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Vitamin A,Lipopolysaccharide and Fetal Injuries

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Abstract
Objectives:*Lipopolysaccharide (LPS) isan important factorthatinduces adversedevelopmentaloutcomes. These includeintrauterine growth retardation (IUGR), intrauterine fetal death (IUFD), embryonic resorption, and preterm delivery inrodents,and wererelatedto LPS-induced oxidativestress. VitaminAisa fat-soluble vitamin thathas someantioxidativeeffects.In this paper, theprotectiveroleofvitamin A was investigatedagainstLPS induced fetaldefectsinrats.*
Methods:*Pregnantratswere selectedanddividedinto4groups.All pregnant rats,exceptfor the controlgroup,receivedanintraperitoneal injectionof LPS (75 µg/kg) daily on gestation days 15–17. The second and third groups received 100 mg/kg of VitaminA(I.M.) dailyoneweekbeforeofthe firstinjectionofLPS.Onday 18, thenumberoflive fetuses, dead fetuses,andresorption siteswerecounted in each respective group.Live fetusesineachlitterwere weighted. Crown-rumpandtaillengthswere examinedandskeletaldevelopmentwasevaluated.*
Results:*AdministrationofLPSsignificantly increases fetal mortality, decreases fetalweight,crown-rumpandtaillengthsoflive fetusesandretarded skeletal ossificationincaudal vertebrae,anteriorand posteriorphalanges andsupra-occipitalbone.*
Conclusions:*Thestudyshowedthat aco-treatmentofVitaminAwithLPScoulddecrease defectsofLPSand improve injuries.Thissuggests vitaminAhasan antioxidant role whichcould protect fetalinjuriesinduced byLPS in rats.*
Keywords: *Vitamin A, Lipopolysaccharide, Fetal Injury, SkeletalDevelopmentRetardation*

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I. INTRODUCTION

During embryogenesis, several environmental signals are involved in the formation of the neuroendocrine system. Several studies, which were carried out on various species, have reported that prenatal stress, inflammation, maternal infection, and inappropriate nutrition of the fetus can result in physiological and behavioral changes in postnatal life (1). Low birth weight (LBW) is one of the indicators of an abnormal uterine environment that may increase the risk of future illnesses (2). Bacterial lipopolysaccharide (LPS) is a special component of the external membrane of gram-negative bacteria, which is not present in gram-positive bacteria and is a toxic substance (endotoxin) with a different biological activity in vivo and in vitro conditions (3). If large amounts of LPS are released in vivo from the contaminating organism, it can lead to severe syndromes such as multiple organ disorders, intravascular coagulation, endotoxic shock (4), and abortion (5). This is mainly caused by biologically active mediators such as tumor necrosis factor (TNF) and nitric oxide, which are released from various cells, particularly by macrophages (4). As aforementioned, although the lipopolysaccharide is in the outer membrane layer, in capsule-free strains, it is placed on the surface of cells. Lipopolysaccharides are predominantly present in the human and animal digestive system. Humans are usually exposed to low levels of lipopolysaccharides because of continuous infections. It has been reported that alcohol consumption and gastrointestinal weakness are the main cause of lipopolysaccharide leakage from the

