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The immunologic and antioxidant effects of L-phenylalanine on the uterine implantation of mice embryos during early pregnancy

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Summary. L-phenylalanine (L-PHE) is a synthetic precursor of catecholamines. Because it cannot be synthesised by an organism, it must be absorbed from the environment. Despite the wide use of L-PHE, whether L-PHE has a negative impact on embryo implantation and development is poorly understood. This study attempted to determine the roles of L-PHE in embryo implantation and development and in the immune response and antioxidant status of the uterus in early pregnancy mice injected intraperitoneally with 320 mg/kg L-PHE. The embryo number of treated mice decreased by 57.6%, and the size of their embryos was reduced by 2.8% ($P>0.05$) along the long diameter and 11.9% ($P<0.05$) along the short diameter at E9 compared with control mice. In addition, L-PHE significantly suppressed B lymphocyte proliferation. L-PHE increased IL-2 secretion but decreased the IL-4 concentration, thereby up-regulating the ratio of IL-2/IL-4 to 1.37-8.45. An analysis of the oxidant and antioxidant status showed that, compared with the control mice, the level of superoxide dismutase activity decreased by 21.54%-39.94% and the glutathione peroxidase activity decreased by 15.27%-18.96% among the L-PHE-treated mice at E1-E9. However, the malonaldehyde content increased by 14.29%-90.11% among the L-PHE-treated mice. Therefore, L-PHE impaired embryo implantation by disrupting cytokine-based immunity and oxidative stress in the uterus.

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Introduction

L-phenylalanine (L-PHE) is the synthetic precursor of catecholamines (Grando et al., 2006; Fernstrom and Fernstrom, 2007) and can be transformed into norepinephrine and epinephrine via dopa decarboxylase (DDC). DDC is found in the central nervous system and in peripheral tissues and organs, such as the vascular wall, stomach, kidney and liver. Because L-PHE cannot be synthesised by organisms and must be absorbed from the environment, it is widely used as a component of aspartame, a medical intermediate, and as an additive in human food and animal forage. However, we have no specific knowledge as to whether side effects emerge when an organism continually absorbs L-PHE, particularly among embryos exposed to L-PHE. The fate of an embryo is determined by specific factors, including its mother's immune responses (Bansal, 2010). The immune responses of the maternal uterus may play an important role in determining whether a pregnancy will be successful (Wegmann et al., 1993; Ho et al., 2001; Piccinni, 2003). Thus, studies of the mechanisms underlying the immunological modulation of the uterus during pregnancy are important for identifying the causes of embryonic loss. During early pregnancy, oxidative stress aids in the development of the placenta and chorion, and the expression levels of antioxidant enzymes increase. These enzymes include catalase (CAT), superoxide dismutase (SOD) and glutathione

peroxidase (GSH-Px), each of which can protect cells from damage due to oxidative stress. Under pathological circumstances, excessive oxygen radicals may occur suddenly, resulting in their accumulation and, ultimately, toxicity (Rao et al., 2003). To date, whether L-PHE plays a role in the regulation of uterine immunity and antioxidant ability remains poorly understood. In the current paper, we investigated the role of L-PHE in the uteri of mice during early pregnancy on embryonic implantation and the particular development of uterine lymphocyte proliferation, cytokine production, antioxidant status and lipid peroxidation.

Materials and methods

Animal treatment

Eighty female (25-32 g) and 20 male (35-40 g) Institute for Cancer Research (ICR) mice were purchased at 8 weeks of age from Vital River Laboratory Animal Technology (Beijing, China). After an adaptation period of 1 week, 40 female mice were injected intraperitoneally for 7 consecutive days with 0.3 mL of a solution containing 320 mg/kg body weight (BW) of L-PHE (Beijing BioDee BioTech Corporation Ltd, Beijing, China), diluted in sterile saline. The remaining control females were treated with vehicle (0.3 mL). After completion of the treatments, the females were paired daily with males of proven fertility following the examination of vaginal smears (Wright's staining). On the following day, the females were confirmed to be pregnant by the presence of a vaginal plug or by vaginal smear, and the day was designated as embryonic day (E1). The mice were sacrificed under deep anaesthesia with Nembutal (50 mg/kg BW) at E1, E3, E5, E7 or E9 on a sterile bench. Uterine samples were immediately removed from the abdominal cavity. The embryo number was counted, and the embryos were removed. All experimental protocols were performed in accordance with the Guidelines for Animal Experimentation of China Agricultural University.

