

L-Carnitine Protect against Cyclophosphamide Induced Skeletal and Neural Tube Malformations in Rat Fetuses

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Abstract- Cyclophosphamide (CP) is a mustard alkylating agent used in the treatment of a number of neoplastic diseases and as an immunosuppressant for the prevention of xenograft rejection. There are many reports that the teratogenic effects of cyclophosphamide can be prevented by application of antioxidant drugs and stimulation of the maternal immune system. Also, there is some evidence that L-carnitine is antioxidant. Therefore, in this study, the prophylactic effect of L-carnitine on teratogenic effects of CP was evaluated. This study was performed on 31 pregnant rats divided into 5 groups. Control group received normal saline and test groups received L-carnitine (500 mg/kg), CP (15 mg/kg), CP (15 mg/kg) plus L-carnitine (250 mg/kg) and CP (15 mg/kg) plus L-carnitine (500 mg/kg) intraperitoneally at 9th day of gestation. 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, spina bifida, and exencephaly incidence were 55.55%, 33.34% and 27.77% in fetuses of mice that received only CP. Cleft palate, spina bifida, exencephaly incidence were 21.42%, 4.76% and 9.52% in the group which received CP plus L-carnitine (250 mg/kg), respectively. However, cleft palate, spina bifida, and exencephaly incidence were 8%, 0% and 8% range in the group received CP plus L-carnitine (500 mg/kg), respectively. In addition, skeletal anomalies incidence including limbs, vertebrae, and sternum defects were decreased by L-carnitine. The mean of weight and length of animals' fetuses received L-carnitine were significantly greater than those received only CP. In conclusion, L-carnitine significantly decreased teratogenicity induced by CP; but this subject needs more detailed evaluation.

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Keywords: Cyclophosphamide; Cleft palate; Exencephaly; L-carnitine; Teratogenicity; Rat

Introduction

Cyclophosphamide (CP), a nitrogen mustard compound is a member of the group of cytostatic alkylating agents used in the treatment of a number of neoplastic diseases and as an immunosuppressant for the prevention of xenograft rejection (1-2). CP has several toxic effects including hemorrhagic cystitis. Metabolites of cyclophosphamide, especially acrolein modulates its toxic effects (3-4). CP is the best known teratogenic drug in human and laboratory animals (4-6).

Previous studies in rodents have shown that exposure to cyclophosphamide during organogenesis caused an embryonic and fetal resorption, growth retardation, or multiple anomalies, including

exencephaly and limb and skeletal defects (7-9)

Several studies show that the stimulation of maternal immune system can decrease or prevent drug-induced embryonic abnormalities (10-11). For example, in one study, macrophage activation decreases the incidence of cleft palate and digital, and tail anomalies in fetuses of mice received urethane and methyl nitrous urea (11). In another study, interferon gamma reduced urethane-induced cleft palate and granulocyte-colony stimulating factor decreased cyclophosphamide-induced distal limb abnormalities in mice (6).

Data from laboratory research and critical trials also suggest beneficial influences of the maternal immune system on pregnancy outcome (12-13). Nonspecific immune stimulation by injection of Freund's complete

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adjuvant (FCA) reduced early embryo loss in CBA/J mice (10). In rodents (14) and humans (13), 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 (15). The mechanism for these effects remains unclear; however, the possible involvement of cytokines produced by immune cells has been suggested (15).

Ivnitsky *et al.*, (1998) found that CP- induced brain and craniofacial anomalies in mice were associated with increased TNF- α in the fetal head and brain. Maternal immunostimulation decreased the severity of CP-induced malformation in these mice, and decreased TNF- α expression in fetal heads (16). In related studies, Savion *et al.*, (1999) reported that maternal dosing with 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 (17).

L-carnitine (4-N-trimethylammonium-3-hydroxybutyric acid) is a natural nutrient that transports long-chain fatty acids into the mitochondria, where they are oxidized to produce adenosine triphosphate (18) and prevent the toxic accumulation of long-chain fatty acids (19). L-carnitine inhibits both the mitochondrial damage induced by oxidative stress and mitochondria-dependent apoptosis in various types of cells (20-21). Recent studies suggest that L-carnitine may play an important role in oxidative/antioxidative balance and has an antiperoxidative effect on several tissues (22-23).