gastrointestinal tract to the blood (6). Other researches have shown that intestinal endotoxins could cause embryonic necrosis, immediate abortion, damage to fetal organs, fetal death, and various malformations in a variety of animal species. Endotoxins cause fetal death, various abnormalities and low birth weight (7). One study reported that administration of E. Coli lipopolysaccharide as an intravenous dose on day 8 of pregnancy led to a decrease in the embryonic weight and an increase in fetal abnormalities and LPS concentration (8). Maternal infections were associated with abortion, premature delivery, and torn premature membrane embryo (9), therefore they can be the cause of the mortality around labor time. It has been reported that administration of LPS to pregnant mice led to abortion in a dose dependent manner (10). It seems that the released anti-inflammatory cytokines such as interleukin-10 (IL-10) (10) and tumor necrosis factor (TNF) (11) are important factors in stimulating intrauterine fetal death by LPS. It has been observed that TNF-α is associated with an increase in the level of oxygenase-1 induced by LPS in the rat liver (12). Moreover, prostaglandins have an important role in fetal toxicity induced by lipopolysaccharides. Based on the results of one study, overproduction of active oxygen species (ROS) due to lipopolysaccharide is one of the main causes of fetal injury and are also effective in intrauterine fetal death and intrauterine growth retardation (13). On the other hand, antioxidants could protect mice against intrauterine fetal death and intrauterine growth retardation due to lipopolysaccharides (14). Enzymes formed are the first and the most important defense mechanism of the cells. Two primary types of antioxidants include; glutathione peroxidase (GPx), superoxide dismutase (SOD), glutathione reductase (GR) and catalases as enzymatic antioxidants and Vitamins A, C, and E as non-enzymatic antioxidants (15). Vitamin A is a fat-soluble antioxidant which prevents bio-molecules and regulates the endogenous activity of scavenging enzymes in oxidative stress induced by cigarette smoking (16). It has been reported that Vitamin A has antioxidant properties and was administered to the mother before accouchement (18). Moreover, one study has shown the positive effects Vitamin A has against oxidative stress due to CCI4 poisoning in the liver (19). Hence, the aim of the present study was to investigate the antioxidant effect of Vitamin A in protecting embryonic lesions induced by lipopolysaccharides.

II. METHODAND MATERIALS

Escherichia coli lipopolysaccharide and Vitamin A were purchased from the Sigma Company. Other laboratory materials were also provided by the Sigma Company. Wistar rats were purchased from Tabriz University of Medical Sciences and had access to standard pellets and water. Their weights were measured on days 1 and 7. Healthy female (weighting of 150-200 g) and male (weighting of 180-220 g) rats without any abnormalities were selected for mating after one week of habituating to the environment. Animals were kept in metal cages at temperatures of 20-26 °C, relative humidity of 40-74%, with ventilation 10 - 15 times per hour, and lighting for 12 hours daily (7 AM to 19 PM). To mate, male and female rats (4:2) were placed in a cage for 24 hours. Then, female mice with a vaginal mucus pluck were considered as pregnant mice (day 0 of pregnancy). At mating, all mice were 11-13 weeks old. The pregnant mice were randomly divided into different groups, and the average weight of mice in each group was 200 to 250 grams. Non-pregnant mice were excluded from the study. Forty-eight pregnant mice were randomly divided into 4 groups; mice in groups 1 and 2 in days 15 to 17 received 75 µg/kg lipopolysaccharide intraperitoneally. Also, mice in groups 2 and 3 received daily and intramuscularly 100 mg/kg Vitamin A for one week before receiving LPS. Mice in group 4 acted as placebo and received normal saline. All female rats were terminated by cervical dislocation to assess the weight of the pregnant uterus. Moreover, in each rat, the number of live embryos and dead embryos was counted. The number of live embryos in each abdomen was weighed and the Crown-rump length (CRL) and tail length were also measured. All embryos were kept in ethanol (95%) for at least 2 weeks in order for the skeletal evaluation to be made (6).

2.1 Fetal Evaluation

The embryo's viscera and skin were removed to allow full coloration. For staining of the skeleton, the embryo was fixed for 3 days in 95% ethanol. Also, the embryos were placed in alcian blue stain, 80 ml of 95% ethanol, and 20 ml of glacial acetic acid for cartilage staining and were later washed and distilled in 95% ethanol for 24 hours. To proceed with bone marrow staining, the embryos were placed in alizarin red S (25 mg/L of 1% KOH) for 2 days. Then, the specimens were placed in 0.5% KOH for one week to make the tissues

clear and afterwards, they were placed in incremental concentrations of glycerol (20%, 50%, and 80%) for 24 hours and before the final storage in glycerol 100% were once again placed in KOH 1%. The bone density of different parts was evaluated according to the method which is described by Aliverti.

2.2 LipidPeroxidationAssay

Lipid peroxidation was measured in terms of MDA. MDA levels were studied using the thiobarbituric acid (TBA) abduction method as described previously. Briefly, 50 µl of sample was 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 mixing, the samples were placed in a water bath and heated for 1 hour at 95-100ºC. The samples were then cooled under running cold water. 25 µl 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.