Lymphocyte preparation from isolated uteri

All left uterine horns without embryos were minced into small pieces and passed through a tissue sieve (200 mesh) to prepare single-cell suspensions in 3 mL of Hank's solution. The suspension was layered onto 3 mL of lymphocyte separation medium (Tianjin Blood Research Centre, Tianjin, China) and separated by density-gradient centrifugation at 1,500g for 30 min at room temperature. The lymphocyte precipitates were resuspended in 3 mL of RPMI 1640 base medium (Gibco BRL, Grand Island, NY, USA) and centrifuged twice at 1,000g at 4°C for 10 min. Lymphocytes were resuspended in 0.5 mL of RPMI 1640 complete medium (Gibco BRL), and the cell concentrations were adjusted to 2×10^5 cells per mL. Cell viability was determined by the 0.1% trypan blue dye exclusion test, and the cells were counted.

Lymphocyte proliferation assay

Mitogen-induced lymphocyte proliferation was selected as an *in vitro* index of immune function. Concanavalin A (ConA)- or lipopolysaccharide (LPS)-induced lymphocyte proliferation was assayed using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay with some modifications. Each cell suspension sample was assigned to ConA (Sigma-Aldrich Co., St. Louis, MO, USA) or LPS (Sigma-Aldrich Co. , St. Louis, MO, USA) treatment and control wells, with three sequential triplicate wells for each combination. Briefly, the 100 μL cell suspension was added to the individual wells of a 96-well microtitre plate (Corning, NY, USA). ConA (or LPS) and RPMI 1640 complete medium were added to the wells containing plated cells to yield the ConA (or LPS) treatment and control wells. The final ConA concentration for both and LPS was 15 μg/mL, and each well was filled with RPMI 1640 complete medium to a final volume of 120 μL. The plates were then incubated at 37°C in 5% CO_2 for 72 h. Subsequently, 10 μL of MTT solution was added to each well to achieve a final concentration of 5 mg/mL in PBS (pH 7.4). The plates were incubated at 37°C for another 4 h. Following incubation, 100 μL of 10% sodium dodecyl sulphate (Shanghai Chemical Factory, Shanghai, China) in 0.01 M HCl was added to lyse the cells and solubilise the MTT crystals. The plates were read at 570 nm using an automated microplate reader (Bio-Rad, USA). The stimulation index was calculated for each sample as the absorbance value for the cells, with the absorbance values for the mitogens divided by the values for the cells without mitogens.

Cytokine measurement by ELISA

All uterine tissues without embryos were homogenised in 10% wt/vol 0.01 M PBS (pH 7.4, 4°C) and then centrifuged at 1,500 rpm for 20 min at 4°C. The supernatants were collected and used to measure the concentrations of interleukin 2 (IL-2) and IL-4 with ELISA kits (Boster Biological Technology, China). The kit sensitivity was 15.6 pg/mL for IL-2 and 7.8 pg/mL for IL-4. Briefly, 100 μL of the samples or standards was applied to polystyrene microplates coated with a specific anti-mouse IL-2 or IL-4 monoclonal antibody. After incubation for 90 min at 37°C, the plates were washed twice with PBS containing 0.05% Tween-20. Next, 100 μL of biotinylated anti-cytokine antibody (anti-IL-2 or anti-IL-4, 1:100) was added for a 60 min incubation at 37°C. After six washes, the plates were treated with 100 µL of avidin-biotin-peroxidase complex (ABC, 1:100) for 30 min at 37°C and incubated with 0.01% 3,3',5,5'-tetramethylbenzidine (TMB, Sigma) substrate plus hydrogen peroxide (0.003%) for 10-15 min at 37°C. After adding 100 µL of 2 N sulphuric acid (H_2SO_4), a reading was taken at 450 nm using an ELISA reader

(Bio-Rad, USA). To determine the protein concentration, the homogenised solutions were measured by UV/VIS (Secoman, French). The concentrations of the cytokines are presented as pg/mg of protein.

