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ORIGINAL ARTICLE**An Investigation on Protective Effects of Vitamin E Against Lipopolysaccharide-induced Fetal Injuries in Rat****¹Masoud Delashoub and ²Seyed Mahdi Banan Khojasteh**¹*Department of Basic Science, Faculty of Veterinary Medicine, Tabriz Branch, Islamic Azad University, Tabriz, Iran*²*Department of Animal Biology, Faculty of Natural Sciences, University of Tabriz, Tabriz, Iran*

Masoud Delashoub, Seyed Mahdi Banan Khojasteh: An Investigation on Protective Effects of Vitamin E Against Lipopolysaccharide-induced Fetal Injuries in Rat

ABSTRACT

Lipopolysaccharide (LPS) has been associated with adverse developmental outcome, including intra-uterine fetal death (IUID), embryonic resorption, intra-uterine growth retardation (IUGR) and preterm delivery in rodents. Induced toxicity in these organs is related to production of reactive oxygen species (ROS). Vitamin E is a fat soluble vitamin that has antioxidant effects. In this study, we investigated the effect of vitamin E on LPS-induced IUID and IUGR in rat. First pregnant rats were selected and were divided to 4 groups. All pregnant rats except controls received an intraperitoneal (75 µg/kg) injection of LPS daily on gestation day (gd) 15–17. The second and third groups received 20 mg/kg⁻¹ of vitamin E (I.M) daily since 7 days before of first injection of LPS. On gd 18, the number of live fetuses, dead fetuses and resorption sites was counted. Live fetuses in each litter were weighted. Crown-rump and tail lengths were examined and skeletal development was evaluated. Also maternal liver, placenta and fetal liver samples were excised for measurement of TBARS and GSH contents. Results were agreed with other researches and showed that maternally administered LPS significantly increased fetal mortality, decreased fetal weight and crown-rump and tail lengths of live fetuses and retarded skeletal ossification in caudal vertebrae, anterior and posterior phalanges, and supraoccipital bone. Biochemical and anatomical results in co-treatment of vitamin E and LPS are close to control group that demonstrate pre-treatment of vitamin E decrease toxicity of LPS in rat fetuses. All of this suggests that vitamin E protects against LPS-induced fetal death and reversed LPS-induced growth and skeletal development retardation via counteracting LPS-induced oxidative stress.

Key words: Vitamin E, Lipopolysaccharide, Fetal injury, Rat**Introduction**

Lipopolysaccharide (LPS) is a toxic component of cell walls of Gram-negative bacteria and is widely present in the digestive tracts of humans and animals [1]. Exposure to this agent is at high range in human and animals but there are differences in levels of infection. Infected animals and humans to gram negative bacteria are at high risk to abortion and many other defects like fetal loss, adverse developmental outcome including embryonic resorption, intra-uterine fetal death (IUID), intra-uterine growth retardation (IUGR) and preterm delivery in rodents [2,3,4].

Numerous studies showed that tumor necrosis factor alpha (TNF-α) is one of the major mediators leading to embryonic resorption, IUID, IUGR and preterm delivery that induced by LPS injection [5,6]. Also maternal LPS exposure significantly increases inducible nitric oxide synthase (iNOS) expression in

decidual and myometrial cells and nitric oxide (NO) production in decidua and uterine [3].

Prostaglandins have also important role in LPS-induced embryo toxicity. It is demonstrated that reactive oxygen species (ROS) involved in LPS-induced IUID and IUGR are one of the important causes for fetal injuries [7,8]. LPS stimulates macrophages to generate reactive oxygen species (ROS) and increases nitrotyrosine, a marker for O², NO formation in macrophage-rich organs [9]. A recent study showed that *N*-acetylcysteine, a glutathione precursor and direct antioxidant, protected fetal death and preterm labor induced by maternal inflammation [2], suggesting that ROS may mediate LPS-induced developmental toxicity.

Several antioxidants have protected mice against LPS-induced IUID and IUGR [5,7]. Vitamin E as a fat-soluble antioxidant is a family member of α, β, γ, and δ tocopherols and corresponding four tocotrienols (11). The most active form of the

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vitamin E homologous is α tocopherol which protects cell membranes from oxidation by reacting with ROS and lipid radicals produced in the lipid peroxidation chain reaction [10].

Ascorbic acid (AA) is an antioxidant and has been demonstrated to be effective on preventing fetal malformation in experimental diabetic pregnancy [11]. In another study, effect of ascorbic acid on IUFD and IUGR in mice was investigated that results indicated that pre-treatment with AA protected against LPS-induced injuries and growth defects, but post treatment with AA had less protective effect on LPS-induced IUFD [5]. LPS induced oxidative stress demonstrated that co-treatment with protective agents like vitamin E is very important. In these study protective effects of vitamin E against LPS induced fetal injuries in rat was investigated.

