Pakistan J. Zool., vol. 45(2), pp. 359-369, 2013.

Behavioral Changes Induced by Prenatal Acute Endotoxemia in Mice Offspring

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Abstract.- This study aimed to investigate septic complications resulted in behavioral changes in mice offspring. Pregnant Swiss female mice were intraperitonealy injected with the acute bacterial lipopolysaccharides (LPS) at a single dose of 2.5 mg/kg of body weight at the 7th day of gestation. LPS increased the oxidative stress and decreased the anti-oxidant glutathione in the tissue of the LPS-treated mothers and pups. This might mediate the behavioral complications responses to the LPS in the pups born to LPS-treated mothers. The experiments indicated that LPS significantly reduced the locomotors activity of mice pups when compared with the saline-control mice. LPS was found to inhibit the sensory motor reflexes in all elements of acts and postures. Significant decreases were observed in the social contacts, threat, attack and number of fights of pups born to LPS-treated mothers. Highly distributed focal areas indicating many phagocytic activities with a marked depletion of lymphocytes was observed in section from thymus of pups born to LPS-treated mice, indicating an intense inflammation. In conclusion, prenatal LPS-induced inflammation enhanced the susceptibility to the development of neonatal systemic and behavioral disorders.

Key Words: Lipopolysaccharide, prenatal exposure of LPS, mice offspring, glutathione, sensory motor reflexes, endotoxin.

INTRODUCTION

Lipopolysaccharides (LPS) are found in the outer membrane of various Gram-negative bacteria and are important component of their ability to cause diseases. Lipid A of the LPS affected mitochondrial respiration and phosphorylation (Kato, 1972). LPS is believed to be the primary trigger of Gram-negative septic shock (endotoxemia), and has been widely used in investigations of bacterial infection-induced inflammatory response (Saluk-Juszczak and Wachowicz, 2005).

During fetal development, signals from the environment program the neuro-endocrine system and behavior of offspring. Investigations have shown that prenatal stress, maternal inflammation or infection, and fetal malnutrition alter physiology and behavior in later life. Response to an inflammatory stimulus such as LPS depends on the integrative activation of the HPA-axis and the immune system (Tilders *et al*., 1994)

In humans, LPS binds to the LPS-binding protein (LBP) in the serum, which transfers it to CD14 on the cell membrane, which in turn transfers it to another non-anchored protein which associates with toll like receptor-4 (TLR4) (Nguyen *et al*., 2002; Blander and Medzhitov, 2004; Doyle *et al*., 2004). CD14 and TLR4 are present in several immune cells, triggering the signaling cascade for macrophage/endothelial cells to secrete pro-inflammatory cytokines and nitric oxide (NO) that lead to septicemic shock (endotoxemia). NO may participate in mechanosensory processing (Ott *et al*., 2000). Oxidative damage plays a key role in septic shock induced by LPS which is known to enhance the formation of reactive oxygen species (ROS) (Ben-Shaul *et al*., 2001), the lipid peroxidation products (MDA) and the suppression of the anti-oxidant glutathione (Zhu *et al*., 2007; Ebaid *et al*., 2012).

It was found that LPS-induced cytokines (Liu *et al*., 2010) are potent stimulators of systemic and

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0030-9923/2013/0002-0359 $ 8.00/0

NO synthesis (Trauner *et al*., 1997) and then tissue injury. For example, impairment of the major determinants of bile acid-independent bile flow by LPS may contribute to the pathogenesis of the cholestasis of sepsis. Moreover, endotoxemia by LPS has been reported to affect gut motility and impaired gastrointestinal smooth muscle activity (Scirocco *et al*., 2010).