Gulcin *et al.*, investigated the antioxidant activity of L-carnitine in vitro and found that L-carnitine is effective in scavenging superoxide anion radical, hydrogen peroxide, and in metal chelating on ferrous ions (24). L-carnitine can also help to prevent and reduce ischemia-reperfusion injury (25-26). However, studies related to the antioxidation capability in healthy human subjects have been scarce.

In the present study, the prophylactic effect of L-carnitine on CP-induced neural tube and skeletal malformations in rat fetuses was evaluated.

Materials and Methods

Male and female healthy rat of Wistar strain, 3-4 months of age, weighing 200-220g were purchased (Joundishapour laboratory animal center, Ahvaz, 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, Tehran, Iran.) and tap water. A 12h light: 12h dark was mentioned. Room temperature was $23 \pm 2^\circ$ C with a relative humidity of 45-55%. This experimental study was done in an animal model in the department of basic sciences of the faculty of veterinary medicine of Shahid Chamram University (Ahvaz, Iran). The animal care was provided under the supervision of a qualified veterinarian.

Females were mated overnight with males. Pregnancy was ascertained the next morning by the presence of a vaginal plug, and this time was designated as gestational day (GD) 1. Pregnant rats (n=31) were randomly divided into 5 groups (25 pregnant rats in treatment groups, 6 pregnant rats in control group) and treated as follow:

Control group received normal saline; test groups received L-carnitine (500 mg/kg) (27-28), CP (15 mg/kg) (29), CP (15 mg/kg) plus L-carnitine (250 mg/kg) (28), and CP (20 mg/kg) plus L-carnitine (500 mg/kg) intraperitoneally, respectively. CP (Baxter, Germany) and L-carnitine (Sigma, Germany) were purchased.

The animals were sacrificed by cervical dislocation at 20th day of gestation. Following laparotomy, the uterus was exteriorized, and the number and location of fetuses and resorption were noted, and then their weight and length (crown- rump length) were measured. Individual fetuses were examined carefully for external anomalies then were stained with a mixture of 0.14% Alcian blue and 0.12% alizarin red S in ethanol and glacial acetic acid. Fetuses were then macerated in 2% KOH, cleared and hardened in 1:1 glycerin and distilled water, and stored in pure glycerin (30) and investigated by stereomicroscope (Nikon, SMZ200, Japan) for skeletal malformations. The incidence of skeletal malformations was determined and compared between 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 deaths were observed throughout the course of this study. Likewise, the dose of CP used in this investigation was well tolerated by the dams, as evidenced by no differences in food and water consumption.

A total of 47 fetuses were obtained from six rats of

the control 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) (Figure 1A). CP-induced cleft palate (Figure 1B), spina bifida (Figure 2B) and exencephaly (Figure 5) at 55.55%, 33.34%, and 27.77% incidence, respectively.

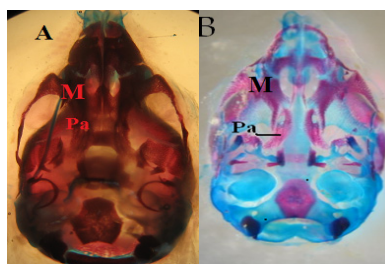


Figure 1. Ventral view of the skull of rat fetuses of GD 20 stained with alizarin red S-alcian blue. A) Normal palatine bone; B) Cleft palate induced by CP (arrow) M: maxilla; Pa: palatine

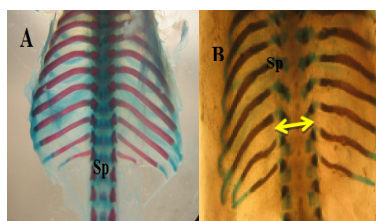


Figure 2. Dorsal view of a vertebral column of gestation 20th-day fetal rat. A) Normal; B) Spina bifida (arrow) induced by CP which was stained with Alizarin red- Alcian blue, SP: spinous process