Materials and Methods

Chemicals:

Lipopolysaccharide (*Escherichia coli* LPS, serotype 0127:B8) and Vitamin E were purchased from Sigma Chemical Co. All other reagents were obtained from Sigma.

Animals:

Female Wistar rats (150-200gr) and males (180-220gr) for mating were acclimated for one week. Wistar rats were obtained from Laboratory Animals Care Center of Tabriz University of Medical Sciences (Tabriz, Iran). Animals were weighed on the day 0 (reception into the facility) and 7, and observed daily (external appearance, nourishment, and behavior).

Based on these observations, healthy animals that characterized by normal weight gain and a lack of abnormalities, were selected for mating. Animals were housed in bracketed metal wire cages at 20–26 °C temperature, 40–74% relative humidity, ventilation 10–15 times per hour, and illuminated for 12 h daily (07:00–19:00). All rats were allowed free access to a standard solid feed and tap water provided in polycarbonate water bottles.

For mating, males and females (2:4) were placed in the same cage overnight. Females were regarded as having copulated when a vaginal plug or sperm on a vaginal smear was confirmed the next morning, which was then designed as day 0 of pregnancy (gd 0). At the time of mating females were aged 11–13 weeks, while males were 12–13 weeks old. Mated animals were assigned to groups by computerized block allocation, so that the body weight of animals was averaged among the groups (200-250 gr). Non-mated female rats in this period were considered infertile and excluded from the study.

Experiment 1:

Forty-eight pregnant rats were divided into 4 groups (N=12). First two groups (A and B) received an intraperitoneal (75 μ g/kg) injection of LPS on gd 15–17. B and C groups received intramuscular injection of Vitamin E (20 mg/kg) daily since one week before LPS administration. Last group (D) was control and received normal saline in same conditions with other groups.

All dams were sacrificed on gd 18 and gravid uterine weights were recorded. For each litter, the number of live fetuses, dead fetuses was counted. Live fetuses in each litter were weighed. Crown-rump and tail lengths were measured. All fetuses were then stored in ethanol a minimum of 2 weeks for subsequent skeletal evaluation.

Experiment 2:

Forty-eight pregnant rats were divided into 4 groups (N=12). Except LPS that administrated as single dose (75 μ g/kg, i.p) on gd 15, all of the injections were same as experiment 1. In this experiment, dams were sacrificed at 6 h after LPS treatment. Maternal liver, placenta and fetal liver samples were excised for measurement of Malondialdehyde (MDA) and GSH contents.

Lipid peroxidation assay:

Lipid peroxidation was measured in terms of MDA. MDA levels were studied using the thiobarbituric acid (TBA) abduction method as described previously. Briefly, 50 μ l of samples were introduced into a tube containing 1 ml of distilled water. After addition of 1 ml of a solution containing 29 mmol/l TBA (Sigma Chemical Co., St Louis, MO, USA) in acetic acid (8.75 mmol/l, pH of the reaction mixture: 2.4-2.6) and mix of them, the samples were placed in water bath and heated for 1 hour at 95-100°C. The samples were then cooled under running cold water. Twenty five μ l of HCl (5 mol/l) was added and the reaction mixture was extracted for agitation with 3.5 ml of n-butanol (Sigma Chemical Co.) for 5 minutes. After centrifugation, the butanol phase was separated and the fluorescence of the butanol extract was measured by spectrophotometry [12, 13].

Determination of GSH contents:

GSH was determined by the method of Griffith [14]. Proteins of 0.4 mL liver homogenates were precipitated by the addition of 0.4 mL of a metaphosphoric acid solution. After 40 minutes, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm at 4 °C for 5 minutes. Four hundred microliters of the supernatant was combined with 0.4 mL of 300mM Na₂HPO₄, and the absorbance at 412 nm was read against a blank consisting of 0.4 mL supernatant plus 0.4 mL

H₂O. Then, 100 μ L DTNB (0.02 %, w/v; 20 mg DTNB in 100mL of 1 % sodium citrate) was added to the blank and sample. Absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. GSH values were expressed as nmol mg⁻¹ protein. Protein content was measured according to the method of Lowry et al. [15].

Fetal assessment:

Fetuses were eviscerated and the skin removed to facilitate stain penetration. For skeletal staining, fetuses were fixed for 3 days in 95% ethanol. The fetuses were then placed in 15 mg alcian blue (80 ml 95% ethanol/20 ml glacial acetic acid) for 2 days for cartilage staining and then washed in 95% ethanol for 24 h, and rehydrated in graded alcohols.