2.3 DeterminationofGSHContent

GSH was determined by the method of Griffith [20]. Proteins of 0.4mL liver homogenates were precipitated by the addition of 0.4mL of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm at 4ºC for 5 min. Four hundred microliters of the supernatant were combined with 0.4 mL of 300 mM Na2HPO4 and the absorbance at 412 nm was read against a blank consisting of 0.4 mL supernatant plus 0.4mL H2O. Then, 100 mL DTNB (0.02%, w/v; 20 mg DTNB in 100 mL of 1% sodium citrate) was added to the blank and sample. The absorbance of the sample was read against the blank 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. GSH values wereexpressed as nmol mg−1 protein. Protein content was measured according to the methodof Lowry et al. [21].

III. STATISTICALANALYSIS

All results were expressed as the mean ± standard deviation. One-way ANOVA and Tukey HSD test were used to compare the studied groups. SPSS software version 16 was also used to verify the data.

IV. RESULTS

Placenta weights, fetal weights, live and dead fetuses, fetal resorption, crown-rump length are summarized in Table 1. Lipopolysaccharide significantly reduced the weight of the pairs, the weight of the fetus, the number of live embryos and the length of the crown of the rats compared to the other groups ($P < 0.05$). Also, the number of dead embryos was significantly increased in the LPS group ($P < 0.05$), while the number of embryo absorption did not show a significant increase in the lipopolysaccharide group relative to other groups ($P> 0.05$). On the other hand, Table 2 shows a comparison of ossified sites of fetuses between all the study groups. Parameters of metacarpus and sternum ossification did not show any significant differences in study's groups ($P >0.05$). But, the parameter of metatarsus in LPS and LPS + Vitamin A groups was significantly lower than two other groups ($P <0.05$). Additionally, parameters of anterior and posterior phalanges in LPS group were significantly decreased compared to other groups ($P <0.05$). In addition, the comparison of GSH content is shown in Table 3. The GSH content in placenta, maternal, and fetal liver was significantly decreased in LPG group as compared with other groups ($P >0.05$). Also, in the LPS + Vitamin A group, the GSH content in fetal liver and placenta showed a significant increase relative to the LPS only group ($P < 0.05$). There is a significant difference between LPS + Vitamin A and control groups ($P < 0.05$). In Table 4, the level of MDA in placenta, maternal, and fetal liver is presented. The level of MDA in LPS group for placenta, maternal, and fetus liver, was significantly increased when compared with the control group ($P <0.05$). Furthermore, although the level of MDA in the LPS + Vitamin A group relative to the LPS group for maternal liver and fetal liver was significantly decreased, there was no significant difference between LPS + Vitamin A and control groups ($P > 0.05$).

V. DISCUSSION

The aim of the present study was to investigate the effect of Lipopolysaccharide on the development of intrauterine embryos and the formation of fetal skeletons, as well as the antioxidant effect of Vitamin A in preventing complications from LPS. Lipopolysaccharide has several complications during embryological development, including embryo absorption, embryo death, and preterm labor. It has been shown that administration of LPS at doses of 25, 50, and 75 μg/kg on days 15-17 of pregnancy led to a significant increase in fetal mortality rates with increasing doses. In the high-dose group, fetal deaths were reported up to 63.2%. However, the administration of LPS did not increase embryo absorption during the late stages of pregnancy (16). Rivera et al. reported that administration of 100 μg/kg LPS to rats on days 14 to 20 of pregnancy caused fetal deaths up to 43% and reduced the growth of live embryos (21). The results in the present study showed that the number of live embryos in the LPS group was significantly reduced as compared with other groups, also the decrease in the number of live fetuses in the LPS + Vitamin A group had a significant difference to the LPS group. Therefore, our results demonstrated that the use of Vitamin A reduces the deleterious effect of LPS. It has been reported that LPS administration to pregnant mice significantly reduced the weight of the fetus and reduced CRL and tail length in the live embryos (16). Moreover, LPS administration before parturition led to reducing the number of live embryos in the uterus on the 18th day of gestation (22). In addition, one study has reported that administration of LPS on day 8 of pregnancy increased the number of embryo uptake as well as decreased the number of embryos (23). On the other hand, LPS prescription to pregnant mice at the end of pregnancy led to reducing bone ossification in the vertebral arch, hands, toes, and supraoccipital region (16). The results of this study also showed that LPS significantly reduced the size of hand and foot fingers as well as metatarsal bones, but Vitamin A was effective in reducing the deleterious effects of lipopolysaccharide. Furthermore, the results showed that the GSH content in placenta, maternal and fetus livers in LPS group was significantly decreased compared to other groups. LPS altered antioxidant capacity and thiol redox state in these tissues. It has been reported that lower GSH levels lead to oxidation and damage of lipids, proteins, and DNA by reactive oxidative stress (ROS). The current results show that Vitamin A could improve GSH content of cells, whilst there was no significant difference between control and LPS + Vitamin A groups. On the other hand, the level of MDA in LPS group in placenta, maternal and fetus liver was significantly increased compared to other groups. It has been reported that increased production of ROS is one of the causes of increase in MDA concentration. One study has shown that LPS could stimulate macrophages to generate ROS and in macrophage-rich organs, LPS could increase nitrotyrosine as a marker for O2•−, NO and formation.