Measurement of antioxidant status and lipid peroxidation

The endogenous antioxidant components SOD, GSH-Px and MDA, which is an end product of lipid peroxidation, were analysed by kinetic and spectrophotometric methods. After removing the embryos, uterine samples were homogenised with ice-cold PBS (pH 7.4) and centrifuged at 3,000g for 15 min, and the supernatant was used immediately to assay the SOD, GSH-Px and MDA levels using the available commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China). The SOD assay was conducted on the basis of the sample's ability to inhibit the oxidation of oxyamine through the xanthine-xanthine oxidase system. GSH-Px activity was detected by the consumption of glutathione, and MDA content was measured by the thiobarbituric acid colourimetric method. Values were calculated using optical density (550 nm for SOD, 412 nm for GSH-Px and 532 nm for MDA) according to the formula described in the manufacturer's instructions. The results are expressed as follows: units (U) per mg protein for SOD and GSH-Px and nmol/mg protein for MDA.

Statistical analysis

All results are expressed as the means ± SD, and statistical analyses were performed with ANOVA or independent samples t-tests using SPSS 16.0. P values of less than 0.05 were considered significant.

Results

Changes in the embryo number and developmental size

The embryos were distinct within the uterus at E7 and E9, and the uterus had a beaded appearance (Fig. 1). In control groups, there were 13.8±1.8 embryos per litter at E7, but this number was reduced to 7.6±3.0 at corresponding days in treated mice, a decrease of 44.9% (P<0.05), and a similar decline of 57.6% (P<0.05) at E9 was observed in treated group. In addition, the embryos of the treated group developed poorly. The long diameter (LD) and short diameter (SD) of the control group embryos were 4.38±0.22 mm × 2.89±0.09 mm at E7 compared with 3.99±0.23 mm × 2.45±0.10 mm in the treated group embryos, with significant decreases of 8.9% in the LD and 15.2% in the SD. The embryos were 4.76±0.12 mm × 3.30±0.13 mm at E9 in the treated group, with decreases of 2.8% (P>0.05) in the LD and 11.9% (P<0.05) in the SD compared with control embryos (Table 1).

Table 1. Changes in embryo numbers and developmental sizes.

	L-PHE-treated group		Control group	
	E7	E9	E7	E9
Number	7.6±3.0**	5.6±2.7**	13.8±1.8	13.2±1.2
Long Diameter (mm)	3.99±0.23*	4.63±0.10	4.38±0.22	4.76±0.12
Short Diameter (mm)	2.45±0.10*	2.67±0.10**	2.89±0.09	3.03±0.13

*: indicates a significant difference between the treated and control groups (P<0.05); **: indicates an extremely significant difference between the treated and control groups (P<0.01). (mean ± SD, n=5).

Effect of L-PHE on lymphocyte proliferation

ConA-induced lymphocyte proliferation

As shown in Fig. 2A, the proliferation of uterine lymphocytes induced by ConA showed no significant changes (P>0.05) at E1 through E9 within the same group, although the proliferation was lowest at E5 in both group.

LPS-induced lymphocyte proliferation

As shown in Fig. 2B, LPS-induced proliferation was decreased among uterine lymphocytes at E5 compared with E1 and E3 (P<0.05) and was increased at E7 and E9 compared with E5 in the control group during early pregnancy. When compared with the corresponding control mice, the LPS-induced proliferation of the uterine lymphocytes decreased to 0.90±0.07 (E5) - 0.99±0.04 (E3), with significantly decreased proliferation at E1, E7 and E9 (P<0.01).

Lymphocyte-secreted cytokine activity in uterine tissue homogenates

IL-2 secretion from lymphocytes

During early pregnancy in the control group, the concentration of IL-2 secreted from the uterine tissue homogenate was lowest at E1 (11.13±2.42 pg/mg), increased to its highest concentration at E5 (43.64±8.69 pg/mg) and decreased to 14.37±1.74 pg/mg at E9 (Fig. 3). Compared with control group mice, the concentration of IL-2 secreted from the uteri of the treated group was significantly increased by 162.17% at E1, 113.02% at E3, 98.61% at E5, 60.06% at E7 and 37.46% at E9.

IL-4 secretion from lymphocytes

In the control group, the concentration of IL-4 was the lowest at E3 (12.90±3.06 pg/mg), it then increased to its highest concentration at E5 (25.83±0.69 pg/mg) and decreased to 18.66±1.75 pg/mg at E9. Compared with the control group, the IL-4 concentration was significantly decreased in the uteri of treated mice by 39.94%, 16.08%, 51.66%, 65.80%, and 41.72% from E1

to E9, respectively. Comparing the two groups, the ratio of IL-2/IL-4 was lower in the control groups (0.6-2.85) and increased to 137-8.45 in the treated group during early pregnancy.

