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Effects of Silymarin after Maternal Exposure to LPS on Histopathologic and Immunologic Profiles in Offsprings

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Abstract

Pre parturition and newborn period events like infectious stresses have important effects on the future life of an offspring. LPS can be a cause of bacterial stress in the body. Silymarin is an herbal substance that has some therapeutic effects on body. According to our literature review there was no any study available about silymarin preservative and useful effects after LPS intervention on fetuses in their future life.

Mice of both sex with 25–30 g weight were kept with each other for breeding and the first day of pregnancy was ascertained by observation of vaginal plaque.

Silymarin was suspended in normal saline and then administered to pregnant mice (100 and 250 mg/kg) by stomach gavage for 5 days. Pregnant mice received LPS by one intraperitoneal injection (300 and 500 µg/kg). Half of the offspring's were used for blood sampling for immunologic tests 1.5 hour after immune system stimulation (IP injection of LPS with the dosage of 500 µg/Kg in the day 67 after their birth) IL-1β, TNF-α and IL-6 were measured in them with indirect ELISA kits .Another half of the litters were killed in 70th day of their life (after 3 days of new LPS injection) and whole brain and a specimen from their left lobe of liver was put into neutral buffered formalin.

LPS has caused hyperemia and inflammation in the brain, meninges and liver of treatment groups. Silymarin managed to control the effects of LPS stress on the second generation (received LPS when they were fetus) in a dose dependent manner.

Stresses of mothers during pregnancy can alter in normal physiology of the offspring's and increase the probability of illness in them in the future. These mothers and their litters has an increased level of proinflammatory cytokines in their serum. Silymarin can be useful for protecting the offsprings from LPS effects in the future life.

Keywords: Brain, liver, LPS, mice, offspring, silymarin

1. Introduction

Preparturition and newborn period events like physiologic and immunologic stresses has an important effects on the disorders of a being in the future life even in the life as an adult [1, 2]. Disorders like behavior perturbations, heart problems, atopic diseases and diabetes [3]. Lipopolysaccharides (LPS) are a part of Gram negative bacterial cell wall that are located at the outer part of the bacterial wall [4]. The usage of LPS in high concentration causes anaphylactic shock, chemotaxic responses, cell membrane damage, vascular permeability enhancement, Disseminated Intravascular Coagulopathy(DIC), leukopenia, decrease in phagocytosis, secondary leukocytosis, thrombocytopenia, hypocalcemia, decrease in blood pressure and…..LPS can cause immense disorder in body tissues [4, 5]. It can be a mitogenic factor for B lymphocytes that cause polyclonal increase in their number [6]. It causes Interleukin-1 (IL-1) and Tumor

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Necrotic Factor (TNF) production from macrophages and mononuclear phagocytes [6].

Different stresses during pregnancy can cause changes in the level of inflammatory mediators in mother serum [7, 8]. Nordentoft et al in 1996 has announced that physiologic stresses of mothers during pregnancy can cause shortening of gestation period and decrease of baby weight in humans [9]. Researches in human showed inflammation in mothers during pregnancy like asthma and infection can cause growth retardation of baby and health problems during adulthood [3,10]. Coussons et al., 2007 showed stresses of mother during pregnancy can enhance the level of proinflammatory cytokines markedly in their serum [11]. Researches on rat showed proinflammatory mediators can cause enhancement in corticosterone level of mother and fetus during pregnancy and decrease in fetus weight in the 20th day of gestation period [2].

Silymarin that is an extract of the dried seeds of milk thistle (*Silybum marianum*) is a mixture of four flavonoids: silychristin, silydianin, isosilybinin and silybin. Some virtue like anticancer effects [12, 13], anti-inflammatory effects [14], antidepressant and sedative effects [15, 16], antioxidant effects [17] is attributed to this substance. Silymarin inhibits the expression of TNF-Alpha, activity of ornithine decarboxylase [18, 19] and production of IL-1B and Prostaglandin E2 [20].

The usage of herbal and biological treatments instead of chemical drugs prophylaxis is going to be more common day by day. According to our literature review there was no any study available about silymarin preservative and useful effects after LPS intervention on fetuses in their future life.

