Oxidative Stress-Induced Prenatal Exposure to Lipopolysaccharides Alters Active Avoidance Learning Behavior in Mice Offspring

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Abstract: The prenatal systemic inflammatory response caused by lipopolysaccharides (LPS) has been proposed to play important roles in the development of organ injury and behavior changes in neonates and in adult life. This study aimed to investigate LPS complications resulted in oxidative stress, impaired cognitive memory, and severely weakened learning processes in offspring. To acheive this aim, pregnant Swiss mice were intraperitoneally injected with LPS at a dose of 2.5 mg/kg of body weight at the 7th day of gestation. Behavioral parameters of offspring were investigated from the first to the thirtieth day ~~of~~ after birth. Results revealed that the endotoxin increased the oxidative stress, and decreased the anti-oxidant glutathione (GSH) in liver tissue of the pups born to LPS-treated mothers mediating the behavioral changes in those pups. The active avoidance training-test indicated that prenatal exposure to LPS was associated with learning impairment in offspring. In conclusion, LPS treatment of the mothers, influenced the passive avoidance performance in their pups suggesting that at least the adaptive response to a LPS-stressful experience that serve as a measure of learning and short-term memory was significantly distorted.
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1. Introduction

During fetal development, signals from the environment program the neuro-endocrine system and behavior of offspring. Prenatal stress, maternal inflammation or infection, and fetal malnutrition alter physiology and behavior in later life of the offspring (Tilders et al., 1994). LPS is a component of the outer membrane of Gram-negative bacteria, and is important to cause disease. LPS is believed to be the primary trigger of Gram-negative septic shock, and has been widely used in investigations of bacterial infection induced inflammatory response (Saluk-Juszczak and Wachowicz, 2005).

LPS stimulates several immune cells to secrete pro-inflammatory cytokines and nitric oxide that lead to septisemic shock. Nitric oxide may participate in mechanosensory processing (Ott et al., 2000; Liu et al., 2010). 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 MDA, and the suppression of the anti-oxidant glutathione (Ebaid et al., 2011; Ebaid et al., 2012). Endotoxemia by LPS has been reported to affect gut motility and persistently impaired gastrointestinal smooth muscle activity (Scirocco et al., 2010). Rats that prenatally LPS-injected produced a significant glutathione (GSH) reduction and increase in oxidized GSH and lipid peroxide (LPO) production (Zhu et al., 2007). Endotoxin-induced circulatory impairments may place the newborn brain at prolonged risk of cerebral endothelium and cerebral blood flow dysregulation and injury as a legacy of endotoxin exposure (Feng et al., 2010). Arimoto and Bing (2003) have demonstrated that inflammation induced by intranigral injection of LPS led to loss of nigral dopamine neurons and extensive activation of microglia. Ling et al., (2002) reported that rat exposed to LPS at embryonic day 10.5 revealed many characteristics seen in Parkinson's disease.

After inducing endotoxemia, here we investigated the behavioral changes in learning processes using the shuttle box in developing pups born to LPS-treated mothers to address whether these endotoxic complications are transferred to the offspring. Results in the current study suggests that endotoximea in mothers may contribute to increased susceptibility to altered offspring behavior in adult life.

2. 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 (CPCSEA) and the National Institutes of Health (NIH) 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 (light : darkness hours?). 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 were assigned to one of two groups: 1) the first group (how many?) was ~~a~~ pregnant control group (LPS-N) that was given phosphate buffered saline via an intra-peritoneal injection on the 7th day of gestation, 2) the second group (C-N) (how many?) was also pregnant ~~and~~ but received a single intra-peritoneal (IP) injection of bacterial lipopolysaccharide (LPS) at a dose of 2.5 mg/kg of body weight on the 7th day of gestation, too. 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

At the end of the experiment, animals were anesthetized with pentobarbital (60 mg/kg body weight) and samples (blood (volume?), the whole livers, and the whole brains) were obtained. Whole blood was drawn from the abdominal aorta. Half of the obtained blood was used to evaluate the complete blood picture and the differential count. Heparinized venous blood was centrifuged at 800 g, for 10 min, and plasma was stored at -20°C until analysis.