Oxidant and antioxidant status

SOD activity

In the control mice, the SOD activity of the uterine tissue increased from E1 to E9, except on E5, reaching its highest level at E9 (Fig. 4). The level of SOD activity in L-PHE-treated mice decreased by 29.66%, 21.99%, 21.54%, 38.65% and 39.94% at E1, E3, E5, E7 and E9, respectively ($P<0.01$).

GSH-Px activity

When mice were treated with L-PHE, the GSH-Px activity decreased by 16.17% ($P<0.01$), 18.96% ($P<0.01$), 18.11% ($P<0.05$), 15.27% ($P<0.05$) and 17.11% ($P<0.05$) compared with control mice at E1, E3, E5, E7 and E9, respectively.

MDA content. In the control group, the MDA content of the uterine tissue increased from E1 to E5 before decreasing from E5 to E9; thus, it peaked at E5

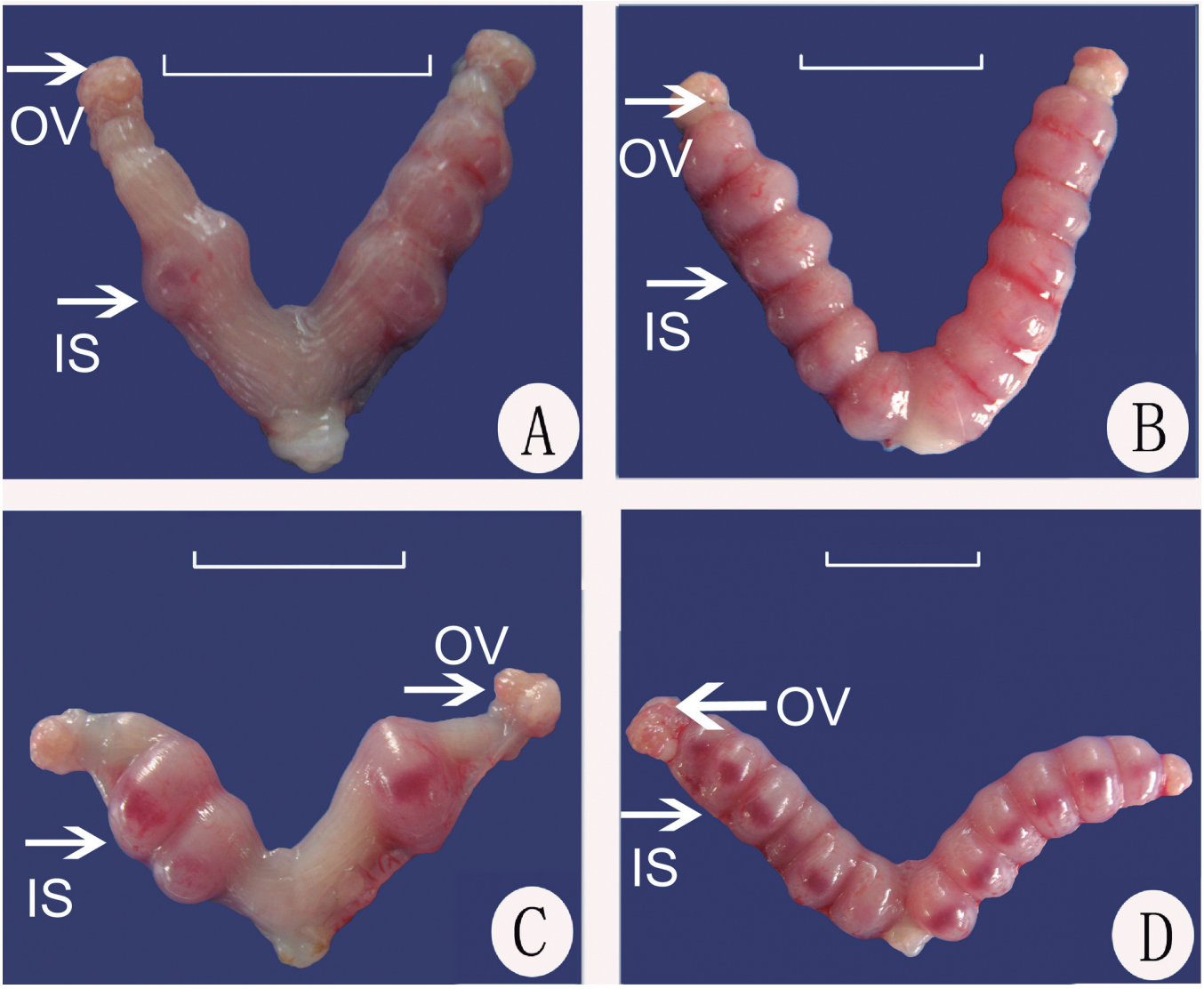

Fig. 1. The morphology of uterus in L-PHE-treated and control pregnant mice. Mice have a bicornuate uterus consisting of two lateral horns, in which the embryos implant. The number of embryos decreased in L-PHE-treated mice (**A**, the seventh day after pregnancy, E7; **C**, the ninth day after pregnancy, E9) compared with the control group, and the embryos were arranged regularly and tightly in the control group (**B**, E7; **D**, E9). IS: implantation site; OV: ovary. Scale bar: 1 cm.

(1.96±0.067 nmol/mg). In the L-PHE-treated group, the MDA content of the uterine tissue was higher than that in the control groups, increasing by 29.69% (P<0.05), 21.12% (P<0.05), 14.29% (P<0.05), 90.11% (P<0.01) and 39.24% (P<0.05) at E1, E3, E5, E7 and E9, respectively.

Discussion

As a widely used additive in human food and animal forage, some researchers have reported that L-PHE has a positive effect on physical function. For example, L-PHE plays a role in controlling blood pressure (BP), and phenylalanine prevents the increases in BP that can occur with increasing age and heart weight (heart/body weight index) (Zhao et al., 2001). Furthermore, this additive exerts a direct and specific anti-proliferative effect on vascular smooth muscle cells, which suggests that this effect may also explain the antihypertensive action of this amino acid that has been observed in spontaneously hypertensive rats (Gao et al., 1998). However, this amino acid is associated with some risks. Pregnant women have been advised to be careful about the total amount of phenylalanine that they ingest. Doses higher than 5,000 mg of the amino acid can cause marked nerve damage in the embryo. Therefore, the negative effects of L-PHE must be studied, particularly during pregnancy. Toxicological hazard assessments have utilised the existing classical test paradigms and a host of innovative approaches (Piersma, 