Role of sympathetic nerves on early embryonic development and immune modulation of uterus in pregnant mice

Yulan Dong a, Yaoxing Chen a,⁎, Zixu Wang a, Jumpei Naito b, Ji-long Chen c

a Laboratory of Veterinary Anatomy, College of Animal Medicine, China Agricultural University, Haidian, Beijing 100094, China
b Department of Animal Science, School of Sciences and Engineering, Teikyo University of Science and Technology, Yamanashi 409–0193, Japan
c Department of Internal Medicine, College of Medicine, University of Iowa, Iowa City, IA 52242, USA

Received 24 October 2005; received in revised form 1 August 2006; accepted 2 August 2006

Abstract

To determine the role of sympathetic nerves in the early embryonic development and the immune modulation of maternal uterus during pregnancy, a model of chemical sympathectomy in mice was established by intraperitoneal injection of 6-hydroxydopamine (6-OHDA). The embryonic development and the distribution of maternal uterine immunocytes were investigated during early pregnancy (E1–E9) with methods of histology, immunohistochemistry and ELISA. Our data showed that in the 6-OHDA-treated group, the number of implanted embryos was only 64.4% of that in the control group at E7, and the development of uterine glands and vessels was poor in pregnant mice. In addition, in uterine tissues of 6-OHDA-treated mice, the number of CD8+ T cells increased ten-fold and the concentration of IL-2 increased 3.6-fold at E5. However, no obvious changes to the number of CD4+ T cells and IL-4 were observed. Thus, the CD4+/CD8+ T cells ratio significantly decreased, while the IL-2/IL-4 ratio significantly increased. These findings indicated that the activation of sympathetic nerves might be favorable to fetal survival and development during early pregnancy through influencing on immune function and decidua formation of uterus.

Keywords: Sympathetic nerve; Pregnancy; Uterus; T cell; Embryonic development

1. Introduction

In mammals, the embryonic and fetal deaths were recognized to be one of the main reasons that caused pregnancy loss (about 40%–60% of all pregnancy losses), while the early embryonic death (early embryonic loss or occult abortions) seemed to account for about 75%–80% of all embryonic and fetal deaths, and resulted in a substantial loss in production (Sreenan and Diskin, 1983). For example, about 25% of pregnancies fail in the first 3 weeks of gestation, representing multimillion pound losses to the industry (Peter, 1996). Furthermore, the early pregnancy loss is not only relevant to species with economical value, but also to humans (Aplin, 2005). Although the exact mechanisms of miscarriage are not fully clarified to date, it has been suggested that it may be associated with maternal immune responses (Talwar et al., 1997). The immune responses of maternal uterus may play an important role in successful pregnancy and pregnancy loss (Wegmann et al., 1993; Ho et al., 2001; Piccinni, 2003). Thus, further studies of the mechanisms underlying the immunological modulation of uterus during pregnancy would be important for clarifying the causes of embryonic loss.

On the other hand, numerous papers have been published to date on the functional interactions between nervous and immune systems in the mammals. It has been found that the lymphocyte immune reactivity was subject to the regulation by the sympathetic nervous system in the lymphoid organs (Murray et al., 1992; Baerwald et al., 1997; Kohm and Sanders, 2001). For example, the sympathetic nervous system could modulate the secretions of IL-1β and IL-6 (De Luigi et al., 1998; Miura et al., 2001), IL-2 and IL-4 (Kruszewskia et al., 1995) in the T cells of lymphoid organs. An adrenergic stimulation increased myelopoiesis in the bone marrow (Yamamura et al., 1996). However, a pregnant
uterus was generally considered to be an immunological privilege site. So far, it has been poorly understood whether sympathetic nerves function in the regulating uterine immunity. Since the neurotoxin 6-hydroxydopamine (6-OHDA) can selectively destroy sympathetic nerve terminals in animals, we have attempted to determine the role of sympathetic nerves in the distribution of uterine lymphocyte populations, the production of cytokines and the development of early embryos using an experimental model system in which 6-OHDA was used to treat mice.