CP plus L-carnitine (250 mg/kg) significantly reduced incidence of cleft palate, spina bifida and exencephaly to 21.42%, 4.76%, and 9.52% range but CP plus L-carnitine (500 mg/kg) significantly reduced incidence of cleft palate, spina bifida, and exencephaly to 8%, 0% and 8% range, respectively. No maternal death or abortion occurred in any experimental groups. There were not any aborted fetuses from total groups but percentage of resorbed fetuses were 4.25%, 35.03%

56.09%, 26.31%, and 3.84% in groups that received normal saline, L-carnitine (500 mg/kg), CP, CP plus L-carnitine (500 mg/kg) and CP plus L-carnitine (250 mg/kg), respectively, so L-carnitine decreased resorption rate.

Open eye and omphalocele, delay ossification in forelimb and hindlimb and several anomalies in sternum were observed (Figures 3-5) which their incidence is shown in Table 1.

Table 1. The incidence of anomalies in fetuses of groups*

Anomaly	Incidence (%)		
	Group 3	Group 4	Group 5
Cleft palate	55.55	21.42	8
Exencephaly	27.77	9.52	8
Spina bifida	33.34	4.76	8
Open eye	27.77	9.52	8
Omphalocele	11.11	9.52	8
Delayed ossification in limbs	33.34	4.76	8
Fused sternbrae	55.56	4.76	8

*Group 3 received CP, Group 4 received CP (15 mg/kg)+ L-carnitine (250 mg/kg), Group 5 received CP+ L-carnitine (500 mg/kg)

L-Carnitine and CP induced teratogenicity

Their incidences were decreased by L-carnitine. These anomalies were not observed in animals treated with L-carnitine. Mean weight and length ($P<0.001$) were significantly decreased in the group which received only

CP. The means weight and length in groups that received CP plus L-carnitine (250 mg/kg) were greater than the group received only CP (Table 2).

Table 2. Mean weight and Crown Rump Length in rat fetuses of groups.

Groups	No. of litters	Implantations	Resorbed fetuses	Live fetuses	Fetal length(mm):(mean \pm SEM)	Fetal weight (g): (mean \pm SEM)
Control	7	49	2(4.25)	47	37.3 \pm 0.31*	4.73 \pm 0.07*
L-carnitine(500 mg/kg)	5	44	20(35.03)	24	35.8 \pm 0.29	3.54 \pm 0.14
CP	6	41	23(56.09)	18	27.8 \pm 1.34**	1.96 \pm 0.2**
Cp+l-carnitine(250)	8	57	15(26.31)	42	34.3 \pm 0.49#	3.34 \pm 0.11#
Cp+l-carnitine(500)	6	26	1(3.84)	25	27.2 \pm 0.83	1.96 \pm 0.12*

Numerals in parentheses are percentages, *: Mean of fetal weight was significantly different when compared with other groups ($P<0.05$) except with L-carnitine 500mg/kg, **: Significant difference when compared with other groups except L-carnitine 500 mg/kg ($P<0.05$), #: Significant difference when compared with other groups ($P<0.05$) except with CP+ L-carnitine 500mg/kg, #: Significant difference when compared with other groups ($P<0.05$) except with CP+ L-carnitine 500mg/kg ($P<0.05$), **: Significant difference when compared with other groups except with CP+ L-carnitine 500mg/kg ($P<0.05$), #: Significant difference when compared with other groups ($P<0.05$) except with L-carnitine 500mg/kg.

The mean weight and length in the group received L-carnitine were significantly decreased in comparison

with normal saline group except with L-carnitine (500 mg/kg) ($P>0.05$).

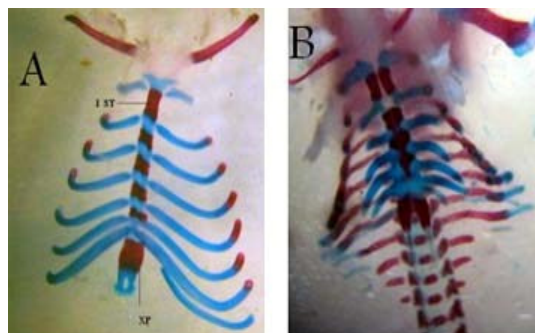


Figure 3. Dorsal view of the sternum of gestation 20th day fetal rat. A) Normal; B) fused sternebrae induced by CP, which stained with Alizarin red-Alcian blue. 1 st: First sternebra; xp: xiphoid process