Glutathione activity upon LPS injection

Glutathione (GSH) assay was carried out on tissues according to Clark *et al*., (2010). Briefly, livers and brains of the tested animals were removed and gently rinsed in physiological saline (0.9% NaCl), and then blotted ~~in~~ on whatman filter paper. The organs fresh weights were recorded ~~and~~ before they were frozen at -20°C until use. 10% of the weight of each organ was homogenized and centrifuged, and the supernatant was used for oxidative stress evaluations. The supernatant was boiled to precipitate and deactivate other proteins. (Clark *et al*., 2010; Beutler *et al.*, 1963 and Zou *et al.,* 1986). GSH concentrations were then measured by adding 100 µl of boiled supernatant to 400 µl PBS [containing 200 mM MCB and 2U/ml glutathione S-transferase (per 100 µl)]. GSH concentrations were then determined by measuring the absorbance (OD) of the reaction mixture after 1 min at 340nm using ~~an~~ a UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech). GSH standards were measured concurrently to obtain a standard curve that was used to calculate GSH concentrations in samples. Results were expressed as ~~µg~~ GSH in µg /g tissue. Statistical comparisons of GSH activities between controls and treatments in each case were performed using Minitab statistical program as detailed below.

Determination of Lipid peroxidation upon LPS injection

Endogenous lipid peroxidation in homogenate was estimated spectrophotometrically following the method described by Okhawa *et al.*, (1979) expressed in nano-moles of malondialdehyde (MDA) per milliliter homogenate (nmole/ml). Tissues were homogenized and centrifuged as mentioned above in glutathione method. An amount of 0.5 ml of the resulting supernatant was shaken with 2.5 ml of 20% trichloroacetic acid (TCA). To the resulting mixture, 1 ml of 0.67% thiobarbituric acid (TBA) was added, shaken, and ~~wormed~~ warmed for 30 min in a boiling water bath and followed by immediate rapid cooling in ice for 5 min. After cooling, 4 ml of n-butyl-alcohol was added and shaken well. The resulting mixture was then centrifuged at 16,000g for 5 min. The resultant n-butyl-alcohol layer was moved into a separate tube and MDA content was determined spectrophotometrically at 535 nm using an UV Visible Spectrometer (Ultrospec 2000, Pharmacia Biotech).

Learning and memory test in automatic reflex conditioner (shuttle box)

Animals of PD35 were tested using an automated shuttle-box (Ugo basile, Italy). The shuttle-box was divided into two chambers of equal sizes with a gate providing access to the adjacent compartment. A light of 60 W for 5 sec was switched alternately in the two compartments and used as a conditioned stimulus (CS), which preceded an electric shock (1 mA for 5 sec) by 5 sec called the unconditioned stimulus. ~~If~~ Whenever the animal avoided the unconditioned stimulus by running into the dark compartment within 5 sec after the onset of the CS, the shuttle box recorded an avoidance response. Each mouse was given 20 trials daily with a fixed intertribal interval of 20 sec. Without shocks, the number of crossings between the chambers was recorded (inter-trial crossing). The automated shuttle box recorded this parameter during the whole experimental period of 20 trials (Hacioglu et al., 2003).

Statistical Analysis

Statistical analysis is undertaken using MINITAB software (MINITAB, State College, PA, Version 13.1, 2002). Data from MDA, glutathione activity and differential counts were first tested for normality (using Anderson-Darling Normality test) and for variances homogeneity prior to any further analysis. Data were normally distributed and thus, one-way ANOVA was used to determine overall effects of treatments followed by individual comparison using Tukey's Pairwise comparison. The data obtained from grip strength, Learning test was compared within the experimental groups by the analysis of variance (ANOVA) using minitab computer program. Data were subsequently analyzed by Student's t-test (Yamane, 1973) for biochemical analyses and Mann-Whitney U tests (Sokal and Rohlfe, 1981) for behavioral analyses. Values of $P > 0.05$ are considered statistically non-significant, while values of $P < 0.05$ and $P < 0.01$ were considered statistically significant and very significant, respectively.

