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The Effects of Interferon-Gamma (IFN-γ) on the Expression of IL-10 in Hypothalamic Pituitary Ovarian Axis and Peripheral Blood

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Abstract: The aim of this research was to confirm the effect of IFN-γ on the expression level of IL-10 in hypothalamic pituitary ovarian axis and peripheral blood of rats during early pregnancy. The rats (gestational day 9) were randomly divided into 3 groups: test group 1 (vaginal muscular injection with 2.5×10^4 U IFN-γ per rat), test group 2 (vaginal muscular injection with 7.5×10^4 U IFN-γ per rat) and control group (vaginal muscular injection with normal saline). About 48 h after injection, the hypothalamus, pituitary, ovary, uterus and peripheral blood were collected to research the effect of exogenous IFN-γ on the expression level of IL-10 by morphological observation, immunohistochemical SP and ELISA. The results showed that injection of IFN-γ (in the dose of both 2.5×10^4 U and 7.5×10^4 U per rat) decreased the expression of IL-10 in hypothalamic periventricular nucleus, hypothalamic supraoptic nucleus, nucleus pre-opticus magno cellularis, medial preoptic nucleus, lateral preoptic nucleus, suprachiasmatic nucleus, nucleus arcuatus hypothalamic, adenohypophysis, corpus luteum, stromal cells in uterine decidua, utricular glands and peripheral blood. These findings suggest that IFN-γ could down regulate the expression of IL-10 in hypothalamic-pituitary-ovarian axis and peripheral blood at a does independent manner.

Key words: IL-10, IFN-γ, immunohistochemical, hypothalamic pituitary ovarian axis, pregnant rats

INTRODUCTION

The embryonic period is an important stage for animal development. Variety of growth factors and hormones play important roles in the regulation of embryonic development and pregnancy mother's organ metabolism. IL-10 was secreted by endometrial glands and endometrial epithelial cells during pregnancy. It plays crucial roles in the immune regulation and the uterine receptivity establishment for embryo implantation (Huang *et al*., 2009; Kim *et al*., 2009; Sallinen *et al*., 2000). IL-10 can down-regulate the maternal immune response to fetus and affect many physiological processes by regulating growth factors and cytokines to maintain normal pregnancy (Vigano *et al*., 2002). IL-10 as a cytokines resisting allograft rejection is secreted by the placental trophoblastic cells during the entire pregnancy process and plays important roles in the regulation of immune tolerance of embryos. IFN-γ belongs to Th1 cytokine is one of the necessary cytokines for pregnancy. Its main function is participation in endometrial stromal cells decidulization and the maintenance of pregnancy (Bulla *et al*., 2004). Studies showed that supraphysiological dose of IFN-γ had antifertility effects (Cao *et al*., 1999; Liu *et al*., 2005). Presently, researches about the IFN-γ or IL-10 focus on the relationship between the expression of IFN-γ or IL-10 in uterus and placenta and gestation while the interaction of the IFN-γ and IL-10 on gestation has not been reported.

In this study, the effect of exogenous IFN-γ on the expression of IL-10 in hypothalamus-pituitary-ovarian axis and peripheral blood during early pregnancy of rat was determined by immunohistochemical SP and Enzyme-Linked Immunosorbent Assay (ELISA). This study would help to clarify the role of IFN-γ in the pregnancy maintenance, IL-10 in the neural immune regulation of

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hypothalamus pituitary ovarian axis and the interaction between IFN-γ and IL-10 on pregnancy. And it would provide morphological evidence for the regulatory mechanism of cytokine in pregnancy.

MATERIALS AND METHODS

Experimental animals: All procedures involving animals were approved by the animal care and use committee at the respective institution where the experiment was conducted.