2013). In the current paper, the roles of 320 mg/kg of BW L-PHE on mouse embryo implantation and development were explored based on the state of local immunity and oxidative stress in the mouse uterus. We initially observed that 320 mg/kg of BW L-PHE, injected intraperitoneally for seven consecutive days before pregnancy, decreased the litter number and size at E7 and E9, whereas 80 mg/kg L-PHE did not.

There are several reasons why 320 mg/kg of BW L-PHE may reduce the litter size. First, previous results and those of the present study suggest that the implantation and development of the embryo are closely related to local immune reactions in the uterus (Wegmann, 1988; Wegmann et al., 1993; Dong et al., 2007). Changes in the total number and distribution of the maternal peripheral blood leukocyte subsets vary throughout the literature. For example, papers have reported a decrease (Matthiesen et al., 1995), no change (Fiddes et al., 1986), or an increase (Kuhnert et al., 1998; Luppi et al., 2002) in the presence of CD8+ T cells during pregnancy. Discrepancies also exist for the CD4+ T-cell subset, which has been shown both to not change (Sabahi et al., 1995; Kuhnert et al., 1998) and to decrease (Luppi et al., 2002; Watanabe et al., 1997) during pregnancy. The frequencies and counts of B cells also appear not to deviate during pregnancy (Watanabe et al., 1997; Kuhnert et al., 1998; Luppi et al., 2002). Therefore, we tested the immune parameters within the local uterus in the current study. The T lymphocyte proliferation induced by ConA reflected no significant difference among the L-PHE-treated mice, but the B lymphocyte proliferation index among the LPS-induced mice was significantly decreased compared with that in untreated mice, except at E3 and E5. These results suggest that L-PHE plays a greater regulatory role in humoral immunity than in cellular immunity, as further demonstrated by the ConA-induced T lymphocyte proliferation. As a catecholamine precursor (Grando et al., 2006; Fernstrom and Fernstrom, 2007), L-PHE may be associated with the activity of sympathetic nerves, which release catecholamine. T lymphocyte proliferation in the spleen is inhibited in adult rats with sympathetic nerve lesions that are treated with 6-OHDA (Madden et al., 1994a,b; Pacheco-Lopez et al., 2003). In 3-month-old rats, when splenic norepinephrine (NE) was maximally depleted, no alterations in spleen cell Con A-induced T cell proliferation were observed up to 15 days after sympathectomy; however, Con A-induced proliferation was reduced in sympathectomised animals by 21 days post-sympathectomy, and then no alterations in T cell function were observed in