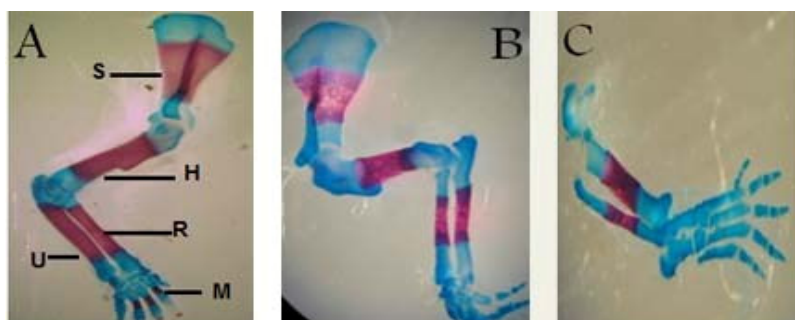


Figure 4. Lateral view of limbs of gestation 20th day fetal rat. A) Normal forelimb; B) delay ossification in forelimb C) delay ossification in hindlimb induced by CP stained with Alizarin red-Alcian blue. S: Scapula; H: Humerus; R: Radius; U: Ulna; M: Metacarpus



Figure 5. Some anomalies in fetuses of rats

Normal fetus (up-left), exencephaly (up-right), omphalocele (left-down), and open eye (right-down)

Discussion

Several studies have reported that the maternal immune stimulation can reduce teratogenic anomalies (31). Mechanisms of this effect remain unclear, but it is thought the fetal gene expression has been modulated (10).

The enhancing antioxidative effects can protect fetuses against drugs teratogenicity (32). Sharova L. *et al.*, showed that interferon-gamma and Freund's complete adjuvant reduced severity of the urethane-induced cleft palate in mice (33).

In the present study for the first time, the prophylactic effect of L-carnitine on CP-induced neural tube and skeletal fetal defects was evaluated in rat fetuses. L-carnitine reduced the frequency of incidence of the neural tube and skeletal fetal defects. L-carnitine with a dose of 500 mg/kg was greater in decreasing the incidence of the neural tube and skeletal fetal defects than 250 mg/kg, but it was not significant.

It is well known that CP causes fetal defects in diverse species of animals including mice, rats, hamsters, and rabbits as well as humans (34-35). In the present study, a single intraperitoneal administration of cyclophosphamide (15 mg/kg) on GD9 caused significant growth retardation and morphological alterations in rat fetuses.

Gibson and Becker (1968) reported CP-induced teratogenicity in mice. They used CP at dose 5 to 20 mg/kg in mice in one of the 9-14th days of gestation. They observed the CP can produce teratogenicity in 67.3% of fetuses with 20 mg/kg (36). They determined fetal defects similar to the current study including cleft palate and exencephaly. These anomalies were decreased by 250 mg/kg and 500 mg/kg L-carnitine,

respectively. They also determined fetal weights and crown rump lengths similar to this study reduced significantly by CP. In the present study, fetal weights and crown rump lengths were increased by 250 mg/kg and 500 mg/kg L-carnitine, respectively.

Sloth and Hales (1986) evaluated the effect of mesna on CP-induced teratogenicity. They used CP at dose 10 and 15 mg/kg in rats in the 13th day of gestation. They observed the CP can produce teratogenicity in 50 and 100% of fetuses with 10 and 15mg/kg, respectively (29). They determined fetal defects similar with this study including cleft palate, exencephaly, open eye and limb defects. These anomalies were decreased by 250 mg/kg and 500 mg/kg L-carnitine, respectively.

Cytokines have been reported to mediate CP-induced neurotoxicity (11). Granulocyte-macrophage colony stimulating factor (GM-CSF) as cytokine and injection of leukocytes decreased CP-induced teratogenicity including limb defects (10,11).

A number of observation suggest that detoxification of a xenobiotic free radical intermediate with antioxidants may provide important embryoprotection (37).