Recent studies have demonstrated that inflammation induced by LPS led to selective loss of nigral dopamine (DA) neurons and extensive activation of microglia (Arimoto and Bing, 2003). Rat that exposed to LPS at embryonic day 10 resulted in a birth of animals with fewer than normal DA neurons (Ling *et al*., 2002) with many characteristics seen in Parkinson's disease (PD). LPS-induced circulatory impairments may place the newborn brain at prolonged risk of cerebral endothelium and cerebral blood flow dysregulation and injury (Feng *et al*., 2010). In the current study, after inducing endotoxemia, we investigated the behavioral changes as critical determinants of endotoxemia in developing pups born to LPS-treated mothers to address whether these complications are transferred to the offspring. The results in the current study highlight the altered locomotion activity, sensory motor reflex, development of hair and eye opening in the animals prenatally exposed to LPS. This may contribute to the increased susceptibility of these animals to a secondary neurotoxin insult.

MATERIALS AND METHODS

Experimental animals

Male and female Swiss-Webster strain mice (8 weeks old) were housed in opaque plastic cages (three females to one male, or three females without a male, in each cage). Animals were obtained from the Central Animal House, Faculty of Pharmacy, King Saud University. All animal procedures were in accordance with the standards set forth in the guidelines for the care and use of experimental animals by the Committee for Purpose of Supervision of Experiments on Animals and the National Institutes of Health protocol. The study protocol was approved by the Animal Ethics Committee of the Zoology Department, College of Science, King Saud University. Animals were kept under reversed lighting conditions with white lights. The ambient temperature was regulated between 18 and 22°C. Food and water were available ad libitum, unless otherwise indicated. The males were removed from the cages after pregnancy was confirmed (appearance of a vaginal plug was considered as day one of pregnancy), and the females were subjected to experimental treatments.

Sepsis model

Females was assigned to one of two groups: the first group was a pregnant control group given phosphate buffered saline via an intra-peritoneal injection; the second group was pregnant and received a single intra-peritoneal (IP) injection of endotoxin (LPS) at a dose of 2.5 mg/kg of body weight. Remick *et al*. (2000) have shown that this model demonstrates sepsis-like symptoms, with pathophysiological responses similar to those in patients with sepsis. Offspring were subjected to developmental and learning investigations from the day of birth (PD1) until day 21 (PD21).

Blood samples

Animals were anesthetized with pentobarbital (60 mg/kg body weight) and samples (blood and liver) were obtained at the end of the experiment. Whole blood was drawn from the abdominal aorta. Half of the obtained blood was used to evaluate the whole blood and measure the differential count. Heparinized venous blood was centrifuged at 800×g for 10 min, and plasma was stored at 20°C until analysis.

Histological sections

Thymus parts was collected from the sacrificed control and treated mice. Tissues were fixed in Bouin's fixative, processed in paraffin, and 4 µm thick sections were prepared. Sections were stained with hematoxylin and eosin (H&E) to investigate general histological architecture. In each group, many sections from different mice were studied and representative slides showing clear morphological changes were photographed.

Standard opponent tests

Male subjects from each litter and in all, 14

male subjects from each group were separately housed for 14 days. After this isolation period, these males from each group were be subjects to the standard opponent test under dim red lighting (ca. 8 lux) as previously described. The docile and age-matched males "standard opponent" were rendered anosomic by applying 25% zinc sulphate tract under ether anaesthesia for 1h prior to encounters. The anosomic "standard opponent" intruders were put into the home cages of the test animals and the standard opponent test of each animal was observed for 500 seconds. The opponents were used only once and the selected elements of behavior were studied. Standard opponent tests were investigated as previously mentioned by Ajarem *et al*. (2011) and Albasher *et al*. (2011).

Developmental motor reflexes

The pups were culled to only eight per dam on PD1 and were left with their mothers until PD21. During the weaning period, three pups of each litter were color marked from the others without any consideration to its sex, and were subjected to various behavioral tests under dim lighting. In all, 21 pups belonging to seven litters were considered. All observations were recorded on PD1 and repeated every other day until PD21 in the same three color marked pups of each litter. Sensory motor coordination reflexes were investigated as previously described elsewhere (Ajarem *et al*., 2011; Albasher *et al*., 2011).

Eye opening and hair appearance

The day at which the body hair fuzz appeared, and the eyes opened were also recorded. These two parameters are also useful morphological indicators of development.