2. Materials and Methods

2.1 Animals

Mice of both sex with 25–30 g weight purchased from Pasteur institute of Iran and were kept in 12–12 light-dark cycle in 23 ± 1 degree centigrade temperature. Food and water ration was *adlibitum*. After 2 weeks acclimation period male and female mice were kept with each other for breeding and the first day of pregnancy was ascertained by observation of vaginal plaque and each pregnant mouse was put in a separate cage [21].

2.2 Treatment

Silymarin was purchased from sigma-Aldrich, USA suspended in normal saline and then administered to pregnant mice by stomach gavage for 5 days (100 and 250 mg/kg body weight). Pregnant mice received LPS (Lipopolysaccharides, from Escherichia coli 0111:B4, Sigma-Aldrich) by one intraperitoneal (EIP) injection (Table 1). There were 6 pregnant mouse in each group.

2.3 Blood Sampling

Half of the offspring's were used for blood sampling for immunologic tests after immune system stimulation (IP injection of LPS with the dosage of 500 µg/Kg in the day 67 after their birth) 1.5 hour post injection [21], the animals were anesthetized with ether and blood samples were collected from their heart directly. After 20 minutes the samples were centrifuged at 5000 rpm for 5 minutes. Serum samples were freezed in

Table 1: Study groups of mothers. There were 6 pregnant mouse in each group

Treatment / Group	Normal saline By gavage For 5 days	Normal saline one intraperitoneal injection	Silymarin (Mg/Kg) By gavage For 5 days	LPS (µg/kg) one Intraperitoneal injection
A	+	+		–
B	+	–	–	300
C	+	–	–	500
D	–	+	100	–
E	–	–	250	–
F	–	–	100	300
G	–	–	100	500
H	–	–	250	300
I			250	500

minus 80 degree centigrade for further studies. IL-1β, TNF-α and IL-6 were measured in them with indirect ELISA kits (Biolegend-USA). Data was analyzed using SPSS-Version19 software with ANOVA test and tukey as *post hoc*.

2.4 Pathologic Sampling

Another half of the litters were killed in 70th day of their life (after 3 days of new LPS injection) and whole brain and a specimen from their left lobe of liver was put in to neutral buffered formalin [22].

Because most of the effects of silymarin is on central nervous system especially the brain and on liver, pathologic study focused on these two parts. After complete fixation of tissue specimens, the brain was cut in two area, 4 mm in front of the fissure between cerebellum and cerebrum and 8 mm in front of that. So hippocampus and its upper cortex were reachable.

3. Results

3.1 Inflammatory Mediators

Inflammatory mediators concentrations are shown in the Table 2 in the form of picogram per milliliter.

3.2 Pathologic Findings

LPS has caused hyperemia and inflammation in the brain, meninges and liver of treatment groups. In the lower dose the only change was hyperemia in central veins and hepatic sinusoids in liver and hyperemia in meninges and brain that was more severe in the meninges (group B). With the enhancement in LPS dose (group C) a meningoencephalitis was observed. Inflammatory cells that were mostly lymphocytes and some neutrophils (neutrophils were present especially around vessels) were very abundant in meninges and scattered in brain parenchyma .Some necrotic neurons, mild gliosis and hyperemia was observable too. In liver some hepatocytes showed necrosis with pyknotic nuclei. There were some necrotic hepatocytes that showed fatty change .This showed they had fatty degeneration before death. A severe portal hepatitis with lymphocyte and neutrophil predominance was observable. Usage of silymarin caused a slight dose dependent hyperemia in liver (Group D and E). Brain was normal and didn't show any change. In the group F the brain and liver were hyperemic and no any other change was observed. In the group G there were hepatocytes necrosis, fatty change, hyperemia and portal hepatitis with neutrophils and lymphocytes.