3. Results

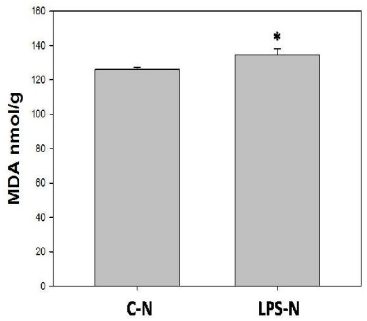

Fig. 1: Malondialdehyde (MDA) in liver homogenates of newborns from control mother (C-N) and LPS-treated mothers (LPS-N). * shows statistically significant differences at $P < 0.05$.

Pregnant mice treated with low doses of LPS gave birth to normal numbers of pups. However, behavior of the offspring was altered. Results of the active avoidance test in shuttle box showed impairment of spatial learning and memory in male offspring born to LPS-injected dams. Detrimental effects of the mother LPS-induced inflammatory response on their newborns in mice models have been applied in this study to mimic Gram-negative infections. In the current study, a single dose of 2.5 mg/kg of body weight ~~at~~ on the 7th day of gestation was used.

During gestation, oxidative stress affected the embryonic developments and organogenesis, which appeared in the delay of different bioactivities of the born pups. Interestingly, we found an increased oxidative stress represented in a significant increase of MDA levels in the liver tissues of pups born to LPS-treated mothers (Fig. 1). Furthermore, glutathione level was significantly suppressed in the liver of pups born to LPS-treated mothers as well (Fig. 2).

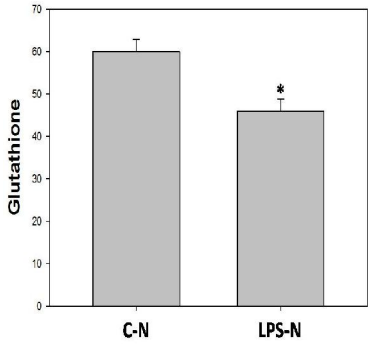

Fig. 2: Glutathione in liver homogenates of newborns from control mother (C-N) and LPS-treated mothers (LPS-N). * shows statistically significant differences at $P < 0.05$.

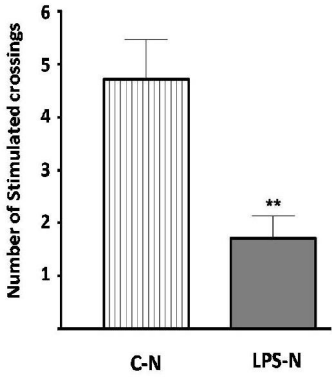

Fig. 3: Number of stimulated crossings of newborns of control mothers (C-N) and the newborns of LPS-treated mothers. LPS was found to significantly suppress the stimulated crossings of LPS-N comparing to C-N. ** shows statistically significant differences at $P < 0.01$.

We studied consequences of behavioral changes on the course of pregnancy after injecting a single dose of 2.5 mg/kg of LPS at the 7th day of gestation. To investigate learning process and memory in shuttle box, a light was switched on and off alternately in ~~the~~ two compartments, and used as a conditioned stimulus, which preceded an electric shock (unconditioned stimulus). The shuttle box recorded an avoidance response whenever the animal avoided the unconditioned stimulus, by running into the dark compartment within 5 sec after the onset of the conditioned stimulus. The ability of the tested animals for learning was addressed. Results of the

ability to learning showed that LPS has impaired the avoidance conditioning in the shuttle box. Impairment of active avoidance learning in pups born to LPS-treated mothers groups was clearly observed in different parameters. The pups born to non-treated mothers (control group) learned to avoid the unconditioned stimulus by running into the other compartment during the unconditioned stimulus on almost all days of the experiment and recorded high success rates of avoidance response (Fig. 3). The pups born to LPS-treated mothers did not learn to avoid the unconditioned stimulus and the number of crossings during the conditioned stimulus decreased over time during the experiment. Therefore, LPS was found to significantly suppress the stimulated crossings of LPS-N comparing to C-N (Fig. 3).