Righting reflex

The time taken by a pup placed on its back to turn over and place all four paws on the substrate was recorded. An upper limit of 2 min is set for this test.

Rotating reflex

The surface used to measure the rotating reflex was the same as that used for righting reflex, except that it was inclined at an angle of 30°. The pups were placed on this surface with their heads pointed downwards. The time elapsed until the pup rotate its body through 180° geonegatively and face its head upwards was recorded as the rotating time. The upper limit of this test was also set at 2 min.

Cliff avoidance

Pups were placed on the edge of a table top with the forepaws and face over the edge. The time taken by the pup to back away and turn from the "cliff" was recorded. Again an upper limit of 2 min was chosen. A latency of 2 min was attributed when the animal fell from the "cliff".

Locomotors activity

Locomotors activities were measured as previously described by Pontieri *et al*. (2001). The animals were placed in the activity cage for at least a 30 min period for acclimatization. Temperature, sound and light conditions were maintained uniform during the course of the experiments. Locomotors activity of the mice was automatically recorded in an activity cage (Basile, Milan, Catalogue No 7400) and the sessions in the activity cage lasted 2 h. Measurements were carried out at 10 min intervals and cumulative counts were recorded.

Statistical analysis

For statistical analysis, the mean of all three color marked pups per litter was considered as a single score. Thus, seven replicates from each treatment category were considered in these observations. Statistical analysis is undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). Results are expressed as mean±standard errors (SE). Values of $P > 0.05$ are considered statistically non-significant, while values of $P < 0.05$ were considered statistically significant.

RESULTS

Early development of sensory motor reflexes were inhibited by LPS

During embryogenic developments, neural tissues attract the bacterial LPS. It was, therefore, expected that a direct change in behavioral elements must be occurred. The sensory motor reflexes were addressed. Prenatal treated mice by LPS had a

significant and dose dependent inhibitory effect on the development of all sensory motor reflexes in the pups. During the first three weeks of the postnatal development, LPS had a significant inhibitory effect on the righting reflex, the rotating reflex and the cliff avoidance activity (Fig. 1).

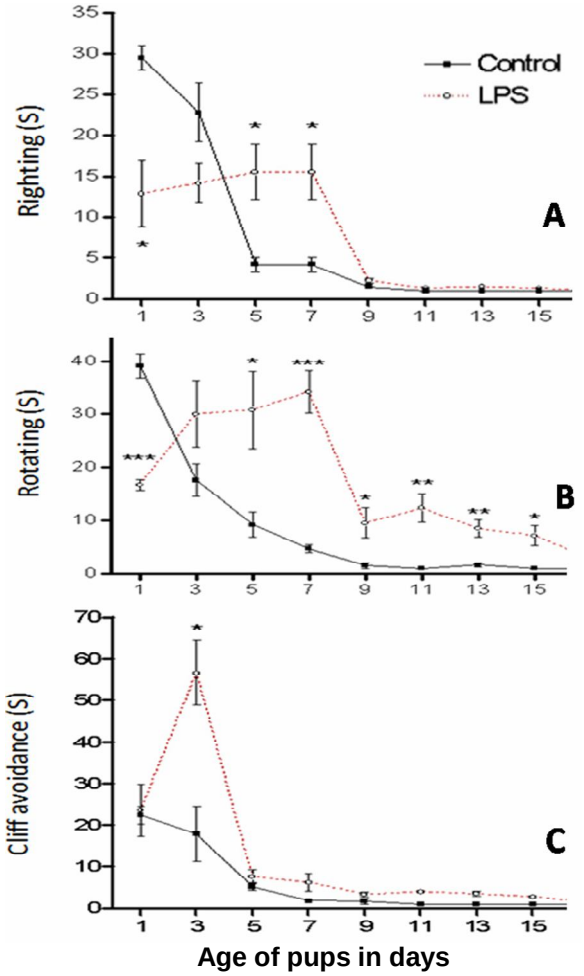

Fig. 1. The early development of the different sensory motor reflexes in the pups born to both control and LPS-treated mothers. A: the mean righting reflex in seconds; B: The rotating reflex; C: The cliff avoidance reflex in seconds. Pups born to treated mothers were inactive comparing to the controls. Results are expressed as mean and standard errors (M±SE). The result was considered highly significant *** when P < 0.01.