Healthy nulliparous female (30) and male (10) SD rats were purchased from the Hena experimental animal center with the weights ranging from 240-250 g. The rats were maintained in an experimental room under controlled conditions of temperature (22±2°C), humidity (50±10%) and a 12 h light/dark cycle with ad libitum access to commercial diet and tap water. After a period of feeding, the rats were mated (male:female = 1:1) based on the estrus confirmation by vaginal smears. The day by which was found a sperm positive vaginal smear was considered as D1 (Day 1) of gestation and thereafter pregnant rats were housed individually. The D9 (pregnant on the 9th day) rats were randomly divided into 3 groups (10 rats per group) using muscle injection at vaginal orifice. Control group: injected with normal saline; group I: 2.5×10^4 U IFN-γ per mouse; group II: 7.5×10^4 U IFN-γ per mouse. Drugs and dosage schedule was performed according to Liu *et al.* (2005).

Reagents and instruments: Recombinant IFN-γ and IGF-1 antibody were purchased from Boster (Wuhan, China). Immunohistochemical SP kit was purchased from Maixin Bio (Fuzhou, China). Bovine serum albumin was purchased from SINO-AMERICAN Biotec (Luoyang, China). The main instruments including Microtome (Leica 2245, Germany) and microscope (Motic, Xiamen, China) were public in the laboratory.

Sections preparation of the rat hypothalamus, pituitary and ovarian: About 48 h after injection, the rats from different groups were anesthetized with 10% chloral hydrate. After chests were opened, the blood was cleaned by saline (37°C) via the aorta. After perfusion with 4% paraformaldehyde phosphate buffer solution (pH 7.4, 4°C), the hypothalamus, pituitary, uterine and ovarian were fixed for 48 h in the same solution. And then the tissues were dehydrated in graded ethanol, diafanized in xylene and embedded in paraffin. And then the 5 μm thick paraffin sections was made from the paraffin blocks and taken out separately. Three sets of sections in total were obtained: the first set was used for IL-10 immunohistochemical staining the second set was used for positive cells positioning by HE staining and the last set was used as negative control.

Immunohistochemical staining of hypothalamus, pituitary and ovarian: Polyclonal antibody of rabbit-anti-mouse IL-10 was used as first antibody (under 100 fold dilution) and PBS buffer was used as the negative control. The procedure of immunohistochemistry SP was performed by the immunohistochemical kit according to the manufacturer's protocol.

Immunohistochemistry staining and observation: Polyclonal antibody of rabbit-anti-mouse IL-10 was used as first antibody (under 100 fold dilution) and PBS buffer was used as the negative control. The procedure of immunohistochemistry SP was performed by the immunohistochemical kit according to the manufacturer's protocol.

Ten different magnitude microscopic vision (x400) from the sections of hypothalamus, pituitary and ovarian were choosen and then the photographs were analyzed by Jiangsu Jetta high-resolution image analysis system. The mean of the staining degree of the positive product for each vision (which was denoted by the average optical density) and the positive area were calculated (Laird *et al.*, 2003). The mean level of the relative expression (μ^2)were calculated via the following equation: μ^2 = Multiples of light microscope x Mean positive intensity x area of the positive (260000) among which 260000 was pixel.

Statistical analysis: The data from each group was described by Mean±Standard deviation and one-way ANOVA performed by Statistical Software SPSS 11.5.

RESULTS AND DISCUSSION

Effects of IFN-γ on the expression of IL-10 in hypothalamus of pregnant rat: The background of sections was color less or light blue after immunohistochemical staining and the positive product was blue or black-blue. There was no positive product detected in the control group. This proved the specificity of the immune response in this experiment. In the control group most of cells in nucleus periventricularis hypothalamic, nucleus supra-opticus, nucleus pre-opticus magno cellularis, medial preoptic nucleus, lateral preoptic nucleus, suprachiasmatic nucleus and arcuate nucleus of optic chiasma were immunoreactive with blue or black-blue, dark blue particles located in the cytoplasm and nucleus. The positive cells showed irregular shapes including round, oval, spindle and their contour were clearly visible (Fig. 1 and 2a).