Fetuses were then placed in alizarin red (25 mg/liter, 1% KOH) for 2 days for staining of ossified bones. The specimens were then placed in several changes of 0.5% KOH until the tissues had cleared (approximately 1 week) and then washed for 24 h in increasing concentrations of glycerol (20%, 50%, 80%) in 1% KOH prior to final storage in 100% glycerol. At this stage the ossification centers and sites of cartilage development were assessed. The counting of the ossification sites was performed according to methodology proposed by Aliverti [16], which determines the degree of fetal development.

Statistical analyses:

All results have presented as mean \pm standard deviation (SD). A one-way analysis of variance (ANOVA) followed by Tukey's HSD test was used to test for significant differences among treatment groups. ANOVA and the Student–Newman–Kelsus post hoc test were used to determine differences between the treated animals and the control and statistical significance. All statistics were analyzed by SPSS 11.0 package program.

Results and Discussion

Placenta weights, number of live and dead fetuses, fetal weights, fetal resorption and crown-rump lengths for all study groups are summarized in Table 1 that shows effect of LPS and co-treatment of LPS and Vitamin E on IUGR and IUFD. Significant differences were found between placenta weights and fetal weights of LPS group and other groups ($P < 0.05$). Also significant differences were found between number of live and dead fetuses of LPS and other groups ($P < 0.01$). There were no significant differences in parameters of fetal resorption between groups. Crown-rump lengths showed significant differences between LPS and other groups ($P < 0.05$). Comparison of ossified sites of fetuses between groups is presented in Table 2. There were no significant differences in parameters of metacarpus and sternum ossification between groups. Significant differences were found between anterior and posterior phalanges of LPS and control groups ($p < 0.01$). Also significant differences were found between metatarsus of LPS and other groups ($p < 0.05$). The level of MDA, which is a major degradation product of lipid peroxidation, was significantly elevated in the maternal and fetal liver, and placenta in LPS groups ($p < 0.01$) (table 3). There were no significant differences between LPS plus vitamin E and control groups. Significant differences were found in maternal and fetal liver contents of MDA between LPS and vitamin E plus LPS groups ($p < 0.01$).

The comparison of GSH contents between groups are presented in Table 4. Results showed that LPS decreased GSH contents in placenta and also maternal and fetal liver. There were significant differences between LPS and control groups. However, this decrease was significantly suppressed when vitamin E co-administrated with LPS and there are significant difference between placenta and fetus liver of LPS and LPS+Vit E groups ($p < 0.01$) but there is no significant difference between maternal liver of LPS and LPS+Vit E groups.

Table 1: Comparisons of placental weights, live and dead fetuses number, fetus weights, and fetal resorption and crown-rump lengths among groups (mean \pm SD)

	Control	Vitamin E	LPS	LPS plus Vitamin E
Placenta weight (g)	0.58 \pm 0.05	0.57 \pm 0.07	0.38 \pm 0.04 *	0.52 \pm 0.02
Live fetuses	12.1 \pm 0.12	11.85 \pm 0.1	6.2 \pm 0.3 **	11.67 \pm 0.1
Fetal weight (g)	4.01 \pm 0.006	3.95 \pm 0.033	3.15 \pm 0.01 *	3.78 \pm 0.31
Fetal resorption	0.58 \pm 0.02	0.59 \pm 0.2	0.65 \pm 0.33	0.59 \pm 0.17
Dead fetuses	0.11 \pm 0.02	0.13 \pm 0.02	0.28 \pm 0.024 **	0.14 \pm 0.11
Crown rump length	3.92 \pm 0.44	3.90 \pm 0.01	3.45 \pm 0.18 *	3.87 \pm 0.14

The values are shown as a mean \pm SD for rats in each group.

* Significantly different from other groups, $p < 0.05$.

** Significantly different from control, $p < 0.01$.

Table 2: Ossificated sites of fetuses in groups.

Groups Ossification site	Control	Vitamin E	LPS	Vitamin E plus LPS
Metacarpus	3.95 +/- 0.01	3.89 +/- 0.07	3.78 +/- 0.1	3.85 +/- 0.23
Metatarsus	4.86 +/- 0.02	4.81 +/- 0.1	4.51 +/- 0.05 *	4.77 +/- 0.04
Anterior phalange	3.89 +/- 0.1	3.82 +/- 0.06	2.9 +/- 0.4 **	3.76 +/- 0.31
Posterior phalange	3.55 +/- 0.24	3.51 +/- 0.03	2.45 +/- 0.01 **	3.5 +/- 0.00
Sternum	5.97 +/- 0.54	5.94 +/- 0.3	5.88 +/- 0.2	5.92 +/- 0.1

Data are shown as mean \pm SD and difference of ($p < 0.05$) considered significant.

* Significantly different from other groups, $p < 0.05$.

Table 3: Comparisons of MDA (nmol/g tissue) levels in tissues among groups (mean \pm SD).