2. Materials and methods

2.1. Animals and 6-OHDA treatments

Eight to nine week old female (25–35g) and male (35–45g) Kun-ming albino mice were purchased from Beijing Biological Produces Company (Beijing, China). Upon arrival, animals were housed in individual cages, maintained under the constant conditions of temperature (25 °C) and illumination (12h light - 12h dark), and fed with a standard pellet diet. After an adaptive period of one week, the females were injected i.p. for five consecutive days with 100mg/kg body weight (BW) of 6-OHDA (Sigma Chemical Co., St. Louis, MO) diluted in a sterile saline solution containing 0.01% L-ascorbic acid (Penttilä Heikki et al., 2001; Klukovits et al., 2002). The control females were treated with vehicle (0.01ml/g BW). After completion of treatment, the oestrous cycles of animals were tested daily by vaginal smears using Wright’s staining. Their oestrous cycles were mostly 4–7 days. The females at oestrus were mated after 1–5 days of treatment cessation. On the next day, animals were confirmed for pregnancy by vaginal plugs. The presence of the vaginal plug was taken as day 1 of pregnancy. The incidences of conception after 6-OHDA injections were delayed to 1–3 oestrous cycles, but about 1–2 oestrous cycles in the control group.

Animals on day 1 (E1), E3, E5, E7 and E9 of pregnancy were killed by cervical dislocation under Nembutal deep anaesthesia (50mg/g BW). The oviduct and uterus were immediately removed from the abdominal cavity, and then embryos were carefully removed from the uterine tissue. The oviducts were flushed with 0.01M phosphate buffered saline (PBS, pH 7.4), and the numbers of fertilized ova were recorded at E1 under an operating microscope (Olympus SZH). On the other hand, the number and size (long diameter and short diameter) of embryos were measured at E7 and E9 using a micrometer under an operating microscope (Olympus SZH). Studies were carried out in accordance with the Guidelines for Animal Experimentation of China Agricultural University.

2.2. Histochemical demonstration of sympathetic nerves

Uterine sympathetic nerves were demonstrated by the glyoxylic acid technique (refer Penttilä Heikki et al., 2001; Klukovits et al., 2002). Uterine tissues without embryo were quickly immersed in 30% sucrose solution in 0.1M phosphate buffer (PB, pH 7.4) overnight and later frozen by embedding medium of O. C. T. (optimal cutting temperature) under −22 °C. Transverse sections (50μm) to the uterine horn long axis were cut using a cryostat (Leica 1800, Leica Instruments GMBH). Sections were immersed three times (5s each) in a 2% glyoxylic acid solution (Sigma) in PBS (pH 7.4), and dried under cold air for 5min and left for 20min at 25 °C. Finally, the sections were heated at 80 °C for 5min and mounted with polyvinyl alcohol 15000. Sections were examined under an Olympus BX51 fluorescence microscope fitted with the appropriate filters (B-filter set, excitation 450nm, absorption 520nm).

2.3. Histology and immunohistochemistry

Uterine tissues were immediately immersed in 4% paraformaldehyde in 0.1M PB (pH 7.4) fixed overnight (4 °C), and dehydrated in graded ethanol series, then embedded in paraffin. Sections (5μm) were mounted on gelatinized glass slides, and were deparaffined, rehydrated and rinsed with 0.01M PBS.

For histological studies, sections were stained with hematoxylin and eosin. The uterine walls (from the cavity epithelium to the serous membrane) were measured from five cross-sections of the uterus of each animal using the software (Scion Image). The ratios of both areas of all uterine glands and total uterus walls were measured from five cross-sections of the uterus of each animal. For immunohistochemistry, sections were treated with 3% hydrogen peroxide in methanol for 30min, and incubated with 2.5% normal rabbit serum (Sigma) for 20min at room temperature. After rinsing with PBS, sections were incubated overnight at 4 °C with monoclonal rat anti-mouse CD4+ or CD8+ primary antibodies (1:200 in PBS, Santa Cruz Biotechnology, Inc., CA). Then the sections were rinsed in PBS and incubated with biotinylated rabbit anti-rat secondary antibody (1:100, Sigma) for 2h at room temperature. After washing, the tissues were then incubated with streptavidin–horseradish peroxidase (1:200, Sigma) for 1h at 25 °C. Immunoreactivity was visualized by incubating in 0.01M PBS containing 0.05% 3’3-diaminobenzidine tetrahydrochloride (DAB, Sigma) and 0.003% hydrogen peroxide for 10min in the dark. After the final rinsing, the sections were mounted. The number of positive cells was counted in the total areas from five cross-sections of the uterus of each animal. The specificity of the immunostaining was checked by omitting incubation in primary antibodies.