In group I most of positive cell of nuclear groups in hypothalamus associated with pregnancy was light blue (Fig. 1 and 2b), the contour of the positive cells was

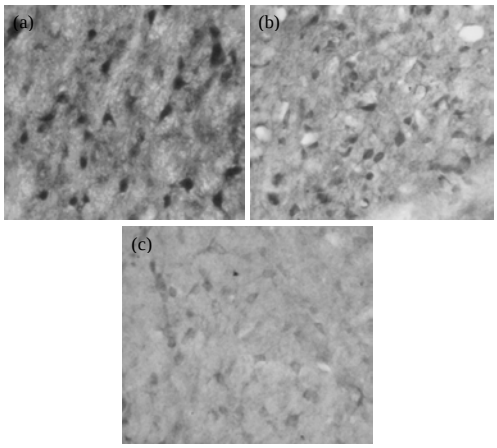

Fig. 1: Immunohistochemical staining of IGF-1 in the nucleus pre-opticus mango celluaris hypothalami rats. a) Control group; b) Experimental group 1 and c) Experimental group 2

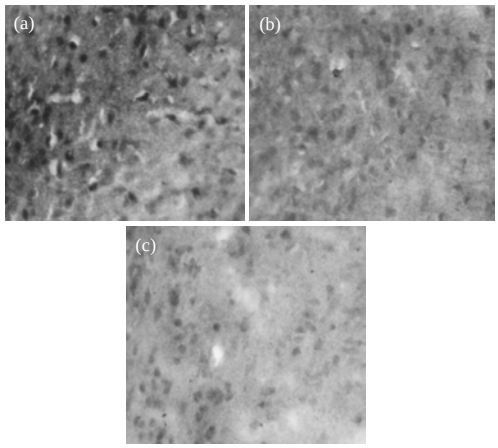

Fig. 2: Immunohistochemical staining of IGF-1 in the nucleus pre-opticus periventricularis rats a) Control group; b) Experimental group 1 and c) Experimental group 2

unclear, light blue particles could be seen at high power lens, compared with the control group, the positive product in nucleus periventricularis hypothalamic, medial preoptic nucleus and hypothalamic arcuate nucleus was decreased, the difference was significantly ($p<0.05$), the difference in other nuclear groups was extremely significantly ($p<0.01$).

In group 2, the number of positive cell of nuclear groups in the hypothalamus was lesser and the positive cell was gray, the contour of the cells was not clear (Fig. 1 and 2c), the expression of positive product was less compared with the control group, the positive expression in the hypothalamic suprachiasmatic nucleus was decreased, the difference was significantly ($p<0.05$); the difference in other nuclear groups was extremely significantly ($p<0.01$, Table 1).

The effect of IFN-γ on the expression of IL-10 in pituitary of pregnant rats: In the control group, IL-10 was mainly expressed in cytoplasm of small round cells from adenohypophysis and it also expressed in the cytoplasm of large round, oval, triangular, polygonal and irregular cells. IL-10 immuno-positive product in the cytoplasm of positive cells was black-blue. The vacuolization nucleus located in the center of the cell and the contour of the positive cell was clear.

In group 1, IL-10 was also mainly expressed in the cytoplasm of adenohypophysis cells with large round, oval, triangular, polygonal and irregular shapes but the IL-10 immuno-positive product in the cytoplasm of positive cells was blue and the positive particles was sparse and expression was significantly lower than control group ($p<0.05$).