Cayır *et al.*, (2009) examined the effects of L-carnitine at a dose of 500 mg/kg (i.p.) on cisplatin-induced oxidative damage in the liver and kidney tissues of rats. Their data showed that L-carnitine elicited significant protective activity in the liver and kidney by decreasing the levels of MDA and elevating the activities of GPX, suggesting an antioxidant effect from this molecule. This finding was most likely caused by a decrease in the damage produced by free oxygen radicals (22). Cetinkaya *et al.*, (38) investigated the effects of L-carnitine at a dose of 500 mg/kg (i.p.) on the oxidant/antioxidant status in acetic acid-induced colitis

and reported that L-carnitine administration to the acetic acid-treated rats significantly reduces the MDA level and MPO activity, whereas the CAT activity increased in colon tissue. In contrast to the results of Cetinkaya, in Yildirim study (39), two different doses of administered L-carnitine significantly induced the MPO activities in liver tissue. The different results might result from the acetic acid-induced inflammatory process. MPO is a heme-containing peroxidase abundantly expressed in neutrophils. In addition, enzymatically active MPO, together with hydrogen peroxide and chloride, produces the powerful oxidant hypochlorous acid and is a key contributor to the oxygen-dependent microbicidal activity of phagocytes.

Therefore, the change in MPO activity can vary depending on whether the number of neutrophil leukocytes increases, as in the case of inflammation (38).

Aleisa *et al.*, (21) reported that the administration of propionyl L-carnitine at a dose of 250 mg/kg (i.p.) significantly attenuated the nephrotoxic effects of cisplatin, which manifested as a normalization of the cisplatin-induced increase in thiobarbituric acid reactive substances, such as MDA and nitric oxide, and the cisplatin-induced decrease in reduced glutathione in rat kidney tissues. In another study, Derin *et al.*, (40) reported that pretreatment with L-carnitine (100 mg/kg, i.p.) increases the tissue catalase activity and protects the gastric mucosa from ischemia-reperfusion injury by decreasing lipid peroxidation via its lipid peroxidation-decreasing activity. As in earlier reports, in the present study L-carnitine supplementation at doses of 100 and 500 mg/kg caused a significant decrease in the hepatic lipid peroxidation and an increase in the activities of GPX, CAT, and MPO in rat liver tissue. However, there was no significant difference in the reduction of the L-thyroxine-induced oxidative stress between the groups supplemented with low- and high-dose carnitine.

In a study, effect of carnitine and antioxidant supplementation on carnitine and lipid profiles in trained and non-trained humans was assessed. The volunteers were divided into four groups; PN (placebo-non exercised), SN (supplement-non exercised), PE (placebo-exercised) and SE (supplement-exercised). The exercised groups were run on a treadmill for 50 min per day at 75% VO₂ max. The supplemented groups were fed carnitine (4g/day), vitamin C (1000mg/day), vitamin E (500 IU/day) and melatonin (0.1mg/kg b.w) for 6 weeks. SN, PE, and SE groups had significantly lower serum total cholesterol and LDL cholesterol but had higher HDL-cholesterol levels than the PN group.

Serum non-esterified (NEC) and acidsoluble acylcarnitine (ASAC) increased in the SN, PE, and SE group. The SN and SE groups had significantly higher urinary excretion of NEC and acid-insoluble acylcarnitine (AIAC) than the PN and PE groups. CPT-1 mRNA expression in skeletal muscle, obtained by biopsy, was enhanced by both supplementation and exercise. These results suggest that both exercise training and supplementation of carnitine and antioxidants improve lipid profiles and carnitine metabolism in people and suggests that carnitine and antioxidant supplementation may improve exercise performance (41).

In conclusion, the present study compared the effects L-carnitine for the first time in teratogenicity of CP in rat. The results showed that L-carnitine produces 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 L-carnitine in CP-induced macroscopic fetal defects in rat may, at least in part, be due to its antioxidant activity, which we believe deserves further investigation.

On the other hand, L-carnitine (500 mg/kg) is more effective than L-carnitine (250 mg/kg) in decreasing incidence of CP-induced neural tube and skeletal defects in fetuses of rats, but it was not significant

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L-Carnitine and CP induced teratogenicity

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