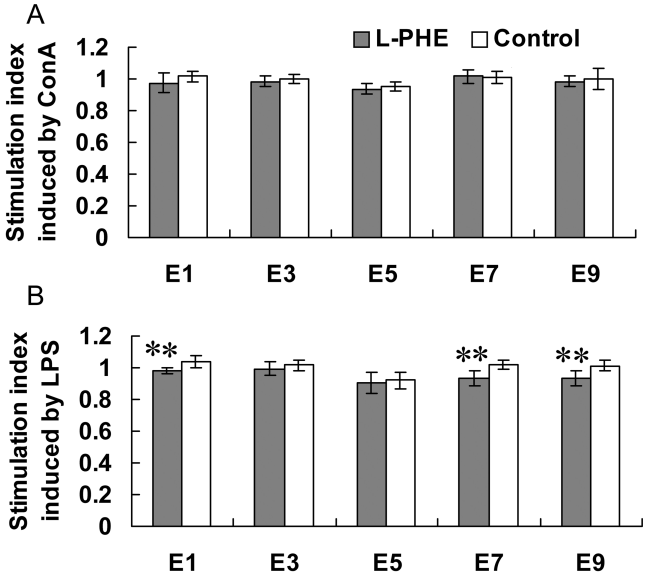

Fig. 2. Proliferation of lymphocytes from the uteri of L-PHE-treated and control mice. **A.** ConA stimulated the proliferation of T lymphocytes from the uteri of treated and control mice. There was no significant difference between the control and treated group, although the ConA-induced proliferative ability of lymphocytes from the uteri of treated mice demonstrated a slight decrease during early pregnancy, except for E7, compared with that of the corresponding control mice. **B.** LPS stimulated the proliferation of B lymphocytes from the uteri of treated and control mice. Note that the proliferation ability of lymphocytes from the uteri in treated mice was decreased to 0.90±0.07 (E5) - 0.99±0.04 (E3); lymphocyte proliferation was significantly lower at E1, E7 and E9 (P<0.01) when compared with the corresponding values in control mice. The values are expressed as the mean ± SD of 5 mice from each treatment on the day indicated. * and ** indicate significant differences (*P<0.05, **P<0.01) compared with the corresponding controls. The following figures are organised similarly.

sympathectomised animals after day 21 post-sympathectomy (Madden et al., 2000). NE, a catecholamine, increases B lymphocyte proliferation in normal mice and a mouse model of chronic mild stress (CMS), but it does not increase T lymphocyte proliferation in normal animals or decrease T lymphocyte proliferation in CMS model mice (Edgar et al., 2003). Based on the above results, we hypothesise that the L-PHE injections at the examined doses inhibit B lymphocyte proliferation during early pregnancy and have specific effects on lymphocyte proliferation at different ages and physiological statuses of mice as well as, on the sympathetic nerve system and catecholamine synthesis.

Lymphocyte activity is associated with lymphocyte proliferation and cytokine secretion. However, L-PHE can has diverse effects on lymphocyte cytokine secretion. The results of this study suggest that L-PHE alters the cytokine secretion pattern of uterine T cells during early pregnancy. When the local immune response in the uterine microenvironment was altered in L-PHE-treated mice, IL-2 production was significantly up-regulated; however, IL-4 production was down-regulated, resulting in a significant increase in the IL-2/IL-4 ratio. In contrast, in pregnant mice, Th1 cells primarily secreted IL-2 and IFN-γ, which are harmful to embryonic implantation and foetal development, whereas Th2 cells primarily secreted IL-4 and IL-10, which support pregnancy and foetal survival (Wegmann et al., 1993; Seder and Paul, 1994; Chaouat et al., 1995; Krishnan et al., 1996; Saito et al., 2010). Therefore, the increase in the IL-2/IL-4 ratio inhibits embryo implantation. Our results suggest that L-PHE regulates the balance of Th1 and Th2 cell in the uterus to