2.4. ELISA

Uterine tissues without embryos were homogenized in 10% wt/vol of 0.01M PBS (pH 7.4, 4 °C), and then were centrifuged at 20,000g for 20min 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 sensitivities of the kits were 15.6 pg/ml for IL-2 and 7.8 pg/ml for IL-4. Briefly, the samples or standards were applied to polystyrene microplates coated with a specific anti-mouse IL-2 or IL-4 monoclonal antibodies, respectively. After the incubation for 90 min at 37 °C, the plates were washed twice with PBS containing 0.05% Tween-20, and then the biotinylated anti-cytokines (anti-IL-2 or anti-IL-4, 1:100) were added for incubation for 60 min at 37 °C. After six times washing, the plates were treated with 100 μl avidin–biotin–peroxidase complex (ABC, 1:100) for 30 min at 37 °C and were incubated with 0.01% 3, 3′, 5′, 5′-tetramethylbenzidine (TMB, Sigma) substrate plus hydrogen peroxide solution (0.003%) for 10–15 min at 37 °C. After adding 100 μl of 2 N sulphuric acid (H₂SO₄), the reading was taken at OD₄₅₀nm using an ELISA reader (Bio-rad, USA). The homogenated solutions of protein density were measured by UV/VIS (Secoman, French), the protein density (mg/ml) = 1.45 × OD₂₈₀nm–0.74 × OD₂₆₀nm. The concentrations of cytokines were represented by pg/mg protein.

2.5. Data analysis

Results are expressed as mean±SD. Mean values were compared using two-tailed Student’s t-test. Values of \( p < 0.05 \) were considered statistically significant.

3. Results

3.1. Identification of sympathetic nerve terminals in uterus treated with 6-OHDA

The histochemical examination with glyoxylic acid-induced fluorescence showed that the typical varicose sympathetic nerve fibers distributed in the uterus of the mice in the control group (Fig. 1A). In contrast, the nerve fibers were few in the uterine tissues of 6-OHDA-treated mice (Fig. 1B). This data

---

Fig. 1. Photomicrographs depicting appearance of the embryos and uterus in different treatment mice on E7. A and B showed the sympathetic terminal nerves in the myometrium by glyoxylic acid-induced fluorescence, and there were numerous fluorescent sympathetic nerves in the control group (A), but not in the treated group (B). Note that the uterine horns were thicker and showed a string of beads in the control group (C) compared with the 6-OHDA-treated group (D). Sections were stained with hematoxylin and eosin (E and F), the uterine glands developed well in the control group (E), but poorly in the treated group (F). Note that the sections were stained with immunohistochemistry (G and K). The number of CD4⁺ T cells in the treated group (H) is similar to that in the control group (G), but the number of CD8⁺ T cells is higher significantly in the treated group (K) than in the control group (J). Scale bar = 100 μm in A for A and B. Scale bar = 1 cm in C for C and D. Scale bar = 200 μm in E for E and F. Scale bar = 200 μm in G for G and K. EB, embryo; OV, ovary; UG, uterine gland.

---
indicated that the sympathetic nerve terminals of uterus in pregnant mice had significantly disappeared when the mice were treated with 6-OHDA. Thus, the experimental model of chemical sympathectomy pregnant mice could successfully be established by intraperitoneal injection of 6-OHDA.