VI. CONCLUSION

Vitamin A regulates the activity of deleterious enzymes with oxidative stress mechanisms. It has antioxidant properties and prevents oxidative stress. The results of the present study showed that vitamin A could prevent lipopolysaccharide complications.

Table1.*Comparisonsofplacentalweight,liveanddeadfetusnumber,fetusweight,fetal resorptionandcrownrumplengthamonggroups(mean±SD).*

Parameters/Groups	Control	LPS	Vitamin A	LPS+ Vitamin A	Levelof significance
Placentaweight (g)	**0.58±0.05**	**0.38±0.04***	**0.55±0.01**	**0.49±0.02**	**0.021**
Fetalweight (g)	**4.01±0.006**	**3.15±0.01***	**4.3±0.112**	**3.54±0.6**	**0.035**
Livefetuses	**12.1±0.12**	**6.2±0.3****	**12±0.11**	**11.23±0.5**	**0.003**
Deadfetuses	**0.11±0.02**	**0.28±0.024 ****	**0.12±0.1**	**0.15±0.12**	**0.009**
Fetalresorption	**0.58±0.02**	**0.65±0.33**	**0.58±0.01**	**0.60±0.21**	**0.062**
Crownrump length	**3.92±0.44**	**3.45±0.18***	**3.95±0.32**	**3.80±0.21**	**0.029**

Table2.*Ossification sites of fetuses in groups*

Ossification site/ Groups	Control		LPS	LPS+ Vitamin A	Levelof significance
Vitamin A					
Metacarpus	**3.95±0.01**	**3.78±0.1**	**3.91±0.02**	**3.83±0.76**	**0.075**
Metatarsus	**4.86±0.02**	**4.51±0.05***	**4.87±0.02**	**4.50±0.02***	**0.036**
Anteriorphalange	**3.89±0.1**	**2.9±0.4****	**3.81±0.01**	**3.5±0.00**	**0.008**
Posterior phalange	**3.55±0.24**	**2.45±0.01****	**3.45±0.43**	**3.54±0.12**	**0.009**
Sternum	**5.97±0.54**	**5.88±0.2**	**5.97±0.11**	**5.94±0.5**	**0.184**

Table3.*Comparisons of GSH (nmol/grtissue)levelsintissuesamonggroups(mean±SD)*

Organs/Groups	Control	LPS	Vitamin A	Vitamin A+LPS
Placenta	**546.1±65.98**	**247.28±51.2****	**551.2±45.2**	**390.93±32.6††**
Maternal liver	**1025.23±24.6**	**724.28±76.9***	**1015.67±56.8**	**732.84±64.9***
Fetal liver	**684.43±25.8**	**305.23±76.5****	**680.87±11.32**	**552± 0.1††**

Table4. *Comparisons of MDA(nmol/grtissue)levels intissuesamonggroups(mean± SD)*

Organs/Groups	Control	LPS	Vitamin A	Vitamin A+LPS
Placenta	**255.1±12.3**	**368.8±24.8***	**2.6±3.4**	**276.94±21**
Maternal liver	**432.45±31.9**	**710.11±54.3***	**436.1±9.4**	**495.10±11.6****
Fetal liver	**271.4±31.1**	**531.86±88.9***	**270± 35.6**	**351.00±33.4****

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