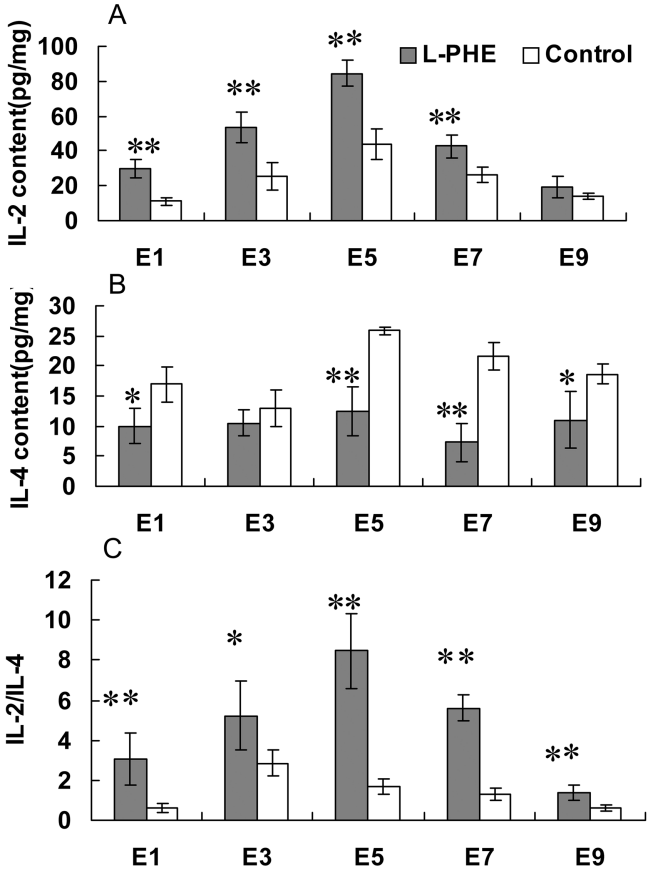

Fig. 3. Cytokine concentrations from the uteri of treated and control mice. Frequency histograms demonstrating the changes in the concentrations of cytokines, including IL-2 **(A)** and IL-4 **(B)**, and the IL-2/IL-4 ratio **(C)** of uteri from mice that underwent different treatments and were at different stages of pregnancy (n=5). In the L-PHE-treated group, the concentration of IL-2 was significantly increased, whereas the IL-4 concentration was decreased markedly, except on E3, increasing in the IL-2/IL-4 ratio compared with the control group during early pregnancy. * and ** indicate significant differences (*P<0.05; **P<0.01) compared with the corresponding controls.