LPS delayed eye opening of the developed pups

Postnatal developments are crucial indicators for the LPS prenatal exposure-stress. From PD1 to PD21, the opening of eyes was daily observed. The eye opening of pups born to mothers injected with LPS lagged behind controls from the day of birth and remained so almost throughout their weaning period until PD21 in a dose-dependent manner and significantly ($p<0.05$) delayed after those of the controls. Furthermore, the postnatal hair appearance of the pups born to LPS-treated dams followed the same trend as the pups born to saline-treated dams even though the pups born to LPS-treated dams had lower birth weight and weighed consistently less than the pups born to saline-treated dams (data not shown).

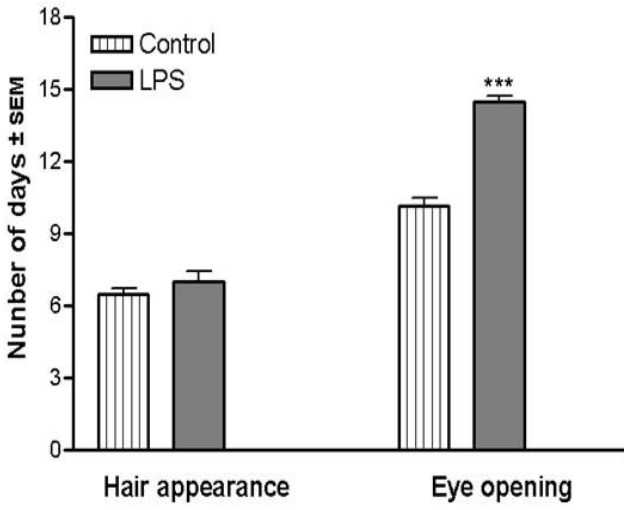

Fig. 2. Eye opening and body hair appearance of the developed control pups and those born to LPS-treated mothers. All observations were recorded on PD1 and repeated every other day until PD21. Results are expressed as mean and standard errors (M±SE). The result was considered highly significant *** when P < 0.01.

LPS delayed the locomotors activity of the developed pups

Mice pups were placed in the activity cage for at least a 30 min period for acclimatization. Temperature, sound and light conditions were maintained uniform during the course of the experiments. The experiments performed with locomotor activity indicated that LPS significantly reduced the locomotor activity of mice pups when compared with the saline-control mice (Fig. 3). This

significant reduction was induced in both vertical and horizontal activities.

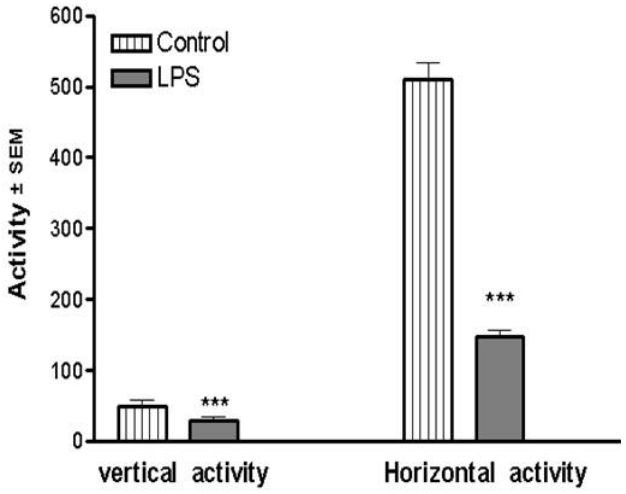

Fig. 3. The vertical and horizontal locomotor activities of pups born to LPS-treated mothers and control mothers. Results are expressed as mean and standard errors (M±SE). The result was considered highly significant *** when $P < 0.01$.

