage colony stimulating factor (GM-CSF) as cytokine and injection of leukocytes decreased CP–induced teratogenicity including limb defects [5, 6].

A number of observation suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide important embryoprotection [23,24]. Vitamin E is a fat-soluble vitamin that acts an antioxidant and free radical scavenger in lipophilic environments and is recognized as a potent chain breaking antioxidant with the particular function of preventing lipid peroxidation in membranes [25]. Toxicity of vitamin E is very low and adverse effects were rarely observed with dosages up to 2000 mg vitamin E/ day in human subjects. At much higher dosages, side effects and intolerance were increasingly noted. The only consistent side effect of vitamin E treatment was coagulation abnormalities in individuals with previous vitamin K deficiency [13]. An association between excess oxygen radical activity and disturbed embryogenesis in diabetic pregnancy has been suggested. Administration of vitamin E to pregnant diabetic animals decreases the rate of embryonic malformations and increases their body weight and enhances their maturation [25, 26].

Boskvic *et al.* reported that consumption of high doses of vitamin E during the first trimester of pregnancy was not associated with an increased risk for major

malformations, but may be associated with a decrease in birth weight [27]. On the other hand, vitamin E supplementation of the ewe resulted in a significant increase in lamb birth weight [28]. However, high dose (500mg/kg) exerted a negative effect on the conceptus, as shown by increased rate of reabsorptions [26].

Silymarin and its related compounds are also found to be scavengers of active oxygen such as the superoxide anion radicals, hydroxyl radicals and hydrogen peroxide [29]. It is suggested that many of properties maybe related to the antioxidant and free radical scavenging activity of silymarin. For example, silymarin increases antioxidant enzymes and reduced glutathione in alloxan induced diabetes in rat pancreas [30].

Recently, several studies have been carried out to elucidate the mechanism of action of silymarin. Accumulated data show that this herbal drug inhibits several isoforms of CYT P450 enzymes [18], potentiates the antioxidant capacity of the liver [31], acts as a scavenger of oxygen free radicals [10], inhibits the synthesis of proinflamatory cytokines and enhances apoptosis.

The present study compared the effects of silymarin and vitamin E for the first time in teratogenicity cyclophosphamide in rats. The results show that silymarin and vitamin E to produce a similar reduction in CP- induced skeletal anomalies in rats. Effect of CP on teratogenicity is mediated indirectly by inducing oxidative stress. The protective effect of silymarin in CP-induces macroscopic fetal defects in rat may, at least in part, be due to its antioxidant activity, which we believe deserves further investigation.

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