Table 2: Inflammatory mediators of treatment group's offsprings (pg /ml). The only mediator that showed significant difference among offsprings was IL-1B. Data is shown in the form of Mean± Standard Error of Mean

Variable / Group	IL-1B	IL6	TNF-α
A	149.50 ± 8.77	181.75 ± 19.74	72.50 ± 10.03
B	183 ± 15.13**	207.25 ± 12.12	121.75 ± 10.60
C	196.25 ± 13.71**	226.25 ± 13.30	143 ± 7.42
D	#¶¶ 153.25 ± 14.34	170.50 ± 15.99	81.75 ± 3.85
E	#¶¶¶ 153.75 ± 16.90	183 ± 9.82	80.50 ± 4.51
F	156.75 ± 15.87	187 ± 13.04	108.75 ± 5.69
G	168.50 ± 6.03	199.25 ± 9.75	107.75 ± 9.29
H	136 ± 13.61¶¶	169.75 ± 12.05	94.05 ± 9.45
I	187.75 ± 9.40¶¶	196.50 ± 19.93	96 ± 9.06

**Statistical significance with control group $p < 0.01$

#Statistical significance with the group B $p < 0.05$

¶¶Statistical significance with the group C $P < 0.01$

¶¶¶ Statistical significance with the group C $P < 0.001$

Severity of the lesions was less than the group C (With the same dose of LPS and without silymarin treatment). In the brain silymarin managed to control inflammatory changes and just hyperemia was observed with the enhancement in the dose of silymarin. The two last group didn't show any important finding. Lesions were similar to the other groups but were not severe. In the group H the only finding in brain and liver was hyperemia. In the group I the brain was normal. In liver the kinds of the lesions were similar to the group G but they were very slighter. Inflammatory cells were scattered and few in number and fatty change was slight .Portal hepatitis was present and most of the cells were mononuclears (Fig.1 and 2)

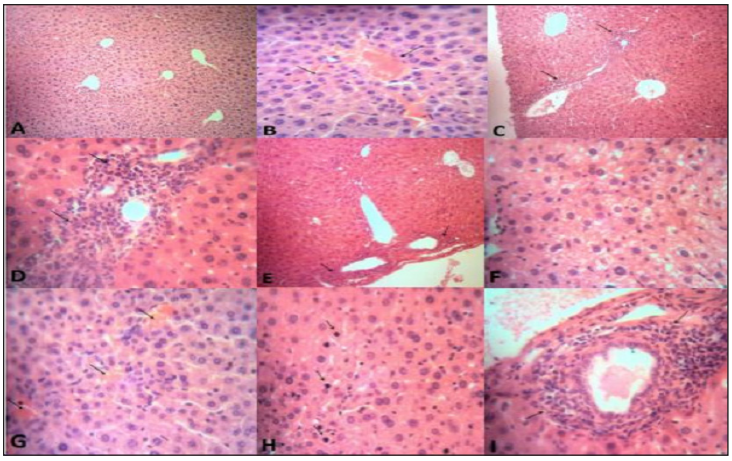

Fig. 1. Hepatic tissue in treatment groups (H&E staining). A: Normal hepatic tissue in control group □80.B: Hyperemia in central vein and sinusoids (arrows). Group B. □800.C: Portal hepatitis (arrows) with the predominance of mononuclear. Group C. □200.D: Portal hepatitis (arrows) with a mixture of neutrophils and lymphocytes. Group C. □800 .E: Portal hepatitis around two bile ducts (arrows) lymphocytes were predominant but some neutrophils were present too. Group G. □80. F: Fatty change in hepatic cells. Group G. □800. G: Hyperemia in central veins and sinusoids (arrows). Group H. □200. H: Necrotic cells with pyknotic nuclei (arrows). Some scattered inflammatory cells are present. Group I. □800. I: Portal hepatitis around a bile duct (arrows).Most of the cells are mononuclears. Group I. □800