LPS suppresses the social contacts of pups

Expectedly, significant decreases were observed in the social contacts of pups from treated mothers. Significant decreases were also found in threat, attack and number of fights of pups born to LPS-treated mothers. From the data presented in Table I, it is shown that the latency to threat was significantly increased in pups born to LPS-treated mothers when compared to the control mice. The threat in the treated groups decreased from 20.1 at the control to 5.6 and at the pups born to LPS-treated mothers. Accordingly, the latency of threat was increased from 10 s. at the control pups to 328.4 s. at the pups born to LPS-treated mothers. Similar results were obtained for the attack activities. Social activities were significantly decreased in pups born to LPS-treated mothers when compared to the control pups. From these results, there is a clear correlation between the LPS and all social, non-social and aggression activities.

Thymus histopathological changes and blood differential count-induced LPS

Thymus sections of pups born to LPS-treated

Table I.- The social behavior of male albino mice induced by the prenatal exposure to the LPS.

	Median number (with ranges) of seconds allocated to behaviors						Median number (with ranges) of acts and postures					
	Nonsocial investigation	Social investigation	Defense	Threat	Attack	Displacement	Latency to threat (sec)	Latency to attack (sec)	Fights	Naso-nasal contacts	Naso-genital contacts	Wall rears	Rears
Control	130.20 (94.30 – 170.60)	224.50 (214.10 – 241.30)	20.10 (15.20– 20.70)	20.10 (13.30 – 30.0)	64.60 (50.40 – 100.20)	40.50 (4.90–56.60)	10.00 (5.00 – 30.00)	60.00 (20.00 – 70 .00)	20.00 (15.00– 21.00)	27.00 (23.00 – 29.00)	18.00 (14.00 – 22.00)	13.00 (10.00 – 16.00)	11.00 (8.00 – 15.00)
LPS	237.70 ** (225.40 – 292.80)	184.30 ** (146.20 – 197.80)	8.20 ** (0.0 – 16.20)	5.60 ** (3.20– 11.5)	5.00 ** (0.0 – 10.10	60.20 (24.80–80.2)	328.40 ** (300.20 –423.40)	0.00 ** (0.00 - 300.20)	0.00 ** (0.00– 1.00)	12.00 ** (10.00 – 15.00)	9.00 ** (7.00 – 119.00)	8.00 ** (5.00 – 9.00)	10.00 (5.00 – 13.00)

Results are expressed as mean and standard errors (M±SE). The result was considered highly significant ** when $P < 0.01$.