Furthermore, LPS was found to significantly suppress the re-enforced crossings of LPS-N pups comparing to C-N pups (Fig. 4). In accordance, figure 5 shows that pups born to LPS-treated mothers showed a significant increase of the latency to avoid the shocks compared to pups born to non-treated mothers, which recorded a short time to avoid the shock over time during the experiment (Fig. 5).

Without shocks, the number of crossings between the chambers were recorded (inter-trial crossing). Moreover, it was found that the number of inter-trial crossings of newborns of LPS-treated mothers was very significantly increased compared to the number of inter-trial crossings of pups born to control mothers (C-N) (Fig. 6) indicating a significant delay in learning process and a clear disturbed behavior.

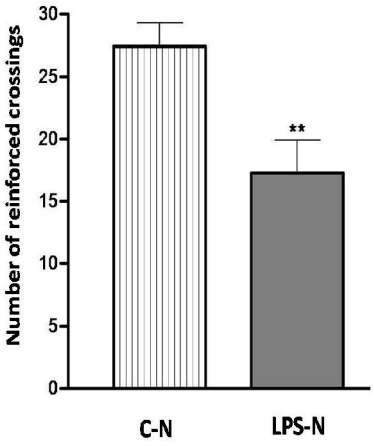

Fig. 4: Number of reinforced crossings of newborns of control mothers (C-N) and the newborns of LPS-treated mothers. LPS was found to significantly suppress the reinforced crossings of LPS-N pups comparing to C-N pups. ** shows statistically significant differences at P < 0.01.

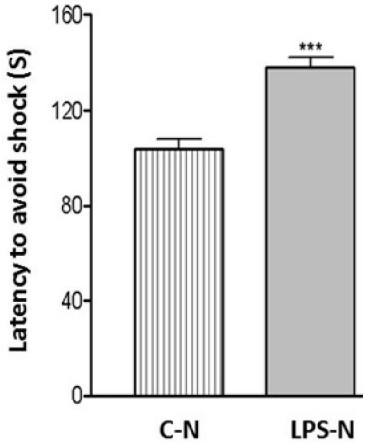

Fig. 5. The latency to avoid electric shock in a shuttle box. A light was switched alternately in the two compartments and used as a conditioned stimulus which preceded an electric shock by 5 sec called the unconditioned stimulus. If the animal avoided the unconditioned stimulus by running into the dark compartment within 5 sec after the onset of the conditioned stimulus, the shuttle box recorded an avoidance response. Pups were tested to determine the time needed to learn how to avoid the electric stimulus by running into the other compartment during an unconditioned stimulus. Results are presented as a mean and a standard errors (SE); ** shows statistically significant differences at $P < 0.01$ when treated compared with control animals.

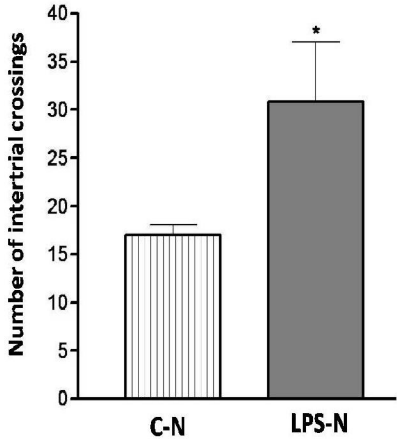

Fig. 6: Number of inter-trial crossings of newborns of control mothers (C-N) and the newborns of LPS-treated mothers. Without shock, the number of crossings between the chambers was recorded (inter-trial crossing). LPS was found to significantly increase the time to the inter-trial crossings of LPS-N indicating a significant delay in learning process. * shows statistically significant differences at P < 0.05.