Table 1: Effect of IFN-γ on the expression of IL-10 in the HPG axis of post-implantation period pregnant rats

Expressiom position	Relative expression of IGF-1 immunological reaction production (μ^2)		
	Control group	Experimental group 1	Experimental group 2
Nucleus pre-opticus periventricularis	46.321±5.091	37.075±4.115^{A}	24.328±4.512^{ab}
Nucleus supra-opticus	44.191±2.398	33.229±5.104^{a}	24.872±5.381^{aB}
Nucleus pre-opticus mango cellularis	31.448±4.508	22.664±3.106^{a}	17.094±5.339^{aB}
Nucleus pre-opticus medialis	55.229±6.364	47.132±3.179^{A}	37.981±4.325^{ab}
Mucleus pre-opticus lateralis	45.271±3.181	35.048±4.198^{a}	24.773±3.823^{ab}
Nucleus pre-opticus suprachiasmatic	46.342±2.971	36.103±3.259^{a}	31.562±5.133^{aB}
Arcuate nucleus of the hypothalamus	50.653±5.732	44.323±5.138^{A}	23.196±3.960^{ab}
Adenohypophysis	34.547±2.277	28.374±3.749^{A}	19.046±5.235^{ab}
Corpus luteum of ovary	39.096±2.355	34.271±5.192^{A}	24.057±4.767^{ab}
Stroma cells of uterus decidua and uterine gland	33.562±3.568	22.797±3.324^{a}	17.009±5.653^{aB}

The data with 'A' means it has significant difference when it compared with control group ($p<0.05$). The data with 'B' means it has significant difference when it compared with experimental group 1 ($p<0.05$). The data with 'a' means it has the difference is very significant when it compared with control group ($p<0.01$). The data with 'b' means it has the difference is very significant when it compared with experimental group 1 ($p<0.01$)

In group 2, the expression of IL-10 in the cells of adenohypophysis was very weak, its expression was significantly lower than group 1 and control group ($p<0.01$, Table 1).

The effect of IFN-γ on the expression of IL-10 in ovary and uterus of pregnant rats: In the control group, IL-10 was mainly expressed in the ovarian granulose lutein cells. The black-blue positive particles fusing into cluster

located at the membrane and cytoplasmic of the positive cells. The nucleuses of the positive cells were vacuolar, the contour of the positive cells was clear (Fig. 3a, Table 1). In group 1, the positive particles uniformly distributed in the membrane and cytoplasmic of ovarian granulose lutein cells (Fig. 3b), the positive product was dark blue, its expression was significantly lower than control group ($p<0.05$, Table 1).

For the rats in group 2, the positive product of IL-10 in the ovarian granulose lutein cell was gray, the contour of the positive cells was unclear, the expression of IL-10 was weak (Fig. 3c). Its expression is significantly lower than control group and group 1 ($p<0.01$, Table 1). The IL-10 expressed in the uterine decidua and uterine glands in all groups but the expression level of IL-10 was decreased with the dose increasing of IFN-γ.

The effect of IFN-γ on the expression of IL-10 in peripheral blood of pregnant rats: The expression of peripheral blood IL-10 in control group was significantly higher than group 1 and 2 ($p<0.01$) and the expression level in group 1 was significantly higher than group 2 ($p<0.05$, Fig. 4).

Pregnancy is a complex and sophisticated physiological process during early pregnancy there are complex cytokine regulatory networks between mother and fetus with these networks fetus can escape from the mothers' immune rejection (Tian, 1999). In recent years with the development of methodology, regulating factors and mechanisms involved in the hypothalamic pituitary ovarian axis arouse people's interest again. IL-10 is a pregnancy protecting cytokine, working in the hypothalamic-pituitary-ovarian axis and mainly secreted by the trophoblastic cells during pregnancy (Xiao and Lin, 2005). In Unexplained Recurrent Spontaneous Abortion (URSA), the expression of IL-10 secreted by the decidual macrophages was significantly lower than that of women with normal pregnancies. In pathogenic process of URSA, IL-10 secreted by the macrophages was decreased and then the balance of cytokines Th1/Th2 would be broken in decidua. The increasing Th1 cytokines would initiate the attack on the fetus from maternal immune system and eventually lead to abortion (Jin *et al*., 2007). Thus, IL-10 plays important roles in the regulation of pregnancy in the local microenvironment (Sun and Wang, 2004).