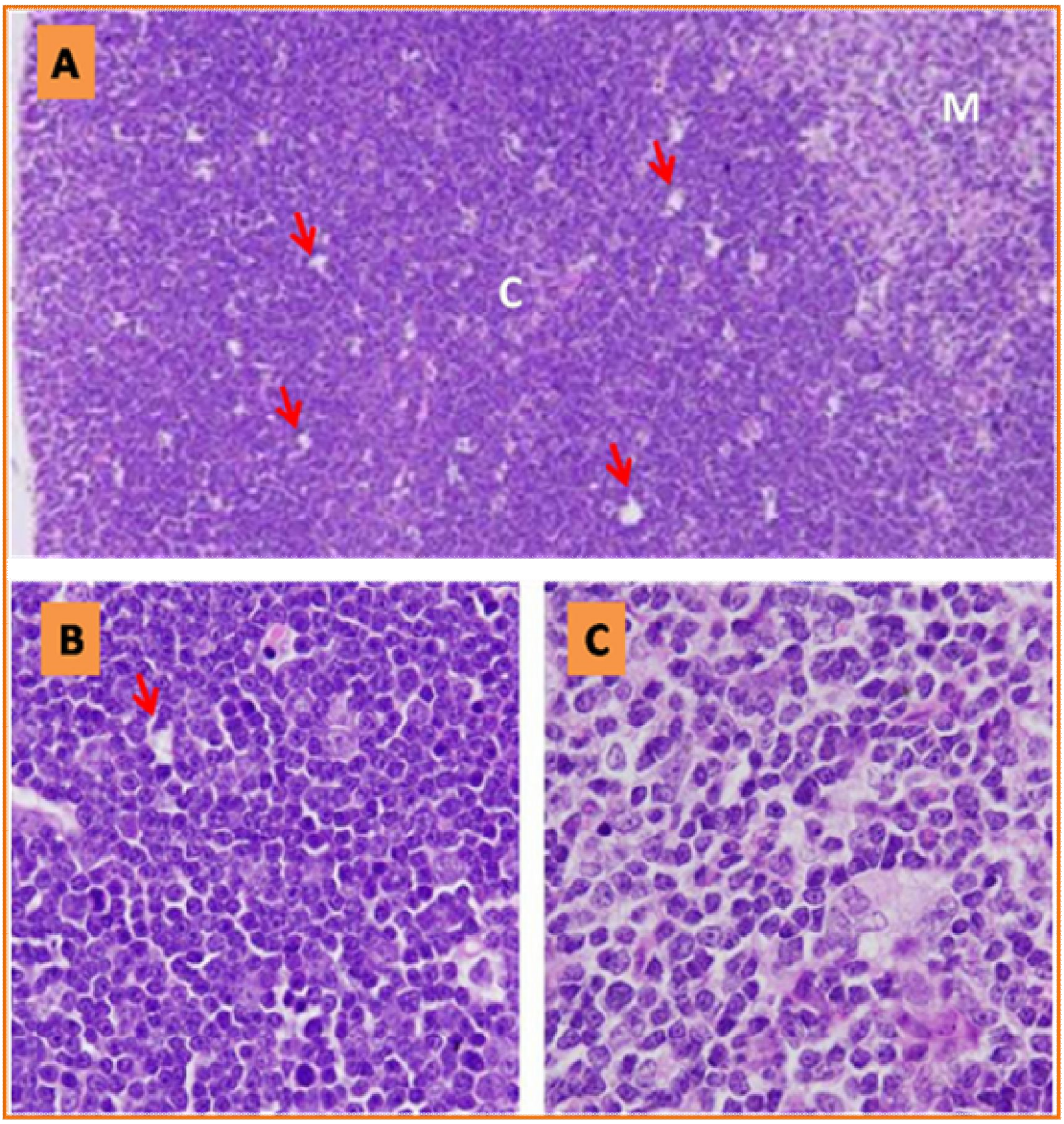

Fig. 4. A: Representative section from thymus of pups born to LPS-treated mothers showing the cortex (C) and medulla (M) cortical and showing the depletion of thymocytes and the focal areas (H&E, X100). The depletion of thymocytes and the focal areas is shown in the magnified section (B) (H&E, X400). C: A magnified section of the medulla region showing the clear thymocytic depletion (H&E, X400).

mice showed highly distributed focal areas indicating many phagocytic activities. The boundaries between cortex and medulla were slightly demarcated. The width of medulla appeared to be predominant over the cortex. The cortical lymphocytes showed a marked depletion. Increased numbers of dilated small vessels were observed in both medulla and cortex (Fig. 4).

In a bacteria endotoxemia model, we have previously detected high antigen presenting cell-activities with a high depletion of neutrophils in peripheral blood (Ebaid and Abdel-Salam, 2006). The differential leukocyte count revealed a significant increase ($p < 0.05$) in the lymphocyte count of the pups born to LPS-treated mothers (78±3.5) comparing to the control pups (62±1.8). A

significant low values ($p < 0.05$) of neutrophils in the LPS-treated pups (18±3.5) comparing to the control pups (30±6) was observed.

DISCUSSION

We studied the prenatal exposure of gestated mice to LPS, on the postnatal development of pups. Results clearly suggest that prenatal exposure to LPS is extremely harmful to the developing pups indicating an active transfer of LPS from mother to embryos. Investigations in a wide range of different species have shown that prenatal stress, maternal inflammation or infection, and fetal malnutrition alter physiology and behavior in later life-response to an inflammatory stimulus such as LPS depends on the integrative activation of the HPA-axis and the immune system (Tilders *et al*., 1994).