4. Discussion

LPS treatment, in this study, did influence the passive avoidance performance, thus suggesting that at least the adaptive response to a LPS-stressful experience that serves as a measure of learning and short-term memory was significantly modified in newborns of treated mothers. This impaired learning and memory was mediated by oxidative stress, which was significantly increased by LPS.

Defense against pathogens is crucial, and involves antibacterial proteins, polysaccharides and other immune-related molecules. Although ~~reactive~~ ROS and free radicals are used by the body as cytotoxic materials against invading pathogens, they can also cause oxidative stress, leading to cellular damage. Cellular responses to oxidative stress are of interest to investigators of a wide variety of human diseases such as cancer, diabetes, atherosclerosis, stroke, Alzheimer's, many auto-immune diseases, and aging (the above statements need to be cited to some references). The current study aimed to obtain further evidence of oxidative stress as a pathogenic mechanism for LPS and its effects on the offspring. Thus, we studied oxidative stress and active avoidance and learning in the pups, in response to mother-LPS.

Although different cellular stresses may have different initial responses, the final pathways are often similar. The mechanisms of cell injury are ATP depletion, disturbed calcium homeostasis, oxidative stress and increased membrane permeability. Oxidative stress, which leads to DNA damage, is a primary driver leading to the accumulation of mutations, which occurs in living organisms. Bautista et al. (1990) observed an increase in O^{2-} generation by blood monocytes and PMN after administration of bacterial endotoxin. Seres et al. (2000) stated that the elimination of invading microorganisms depends on the generation of ROS during the phagocytosis-associated respiratory burst. The oxidants created in this process therefore carry the potential to damaging the phagocytes themselves as well as other cells at sites of inflammation (Henson and Rjb, 1987). Thus, bacterial agents activate neutrophils to secrete NO.

Here we found an increased oxidative stress represented in a significant increase of MDA level and a significant decrease of glutathione level in the liver tissues of pups born to LPS-treated mothers. Previously we had an increased oxidative stress in the tissues of mothers exposed to LPS during gestation period (Ebaid et al., 2012). This clearly indicates that during gestation, oxidative stress affected the embryonic developments and organogenesis and then ~~continue~~ continued with high levels in newborns.

We have previously induced endotoxemia in adult mice using whole bacteria, and observed markedly increased NO synthesis, and decreased catalase, total peroxidase and superoxide dismutase activities (Ebaid and Abdel-Salam, 2006). With LPS, (Ebaid et al., 2012) observed markedly increased MDA synthesis, and decreased glutathione. ROS are radicals that can cause cellular damage such as lipid peroxidation, which disrupts membrane fluidity; degradation products can initiate cellular apoptosis (Horton and Fairhurst, 1987; Halliwell and Gutteridge, 1999; Kannan and Jain 2000; Lopes et al., 2008; Golbidi and Laher, 2010). In the current study, oxidative stress is a potential cause of the impairment of various biochemical reactions in the body of both mothers and offspring. We found that MDA was significantly elevated in developing pups born to LPS-treated mothers. Similar results were observed by Yazar et al., (2010) who found that LPS increased MDA and organ damages markers. Functional studies in mammals have shown that glutathione prevents lipid peroxidation and protects bio-membranes against oxidative stress (Ursini et al., 1982). This is because glutathione is an antioxidant enzyme that can directly reduce peroxidized phospholipids and cholesterol within membranes (Ursini et al., 1985; Thomas et al., 1990). Based on these studies, the observed significant increase of lipid peroxidation after LPS treatment in the current study was associated with LPS-mediated suppression of glutathione production in the body of the treated mice and their pups. Thus, an oxidative imbalance (the formation of reactive oxygen species together with the depletion of GSH content) is the result of exposure to LPS, as has previously been shown (Scirocco et al., 2010). In the present study, total levels of the anti-oxidant enzyme glutathione were significantly reduced in pups from LPS- treated mothers.