3.2. Changes in number and size of embryos in pregnant mice treated with 6-OHDA

Compared to the control group, the number of fertilized ova did not have a significant reduction (12–13 ova in the 6-OHDA-treated group vs 14–15 ova in the control group) at E1, but the number of embryos was significantly reduced to 4.80 (E7)–3.70 (E9) \((P<0.05)\) (Table 1), and decreased by 64.4% (E7)–69.9% (E9) in 6-OHDA-treated pregnant mice. Meanwhile, the embryos of the treated group developed poorly, and their sizes were 3.05±0.25 mm (long diameter) and 2.15±0.43 mm (short diameter) at E7 in the 6-OHDA-treated group. They were significantly smaller than those of the control group which were 3.56±0.64 mm and 2.71±0.42 mm in long and short diameters at E7, respectively (Fig. 1C and D).

3.3. Morphological changes of pregnant uterus treated with 6-OHDA

After pregnant mice were treated with 6-OHDA, the uterine walls of nonpregnant sites became thinner, but not significantly different (454.2±62.2 μm in treated mice, 553.4±40.3 μm in control mice) in the statistics. However, the decidua formations of pregnant sites were poor. Fibrosis occurred in the uterine endometrium, and the blood vessels and uterine glands greatly reduced (Fig. 1F). In contrast, the uterine decidua of controls was well developed and contained a great number of vessels and glands (Fig. 1E). As shown in Table 2, comparing the data for both groups, the percent of uterine glands was greatly lower in the 6-OHDA-treated group (4.1%–1.0%) than in the control group (5.4%–1.2%) from E1 to E9.

3.4. Changes in T cell subsets in pregnant uterus treated with 6-OHDA

The shapes of CD4+ T cells in uterus were various. Most of them were rotundity, and a few were spindle. They were mainly distributed in the myometrium and endometrium of the uterus in a clustered pattern (Fig. 1G and H). As shown in Fig. 2A, the number of CD4+ T cells in the uterus increased from 8.2±2.5/mm² in the control group and 8.3±1.8/mm² in the 6-OHDA-treated group at E1 to the maximal 22.4±4.8/mm² in the control group and 24.1±

![Fig. 2](image-url)

Table 1

<table>
<thead>
<tr>
<th>Embryo number</th>
<th>Long diameter (mm)</th>
<th>Short diameter (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>E7 ((n=8))</td>
<td>E9 ((n=7))</td>
</tr>
<tr>
<td>Embryo number</td>
<td>13.50±0.70</td>
<td>12.30±1.50</td>
</tr>
<tr>
<td>Long diameter</td>
<td>3.56±0.64</td>
<td>4.55±0.19</td>
</tr>
<tr>
<td>Short diameter</td>
<td>2.71±0.42</td>
<td>2.49±0.20</td>
</tr>
</tbody>
</table>

The sign * means statistically significant difference \((P<0.05)\) comparing with the corresponding control group.

Table 2

| Ratio of both areas of all uterine glands and total uterus wall in both groups \((\%, n=5)\) |
|---------------------------------|-----------------|-----------------|
| E1                              | E3              | E5              |
| Control group                   | 5.4±0.4**       | 4.5±1.2*        |
| 6-OHDA-treated group            | 4.1±0.3         | 3.1±0.2         |
|                                 | 2.0±0.2         | 1.3±0.2         |
|                                 | 1.0±0.04        |                 |

The signs * and ** means statistically significantly differences (*, \(P<0.05\); **, \(P<0.01\)) comparing with corresponding control.
7.3/mm² in the 6-OHDA-treated group at E5. Comparing the data from both groups, the number of CD4⁺ T cells was slightly larger in the 6-OHDA-treated group than in the control group during early pregnancy (up to 7.7% at E5), but their differences were not significant statistically (P > 0.05). At E9, however, there was a statistically significant difference (P < 0.01).

In CD8⁺ T cells, the shape and distribution pattern were similar to CD4⁺ T cells in the uterus of E1–E9 (Fig. 1J, K). However, the cell number was considerably fewer in the control group, whereas it was larger in the 6-OHDA-treated group than that of CD4⁺ T cells (Fig. 2B). Comparing the data of both groups, the CD8⁺ T cell number significantly increased to ten-fold at E5 (35.0±6.4/mm²) in the 6-OHDA-treated group (Fig. 2B).