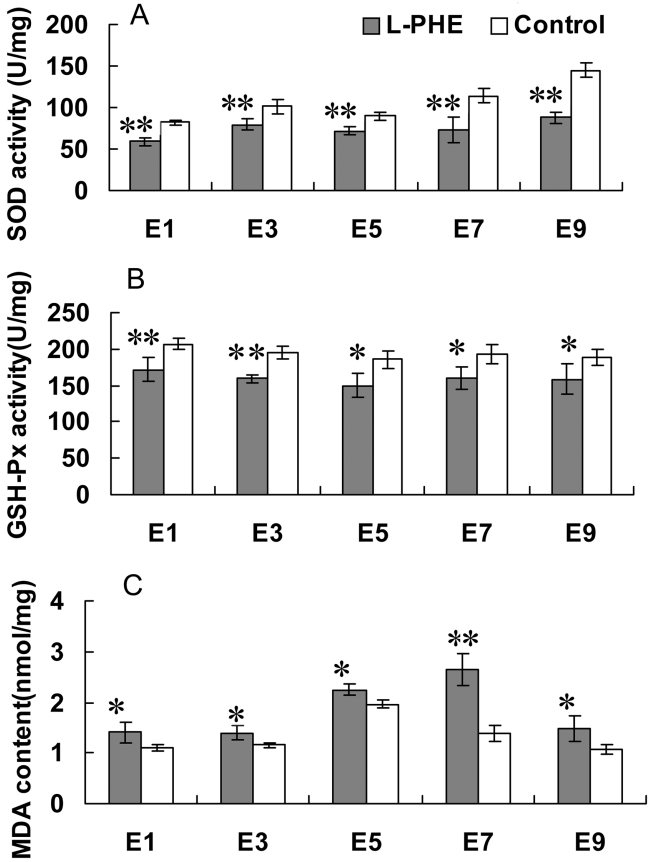

Fig. 4. The effect of L-PHE on the oxidative stress status within the mouse uterus during early pregnancy. Frequency histograms demonstrate the effect of L-PHE treatment on SOD activity **(A)**, GSH-Px activity **(B)** and MDA content **(C)** in the uteri of pregnant mice. In L-PHE-treated mice, the level of SOD activity decreased significantly by 21.54-39.94% and the GSH-Px activity decreased by 15.27-118.96% during early pregnancy. However, the MDA content of the uterus was higher in L-PHE-treated mice than in the control group, showing increases of 29.69% (P<0.05), 21.12% (P<0.05), 14.29% (P<0.05), 90.11% (P<0.01), and 39.24% (P<0.05) at E1, E3, E5, E7 and E9, respectively.

influence embryo implantation and development during early pregnancy, similar to previous findings showing decreases in norepinephrine secretion due to chemical sympathectomy (Dong et al., 2007). L-PHE is a neurotransmitter and is thought to have marked medical benefits, which should be transferrable to norepinephrine and epinephrine. L-PHE might activate sympathetic nerves, stimulating norepinephrine and epinephrine secretion. We deduced that the 320 mg/kg dose of L-PHE played a role in the local immune response in the uterus that was similar to that of chemical sympathectomy.

Lymphocyte activity has a close relationship with oxidative stress, and pregnancy is a physiological state that is marked primarily by oxidative stress (Casanueva and Viteri, 2003). Low levels of reactive oxygen species (ROS) are required for normal cell functions. However, elevated ROS production can cause oxidative stress and cellular damage, generating both physiologic and pathologic effects within the placenta (Burton, 2009), embryo (Symonds et al., 2007), and foetus (Herrera et al., 2012). Increased maternal oxidative stress in early human pregnancy is associated with preeclampsia, a shortened gestation duration and lower infant birth weight (Stein et al., 2008). Hansen reviewed a number of teratogens that elicit their deleterious effects on embryos through mechanisms involving the generation of ROS and oxidative stress (Hansen and Harris, 2013). Therefore, identifying the impact of L-PHE on intrauterine oxidative stress will be important for understanding cell-specific responses to L-PHE and for exploring the mechanisms of embryo implantation after exposure to L-PHE. In our study, the activities of SOD and GSH-Px were decreased ($P<0.05$) in the maternal uterus during early pregnancy after L-PHE treatment. Thus, the antioxidant function of the pregnant mouse uterus was impaired by L-PHE treatment. In contrast, the MDA content of L-PHE-treated uteri was increased compared with that found in the control group during early pregnancy ($P<0.05$). These results suggest that more free radicals were produced by L-PHE treatment, which may cause significant damage to cells. Our results demonstrated that L-PHE had a modulatory effect on maternal lipid peroxidation by adjusting the SOD and GSH-Px activities and MDA content during early pregnancy. Chemical sympathectomy has a similar effect on the SOD, GSH-Px and MDA activities in the spleen during early pregnancy (Bai et al., 2011). Macarthur et al. confirmed that oxidative stress is indeed involved in the changes that occur in sympathetic neurotransmission in hypertension (Macarthur et al., 2008).

Based on the current study, we speculate that L-PHE may regulate embryo implantation through the following routes. (1) L-PHE may regulate the proliferation of local B lymphocytes, instead of T lymphocytes, in the uterus. (2) L-PHE may modulate cytokine secretion, predominantly through the up-regulation of IL-2, which is harmful for embryo implantation, and through the down-regulation of IL-4, which is beneficial for the physiological state of pregnancy. (3) L-PHE may also attenuate oxidant stress states and antioxidant functions in the pregnant mouse localised uterus. A dose of 320 mg/kg L-PHE can decrease the SOD and GSH-Px activities, even while increasing the MDA content in the uterus during early pregnancy.

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Conflict of interest statement. The authors disclose that no conflicts of financial interest that could inappropriately influence the work.

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