We have previously induced endotoxemia in adult mice using whole bacteria, and observed markedly increased NO synthesis and MDA, and decreased catalase, total peroxidase, superoxide dismutase and total glutathione (Ebaid and Abdel-Salam, 2006). ROS causes cellular damage such as lipid peroxidation, which disrupts membrane fluidity and the degradation products can initiate cellular apoptosis (Horton and Fairhurst, 1987; Halliwell and Gutteridge, 1999; Kannan and Jain 2000; Golbidi and Laher, 2010, Ebaid *et al*., 2011). Thus, it seems more likely that oxidative stress is a potential factor of the tissue damage and impairment of various physiological and biochemical reactions in the body of both mothers and offspring in the current study. Oxidative imbalance with high levels of pro-inflammatory cytokines are the result of exposure to LPS, as has previously been shown (Ebaid *et al*., 2012; Scirocco *et al*., 2010).

The overproduction of pro-inflammatory cytokines contributes to manifestation of the systemic inflammatory response and development of organ failure (Kotb and Calandra, 2003). Pro-inflammatory cytokines cause centrally mediated effects, such as changes in body temperature (Linthorst *et al*., 1994; Pauli *et al*., 1998), sleep (Pollmächer *et al*., 1993) and neuroendocrine function (Anisman *et al*., 1998; Hanisch *et al*., 1997; Pauli *et al*., 1998; Zalcman *et al*., 1998). It is known that maternal exposure to LPS increases the levels of TNF-α, IL-1β, and IL-6 in the maternal blood (Chen *et al*., 2005), and in the placenta (Ashdown *et al*., 2006; Beloosesky *et al*., 2006; Urakubo *et al*., 2001). These cytokines may cross placenta and enter fetal circulation (Dahlgren *et al*., 2006; Kent *et al*., 1994). Beside an inflammatory response, maternal exposure to LPS elicits other changes in the mother and fetuses, such as fever, in utero hypoxia, hypotension, and oxidative stress (Cambonie *et al*., 2004; Coumans *et al*., 2005; Dalitz *et al*., 2003).

Pups born to LPS-treated dams display many characteristics seen in patients with PD (Ling *et al*., 2006). LPS increases thickness of the cortical plate and hippocampus together with abnormal distribution of immature neuronal markers and decreased expression of markers for neural progenitors and elevated levels of cytokines in fetal forebrains that lead to neurobehavioural deficits (Ghiani *et al.*, 2011). These harmful effects of LPS on the neurons can explain the significant depletion in the sensory motor reflexes observed in the current study. On the other hand, the possible DNA damage by LPS during pregnancy could also affect the sensory motor reflexes of the early developmental stages (Jaiswal *et al*., 2009). This obviously reflected on the different inhibited reflex activities in the current study. However, when Poggi *et al*. (2005) use a single injections of LPS on GD15, there were no alterations in sensorimotor skills. The activation of microglia, which are sensitive to endotoxins (Frank *et al*., 2007) and are a major source of IL-1 β in the brain (Aloisi, 2001; Kreutzberg, 1996) remains in response to infection (Chew *et al*., 2006; Cunningham *et al*., 2005).

The reducing effect on the locomotion activities induced by LPS is very similar to that induced by *Valeriana officinalis* extracts that are able to induce a reduction of locomotor activity even at the low dose (Capasso *et al*., 1996). As already reported, LPS induced dose- and time-dependent decreases in locomotor activity, food intake, social interaction, and exploration for novel objects, and an increase in immobility in the forced-swim test. This suggests that LPS most prominently affects object exploratory behaviors by impairing cognition as we previously found (Ebaid *et al*., 2012) and/or motivation including continuous attention (Haba *et al*., 2012).