Consequently, the changes in CD4⁺ and CD8⁺ T cell number were various in the uteri when pregnant mice were differently treated. In 6-OHDA-treated mice, the CD4⁺/CD8⁺ T cell ratio was significantly lower than that of the control group, and decreased by 90% at E5 and 73% at E7, respectively (Fig. 2C).

3.5. Changes in cytokine production in pregnant uterus treated with 6-OHDA

To examine the effect of 6-OHDA on cytokine production, two cytokines, IL-2 and IL-4, which were respectively secreted by Th1 and Th2 cells, were measured using ELISA. Results showed that the IL-2 concentration increased markedly in the pregnant uteri of 6-OHDA-treated mice, reaching a maximum (113.6±15.3 pg/mg) at E5 (Fig. 3A). That is, the IL-2 concentration in the 6-OHDA-treated group was about 355.7% (P < 0.01) of that in the control group (31.9±5.3 pg/mg) at E5. In contrast, no significant difference of IL-4 concentration between the 6-OHDA-treated group and the control group was observed during E1–E3 and E7–E9. The IL-4 concentration was significantly larger in the 6-OHDA-treated group (34.2±6.4 pg/mg) than in the control group (18.6±2.0 pg/mg) at E5 (P < 0.05) (Fig. 3B).

As shown in Fig. 3C, the ratios of IL-2 and IL-4 were significantly larger at E3 (3.4±0.5) and E5 (3.7±0.7) in the 6-OHDA-treated group than those in the control group at E3 (1.3±0.06) and E5 (1.8±0.4) (P < 0.05), and there were significantly differences at E1 and E7–E9.

4. Discussion

4.1. Role of sympathetic nerve in the fetal survival and development in mice during early pregnancy

Recently, it was reported that the monoamines might modulate several steps of the reproductive processes such as embryo implantation and decidua formation (Bottalico et al., 2003). However, the occurrence of a natural axonal degeneration of sympathetic nerves was found in the middle and later periods of normal pregnancy in rat (from day 15 of pregnancy, Klukovits et al., 2002) and rabbit (at day 18 of pregnancy, Chen et al., 2000). Therefore, the role of the sympathetic nerves of the uterus in pregnancy might be only confined in the early period of pregnancy. This study showed that the number of implanted embryos decreased to 64.4% (at E7) and the size of embryos was small in mice during early pregnancy after sympathetic denervation. A similar finding was also reported in the rat (Mac Donald and Airaksinen, 1981). Therefore, the role of the sympathetic nerves of the uterus in pregnancy might be only confined in the early period of pregnancy. This study showed that the number of implanted embryos decreased to 64.4% (at E7) and the size of embryos was small in mice during early pregnancy after sympathetic denervation. A similar finding was also reported in the rat (Mac Donald and Airaksinen, 1981). However, some previous studies recognized that sympathectomy did not have any significant effect on the fertility as reported in mice (Johns et al., 1975) and in rat (Lara et al., 1989; 1990). The different results between Lara’s and our study were probably caused by different methods, since Lara employed methods of immunosympathectomy with nerve growth factor antibodies and of chemical sympathectomy with guanethidine treatment. However, we did not have a clear answer to the question why there were discrepancies.
in our data and those of Johns who used a similar treatment of 6-OHDA administrations. In addition, our experiments showed that there were no significant differences between the 6-OHDA-treated (12–13 ova) and control (14–15 ova) groups in the number of fertilized ova at E1. In fact, Johns also observed a few reduction in the average number of fetuses in 6-OHDA-treated pregnant mice. Therefore, our findings may indicate that the sympathetic denervation led to a reduction in the number of implanted embryos during early pregnancy.