The reproductive activities are regulated by the endocrine and immune systems. Human reproductive system can synthesize a number of cytokines to regulate the immune system. IFN-γ is one of the immune cytokines, it can regulate the nervous system and endocrine system

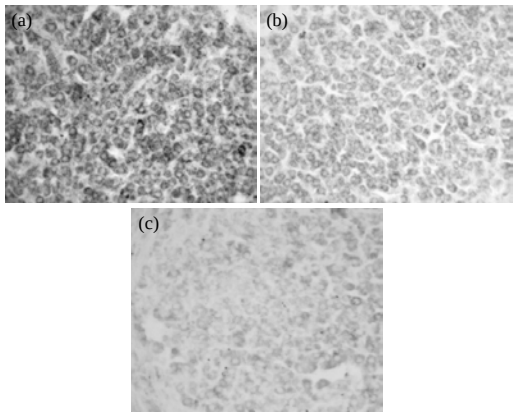

Fig. 3: Immunohistochemical staining of IGF-1 in the granular lutein cell rats a) Control group; b) Experimental group 1 and c) Experimental group 2

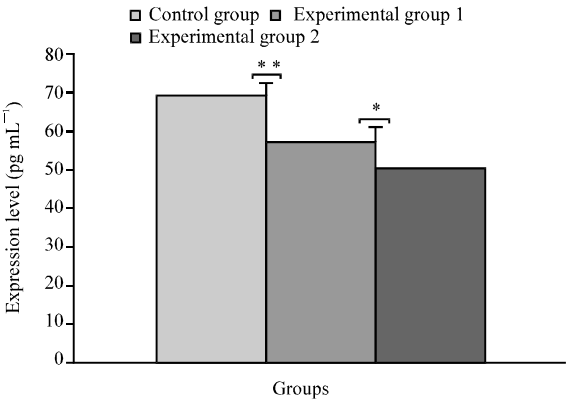

Fig. 4: IL-10 levels in peripheral blood of each experimental group *$p<0.05$,**$p<0.01$

and it is also important reproduction regulation (Chen *et al*., 2000; Vigano *et al*., 2002; Yang *et al*., 2003; Yui *et al*., 1994). For example, IFN-γ is mainly involved in the reconstruction of decidua vessels during pregnancy, it is indispensable for the integrity of decidual (Ashkar and Croy, 2001). And the IFN-γ receptor was expressed in many cells in the implantation site (Chen *et al*., 1994). IFN-γ can protect cells against tachyzoites injection in mouse. It can improve the immunity levels during early pregnant (Jin *et al*., 2008). It would appear that a certain dose of IFN-γ in the body is indispensable for the success of pregnancy.

But Liu *et al*. (2002) reported that high doses of IFN-γ could inhibit the secretion of progesterone, induced the apoptosis of placental cells and induced the expression of Major Histocompatibility Complex class II (MHC II) antigen, MHC II antigen is one of the major antigen associated with the maternal-fetal immune rejection during pregnancy and it is unfavorable to pregnancy.

CONCLUSION

In this study, the impact of IFN-γ on the expression of IL-10 in rat hypothalamus-pituitary-gonadal axis and the peripheral blood during the early pregnancy was studied using immunohistochemical SP and ELISA. The results showed that exogenous injection of IFN-γ at the dose of 2.5×10^4 U or 7.5×10^4 U per rats could reduce the expression level of IL-10 in hypothalamus pituitary gonadal axis and the peripheral blood. Researchers speculated that high dose of IFN-γ may affect pregnancy through the regulation of the level of IL-10 in hypothalamus pituitary gonadal axis and the peripheral blood. The regulatory role of IL-10 on early pregnancy was affected by IFN-γ and low level of IL-10 in hypothalamus-pituitary-gonadal axis and the peripheral blood may be the reasons for IFN-γ's unfavorable influence on pregnancy.

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