Results of the active avoidance test in shuttle box showed impairment of spatial learning and memory in male offspring born to LPS-injected dams. We proved that oxidative stress was transferred and that it continued in tissues of pups born to mothers treated with LPS during gestation. It is more likely that oxidative stress not only affected the embryonic developments and organogenesis but also delayed different bioactivities of the born pups. The active avoidance training-test indicated that LPS exposure during gestation was associated with learning impairment. Similar results were obtained for gestational exposure with aluminum (Abu-Taweel et al. 2010) and selenium (Ajarem et al., 2011; Al-Basher et al., 2011). A growing body of evidence suggests that reactive ROS play a crucial role in the development of these impairments (Yamada et al., 1995; Kucukatay et al., 2007). The increased production of oxidants altered neurotransmitter

release and increased membrane permeability (Gupta et al., 1991). Besides, free radicals may degrade nitric oxide (NO), which plays an important role as a diffusible intercellular signaling molecule (a neurotransmitter in the central nervous system) (Geyer et al., 1997). Chlodzinska et al., (2011) found that, in the nest building test, adult mice born from LPS challenged pregnancies constructed worse quality nests, which points to the presence of hippocampal dysfunction. LPS was found to markedly induce brain inflammatory response, disturbed myelin basic protein expression, and lateral ventricle dilation (Pang et al., 2012). Deng et al., (2012) examined the expression of Pir-B in the rat brain following intra-cerebral application of LPS. They indicated that hippocampus-dependent spatial learning and memory were impaired in LPS-treated animals. Their findings added new data for ~~an~~ up-regulation of immune proteins in neuronal and glial cells in the brain in a model of endotoxin-induced neuro-inflammation, synaptic alteration, and cognitive decline. Cloutier et al., (2012) suggested that learning impairments by bacterial endotoxin treatment may have stronger central effects on learning and memory behavior, relative to peripheral effects on body weight and other sickness-related responses. Furthermore, data obtained by Choi et al., (2012) showed that obovatol mitigates LPS-induced amyloidogenesis and memory impairment via inhibiting NF-κB signal pathway. The current data are further confirmed by the results obtained by Lee et al., (2008). They found that intra-peritoneal injection of LPS, induced memory impairment determined by passive avoidance and water maze tests in mice. This study suggests that neuro-inflammatory reaction could contribute to Alzheimer's disease pathology, and anti-inflammatory agent could be useful for the prevention of Alzheimer's disease.

Cytokines play a role in the pathogenesis of LPS. Changes in brain cytokines of fetuses depend on the gestational day of LPS administration and on its dose. The level of cytokines in the placenta and amniotic fluid is increased starting from 2 to 24 hours after treatment of pregnant rats with high doses (2.5 mg/kg) of LPS (Urakubo et al. 2001, Ning et al. 2008). Two cytokines are particularly important: IL-1 and TNF-a. Although we did not address the cytokine increase in this study, it is known that oxidative stress initiated the pro-inflammatory cytokine secretion especially IL-1 and TNF-a. In critically ill patients with sepsis syndrome or septic shock, the transient overproduction of pro-inflammatory cytokines likely contributes to manifestation of the systemic inflammatory response, development of organ failure, and even death (Kotb and Calandra, 2003).

Data of the present study indicated a strong and persistent impairment in learning activity in newborns exposed to LPS during embryogenesis, and possibly, in the functioning of neuronal pathways governing this task. Endotoxin-induced elevation of oxidative stress and suppression of glutathione may place the embryos and the developing pups at prolonged risk of tissue damage by free radicals. The imbalance of the oxidative stress response may lead to major subsequent damage in a variety of tissues in mothers and pups. Taken together, these results showed a positive correlation between oxidative stress imbalance resulting from endotoxemia, and impairment of learning process of the pups born to LPS-treated mothers.

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