4.2. Role of sympathetic nerve in the modulation of uterine immunocytes and cytokines in mice during early pregnancy

The relevant activation of uterine immunocytes was known to be essential for the maintenance of normal pregnancy. Of these immune cells, the T cells may play an important role in early pregnancy (Leung et al., 2000; Piccinni, 2003). For example, some papers reported that the number of CD4+ and CD8+ T cells decreased to a lower level in the uterus during early pregnancy (Degenne et al., 1988; Castilla et al., 1989; Vassiliadou and Bulmer, 1996). In contrast, the increases of CD4+ and CD8+ T cells were observed in the uterus of abortion rat (Talwar et al., 1997). Additionally, the increases or decreases of ratios of CD4+ to CD8+ T cells were involved in abnormal pregnancy (Wongweragiat et al., 1999) and fetal malformations (Seniz et al., 1993). Subsequently, Minagawa and co-workers reported that the decrease of the proportion and absolute number of T cells in the uterus during pregnancy possibly associated with sympathetic nerve activation (Minagawa et al., 1999). However, present study indicated that chemical sympathectomy by 6-OHDA could significantly increase CD8+ T cell number (up to ten-fold at E5), but only slightly increase CD4+ T cell number (up to 7.7% at E5), and then resulted in a significant reduction of CD4+/CD8+ ratio. Therefore, activation of the sympathetic nerves conduced to the maintenance of a normal level of T cell subsets in the uterus during early pregnancy.

This study also suggested that the chemical sympathectomy by 6-OHDA could alter the secreting pattern of cytokine in uterine T cells during early pregnancy. When the sympathetic nerve was chemically destroyed in pregnant mice by 6-OHDA, the production of IL-2 was significantly upregulated but no large changes in the IL-4, which resulted in a significant increase of the IL-2/IL-4 ratio. In mice, however, the Th1 cells secreted mainly IL-2 and interferon-γ (IFN-γ) which are harmful to embryonic implantation and fetal development, while Th2 cells secreted mainly IL-4 and IL-10 which are favorable to supporting pregnancy and fetal survival (Wegmann et al., 1993; Seder and Paul, 1994; Chaouat et al., 1995; Krishnan et al., 1996). Therefore, our results suggested that the sympathetic nervous system could regulate the balance of Th1 and Th2 in the uterus to influence the implantation and development of embryos during early pregnancy.

Additionally, our results showed that the number of T cells and cytokine levels increased in the early period of embryonic implantation age (E1–E3), and reached the maximum at E5, which was a stage of embryonic implantation in mice. After that, it continued to decrease through the latter period of embryonic implantation time (E7–E9). Furthermore, it is well known that there is a positive role for IL-2 in promoting CD8+ T cell expansion in both mouse models and human patients (Blattman et al., 2003; Cheng et al., 2002). Consequently, the sympathetic denervation might upregulate T cell numbers and cytokine levels in the uterus to alter its immune environment during early pregnancy. It was possibly one of the reasons why there were fewer embryos in denervated pregnant mice.

4.3. Role of sympathetic nerve in the decidua formation in mice during early pregnancy

To accommodate the growing fetus, the structure and function in the maternal organ systems should be altered during pregnancy, especially in the uterus. However, a little was known about the role of the peripheral sympathetic nerves in structural alterations of the uterus during pregnancy. Several studies indicated that the uterine sympathetic nerves mainly influenced the excitability of the myometrium and uterine motility (Legrand and Maltier, 1986; Legrand et al., 1987; Klukovits et al., 2002), while other studies suggested that the sympathetic nervous system regulated primarily uterine blood circulation to continue redistribution of blood flow (Hart et al., 1986; Greenwood et al., 2001; O’Hagan and Alberts, 2003). In the current study, our results showed that the chemical sympathectomy by 6-OHDA was harmful to the development of the uterine gland and vessel in the endometrium of mice during early pregnancy. The activation of the sympathetic nerves was necessary for the decidua formation in the uterus in early pregnancy.

Additionally, it was reported that endometrial glands not only secreted proteins to support nutrition for embryo implantation and development, but also secreted cytokines, such as IL-6 and leukemia inhibitory factor, which was advantageous to implantation (Perrier d’Hauterive et al., 2005). Therefore, the development of endometrium would influence the concentrations of cytokines.

In summary, our study has shown that peripheral sympathetic nerves activation may be essential for the maintenance of early pregnancy. Peripheral sympathetic nerves have favorable impact on embryo implantation and fetal survival and development in early pregnancy through regulating uterine immune function and influencing decidua formation.

Acknowledgment

This work was funded by grants from the National Nature of Science Foundation of China (No. 39500108, 30270960, 30471247).
References