A significant increase in the lymphocyte count of the peripheral blood was observed in the current study. LPS affects the metabolic processes via action of endocrine and immune functions which controls metabolism by increasing the level of cortisone and cause high lymphocyte proliferative and increase the ratio of $CD3^+$ T cells, $CD4^+$T cells (Jian-tao *et al*., 2006). This may explain the clear depletion of the thymocyte population in the cortex of thymus gland. Thymus-derived the T-cell have important function of the recognition of the self antigens and the foreign (non-self) antigens which are represented in the current study by LPS. Thus, chemoattraction of the lymphocytes including T-cells to the peripheral blood is necessary to cellular and humoral immunity against LPS. Establishing a link between innate and specific immunity, interleukin-12 is an essential cytokine of the inflammatory response (Ethuin *et al*., 2004). The pro-inflammatory cytokines which is extensively increased by LPS (Suarez *et al*., 2002), mediated this lymphocyte migration to blood. On contrary, LPS-induced inflammation is accompanied by an increase of the phagocytic activity in particular, in the lymphoid organs. The immune response against a bacterial aggression involves the monocytes macrophages and polymorphonuclear neutrophils (PMN) in the first line of defense (Ethuin *et al*., 2004). The phagocytic activity in the thymic gland is usually represented by focal areas detected in the cortical region as found in this study. IL-12 potentiates the effect of LPS on the production of IL-8 by stimulated PMN, the main chemotactic and activating cytokine of neutrophils (Ethuin *et al*., 2004). This suggestion may explain the depletion of the neutrophil count in the peripheral blood and may indicate the strong inflammation caused by the LPS in the pups tissues.

Significant decreases were found in threat, attack and number of fights of pups born to LPS-treated mothers. In accordance, Abu-Taweel *et al*. (2010) found that aluminum exposure resulted in significant decline in aggression and levels of neurotransmitters. LPS challenge shares with psychological stress some common physiological adaptations (Gasparotto *et al*., 2007). Delay of aggression by LPS was proved by increasing the latency of aggression. Many studies showed a close relationship between testosterone and different behavioral changes, especially aggression (Schlinger and Callard, 1990; Zitzmann, 2006; Lynn, 2008). Animal studies provide a strong evidence of change, serotonergic neurotransmission, being associated with change aggression (Manuck *et al*., 2002; De Almeida *et al*., 2005; Ferrari *et al*., 2005). In addition, our previous data obviously revealed that the dopaminergic and serotonergic system was modulated by aluminum decreasing the aggression rate in adult mice (Abu Taweel *et al*., 2010). Thus, LPS may affect the testosterone level with some other neutransmitters such as acetylcholine, serotonin and dopamine.

Concerning the increase in lymphocyte number, Genhong *et al*. (2003) mentioned that androgens influence some immunological processes, including alternation of the number and function of the circulating lymphocytes and monocytes. Extracellular acetylcholine levels in the hippocampus are known to increase during stress (Imperato *et al*., 1991; Tajima *et al*., 1996; Mizuno and Kimura, 1997). It is logical that the decrease of blood parameters can directly affect the animal activities and in particular, the animal aggression behavior.

It took the pups born to LPS-treated dams an average of two days longer to develop a hair coat than the pups born to control dams (Lasala and Zhou, 2007). Additionally, the delay in hair appearance might be due to the delay in the collagen protein-synthesis by oxidation. The retardation in the eyes opening as it was attributed to an abnormality in the neural developments may be due to the total number of tyrosine hydroxylase immunoreactive (TH-IR), dopaminergic amacrine cells was reduced and the myelin sheath became thinner in LPS-exposed fetuses that results in alterations to the optic nerve (Loeliger *et al*., 2007).

This study, confirmed the placental transfer of LPS to the tissue of embryos from treated mothers in a prenatal exposure. LPS strongly affected the, locomotion**,** sensory motor reflexes, hair and eye development, and the histological architecture of the thymic gland . Thus, LPS is highly toxic to the tissue of embryos and based on this study and other previous studies (Ebaid *et al*., 2012; Jaiswal *et al*., 2006), we expect that LPS can

cause serious effects especially abnormal functioning of the nervous system and its sequential behavior.

ACKNOWLEDGMENT

The authors extend their appreciation to the Deanship of Scientific Research at king Saud University for funding the work through the research group project No. RGPVPP-028.

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(Received 6 September 2012, revised 6 February 2013)