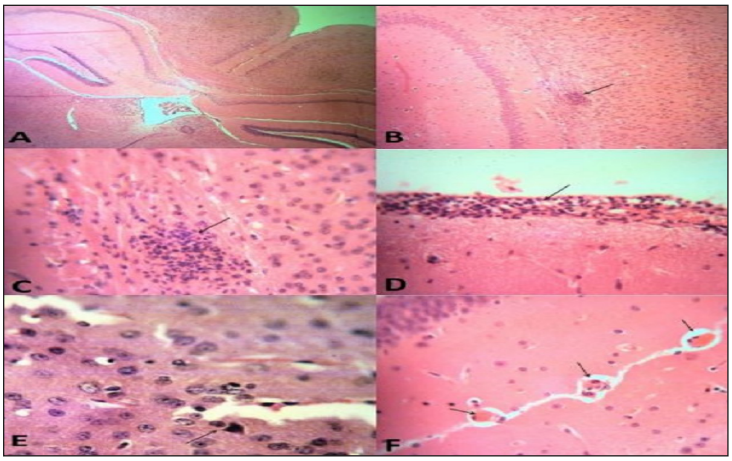

Fig. 2. Hippocampus of treatment groups. (H &E staining).A: Normal hippocampus tissue in control group. □80. B and C: Focal gliosis of microglia (arrows). Group C. □200 (B) and □800 (C). D: Meningitis with mononuclears (arrow) and hyperemia. Group C. □800. E: Neuronophagia (arrow) a part of neuron has been phagocytosed by an oligodendrocyte. Group C. □1000. F: Capillary hyperemia in hippocampus (arrows). Group F. □800.

4. Discussion

In this study LPS caused hyperemia in lower dose and with the enhancement in dosage a marked inflammation was observed in the meninges and brain, scattered neuronal necrosis and gliosis was observed too. In liver inflammation especially in portal area, necrosis of hepatocytes and hyperemia was observed.

LPS causes production of inflammatory mediators from hepatocytes. It stimulates repair mechanisms in liver too [23]. LPS can be used as a model of bacterial infection and immunologic stress. Necrosis of hepatocytes and inflammation of different organs following treatment with LPS has been reported. LPS can be mitogenic for B-lymphocytes and can cause their polyclonal and unspecific mitosis. Some researchers showed LPS has broad spectrum effects on body and causes hyperemia, necrosis and inflammation in liver and hippocampus of the brain [4, 5].

In this research concentration of IL-1B, TNF-α and IL-6 enhanced after LPS treatment .This enhancement was statistically significant about IL-1B.This is in agreement with other researches. LPS can irritate production of TNF and IL-1 from macrophages and mono nuclear phagocytes [6]. Researches showed enhancement in cytokines like IL-1 production after LPS irritation from different sources like macrophages can cause increase of neutrophil and platelet production from bone marrow [24, 25]. Hepatocytes produce a protein that bonds with LPS. This union is recognizable by a receptor on kupffer cells. Bonding of LPS with kupffer cells induces cytokine and prostaglandin release from these cells and induction of inflammation [26]. In the present study the usage of silymarin managed to control the effects of LPS on cytokines level in offsprings and tissue injuries in liver and hippocampus after confrontation with that. Silymarin in rats has caused inhibition of hepatic enzymes like Alanine transaminase (ALT), Aspartate transaminase (AST) and Gamma-glutamyl transpeptidase (GGT) after liver toxicity with alcohol. Silymarin can inhibit hepatic fibrosis after bile ducts obstruction [27]. Milk thistle extract that contains silymarin can inhibit inflammation in liver after LPS usage and can preserve hepatocytes [26, 28]. Silymarin with different mechanism like stimulation of DNA polymerase, stabilizing the cell membrane, free radical neutralization and increase in glutathione concentration can preserve hepatocytes [29–31] DNA polymerase stimulation can increase ribosomal RNA production and renovation of hepatocytes. Silymarin with inhibition of lipooxygenase cycle and leukotrienes production and inhibition of lipid peroxidase can inhibit cell injury in murine hepatocytes [29]. Silymarin treatment can prevent liver from injury after consumption of hepatotoxic substances in pregnant women [32, 33].

Inflammation in the brain is one of the important cause of neurons injury. Silymarin can control inflammation of the brain and save the neurons [34]. It can preserve the brain from injury after obstruction of its vessels with thrombus or thromboembolisms [35]. It can improve impulse conduction in neurons of diabetic patients too [36].

Murphy et al in 2003 announced that inflammatory conditions like asthma and infection in pregnant women can cause decrease in fetus growth and increase of inflammation probability after birth and health problems even in adulthood. Stresses of mothers during pregnancy can alter in normal physiology of the offspring's and increase the probability of illness in them in the future [3]. These mothers and their litters has an increased level of proinflammatory cytokines in their serum [11]. Silymarin can be useful for protecting the offsprings from LPS effects in the future life.

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