	Placenta	Maternal liver	Fetal liver
Control	255.1 +/- 12.3	432.45 +/- 31.9	271.4 +/- 31.1
Vitamin E	264.4 +/- 5.7	447.39 +/- 10.0	279.6 +/- 21.87
LPS	368.8 +/- 24.8 *	710.11 +/- 54.3 *	531.86 +/- 88.9 *
Vitamin E plus LPS	269.12 +/- 15.2	479.58 +/- 23.2 **	321.01 +/- 10.97 **

Data are shown as mean \pm SD and difference of ($p < 0.01$) considered significant.

* Significantly different from control groups, $p < 0.01$.

** Significantly different from LPS groups, $p < 0.01$.

Table 4: Comparisons of GSH (nmol/g tissue) levels in tissues among groups (mean \pm SD).

	Placenta	Maternal liver	Fetal liver
Control	546.1 +/- 65.98	1025.23 +/- 24.6	684.43 +/- 25.8
Vitamin E	537.34 +/- 31.7	1000.43 +/- 15.7	678.21 +/- 14.6
LPS	247.28 +/- 51.2 **	724.28 +/- 76.9 *	305.23 +/- 76.5 **
Vitamin E plus LPS	410.26 +/- 47.8 ††	754.4 +/- 127.2 *	570.23 +/- 41.6 ††

Data are shown as mean \pm SD.

* Significantly different from control groups, $p < 0.05$.

** Significantly different from control group, $p < 0.01$.

†† Significantly different from LPS groups, $p < 0.01$.

In the present study, administration of LPS in rats resulted in development of oxidative stress damage in mothers and fetuses. This effect was indicated by an increase in the concentration of MDA, lipid peroxidation indices and decrease in the concentration of the total GSH and by effects on IUGR and IUFD.

Administration of LPS significantly reduced fetal weights and crown-rump lengths and retarded skeletal ossification in anterior and posterior phalanges, and metatarsals. These results are agreed with study of Rivera *et al.* [17].

LPS perfusion induced multiple organ oxidative damages characterized by increase of MDA concentrations in serum and in examined tissues (heart, liver and kidney). Our results were in good agreement with previous studies which reported increases of MDA concentrations in plasma and liver [18,19,20].

Some studies demonstrated that LPS stimulates macrophages to generate ROS and increases

nitrotyrosine, a marker for O²•, NO and ONOO⁻ formation, in macrophage-rich organs [9]. It has found that LPS enhanced placental expression of 4-hydroxy-2-nonenal (HNE)-modified proteins, markers of oxidative stress [21]. In other study, maternally administered LPS increased MDA levels in fetal liver [8].

Decrease of the contents of -SH groups in LPS-treated rats also suggests increased formation of ROS or nitrogen species in the tissue. Similarly, it has revealed that the reduced contents of sulfhydryl groups after endotoxemia may be attributed to overproduction of oxidants and NO, since high levels of NO were associated with oxidation of the sulfhydryl groups [22]. The reduction in the contents of -SH groups may be a result of a decrease in the synthesis of protein containing -SH groups, a decrease in GSH synthesis and in the activity of glutathione reductase [23], transferase, or an increase of GSH peroxidase [24].

Glutathione is known to be a major low molecular weight scavenger of free radicals in the cytoplasm. In present study, decrease of maternal and fetal liver GSH contents after LPS administration indicates that LPS altered antioxidant capacity and thiol redox state in these tissues. GSH is essential for the protection of thiol and other nucleophilic groups in proteins from the toxic metabolites and lower GSH levels under conditions of intracellular stress lead to oxidation and damages of lipids, proteins and DNA by ROS [25]. Other studies show a decrease in the tissue levels of GSH during the initial phase of septic shock [26].

Vitamin E is a fat soluble antioxidant and directly reduced membrane peroxides into hydroperoxides by promoting the termination phase during radical reactions, and consequently facilitated the local action of membrane GSH peroxidases on hydroperoxides.

The results of this study showed that co-treatment of vitamin E reduced LPS-induced IUGR, and reversed LPS-induced skeletal ossification retardation in anterior and posterior phalanges and metatarsals. Furthermore, it has found that vitamin E significantly attenuated LPS-induced increase in MDA level in maternal and fetal liver, and placenta. Also LPS induced decrease of GSH contents in placenta and fetal liver is recompensed significantly by vitamin E. All these results suggest vitamin E protection against LPS-induced IUFD and IUGR that is at least partially, associated with important antioxidative effect of this vitamin and decreased lipid peroxidation and increase of GSH contents. As a conclusion, antioxidant administrations such as vitamin E can protect against LPS induced injuries in fetal development.

Acknowledgement

The authors would like to thank Tabriz Branch, Islamic Azad University for the financial support of this research, which is based on a research project contract.

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