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Full Length Research Paper

The effects of interferon-gamma (INF-γ) on the expression of insulin-like growth factor 1 (IGF-1) in uterus, ovaries and peripheral blood of rats in early pregnancy

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To explore the immunological regulatory mechanisms of interferon-gamma (IFN-γ) on insulin-like growth factor 1(IGF-1) and its effect on pregnancy, the dynamic expression of mRNA and cellular localization of IGF-1 protein in the early pregnant (Day 9) rats after the injection of different doses of IFN-γ, their ovaries and uterus were collected and processed by RT-PCR and immunohistochemistry. ELISA assay was also applied to investigate the effect of exogenous IFN-γ on the level of IGF-1 in the peripheral blood. The results indicate that both uterine and ovarian IGF-1 mRNAs and IGF-1 immunoreactivity were the highest in the normal saline group and the lowest in the 300IU IFN-γ group ($P < 0.01$). When compared with the normal saline group, the IGF-1 expression was also lower in the 100IU IFN-γ-treated group ($P < 0.05$). The level of IGF-1 in peripheral blood of the 300IU IFN-γ group was the lowest, which was significantly different from normal saline group ($P < 0.01$) and 100IU IFN-γ group ($P < 0.05$), respectively. The results suggest that exogenous IFN-γ might regulate pregnancy by controlling the expression of IGF-1 in the uterus and ovaries in early pregnancy.

Key words: Insulin-like growth factor 1(IGF-1), interferon-gamma (IFN-γ), uterus, ovaries, early pregnant rats.

INTRODUCTION

Embryonic period is an important stage for animal growth and development. A variety of growth factors and hormones play important regulatory roles in embryonic development and pregnancy, in which insulin-like growth factor1 (IGF-1) has been shown to be an important regulatory factor between fetal and maternal during pregnancy (Irwin et al., 1999; Akercan et al., 2004; Erdemoglu and Mungan, 2004; Ohkawa et al., 2010).

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Abbreviations: **IGF-1**, Insulin-like growth factor 1; **IFN-γ,** interferon-gamma; **D9**, pregnant on the 9th day; **MHCII**, major histocompatibility complex class II.

IGF-1 is one of the peptide hormones containing 70 amino acids, which is located on the short arm of chromosome 12, and 45% of its amino acid sequence are homologous with the insulin which has implicated IGF-1 insulin-like effect. IGF-1 was produced in a variety of organisms and acts through autocrine and autocrine-paracrine mechanisms. IGF-1 has been recognized as one of the important hormones in the hypothalamus – pituitary - gonadal axis in reproductive activities, which work together with several factors to maintain the appropriate microenvironment between the maternal and fetal in the late embryo implantation.

Interferon-gamma (IFN-γ) belongs to the Th1 type cytokines and has an important role during pregnancy. Its main function is participating in the decidualization process of endometrial stromal and the maintenance of

pregnancy (Bulla et al., 2004). Studies showed that super-physiological doses of IFN-γ produced anti-fertility effect (Cao et al., 1999). Currently, researches involving the role of IFN-γ and IGF-1 focus their specific effects and its expression in uterus and placenta, the interaction between the IFN-γ and IGF-1 in pregnancy are not yet fully understood.

This study aimed to investigate the effect of exogenous IFN-γ injection on the expression of IGF-1 in uterus and ovaries as well as the IGF-1 levels in peripheral blood of rats during early pregnancy.

MATERIALS AND METHODS

Experimental animals

Healthy female SD rats were purchased from the Hena Experimental Animal Center. SD rats (240 to 250 g) were used in this experiment.

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 1 week feeding, the rats were mated based on the estrus confirmation by vaginal smears. The day in which a sperm positive vaginal smear was found was considered as D1 (day 1) of gestation, and thereafter the pregnant rats were housed individually.

Sample collection

The D9 (pregnant on the 9th day) rats were randomly divided into 3 groups (10 rats per group). Control group, injected with normal saline; group I, 100 IU/g IFN-γ; group II, 300 IU/g IFN-γ. Drugs and dosage schedule were performed according to Liu et al. (2005). Rats used for the immunohistochemistry of streptavidin-peroxidase (SP) were anesthetized with 100 ml/L chloral hydrate, then their chests were opened and the blood was cleaned by saline via the aorta at 37°C. The ovary and uterus tissue was obtained after a cesarean section, the mesenteric tissues were removed and washed three times with sterile PBS and frozen at -80°C. After perfusion with 40 ml/L paraformaldehyde phosphate buffer solution (pH 7.4) at 4°C, the ovary and uterus were fixed for 48 h in the same fixative. Then the tissues were dehydrated in graded ethanol, diafanized in xylene and were paraffin-embedded. Three sets of the serial 5 µm-thick paraffin sections were obtained to detect the positive and negative cells.

Blood obtained from the heart on D9 was kept at 37°C for 1 h, then overnight at 4°C. Serum was separated and kept in different EP tubes at -20°C for further ELISA assay.

Total RNA extraction and RT-PCR

The total RNA of the uterus and ovaries were extracted by TRIzol-Phenol-Chloroform method and dissolved in RNase-free water. The samples were quantified and read at 260 nm. Amplification of IGF-1 and β-actin genes was performed by RT-PCR using an Access RT-PCR System Kit (Promega, USA). Amplification of specific fragments and reverse-transcriptions were carried out in the same tube. The following primers were used: the forward primer of IGF-1, 5'-TTCAGTTCGTGTGTGGACCAAG-3'; the reverse primer of IGF-1, 5'- GATCACAGCTCCGGAAGCAA-3'. The amplification was 120 bp. The forward primer for the β-actin was: 5'-AGCCATGTACGTAGCCATCC-3′, the reverse primer for the β-actin was: 5'- ACCCTCATAGATGGGCACAG-3', and the length of the PCR fragment was 115 bp. The cycling protocol of the RT-PCR for IGF-1 was 94°C for 5 min, followed by 35 cycles (94°C for 45 s, 54°C annealing for 45 s, 72°C for 45 s), and a final extension of 10 min at 72°C. The protocol for the amplification of β-actin was the same as IGF-1. In order to confirm the specificity of amplification, three kinds of control were used in each group (RNA was substituted by nuclease-free water, PCR without reverse transcriptase, while other conditions remained). The PCR products were detected by electrophoresis in 2.0% agarose gel. After normalizing by the *β*-actin, an A value of the electrophoretogram obtained by Quantity One software (Bio-Rad Inc) was statistically analyzed. The data from each group was described by Mean ± Standard Deviation and performed by the Normal test before statistical analysis. *T* test were used to compare the differences among these data, $P < 0.05$ and $P < 0.01$ indicated significant difference. The analysis was complemented by statistical software SPSS 11.5.

Procedures of the immunohistochemistry and the results of observation

The procedure of immunohistochemistry SP was performed by the immunohistochemical kit according to the manufacturer's protocol. However, 10 different magnification (×400) from the same field of the different ovaries and uterus were chosen and 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 (Hu and Gui, 2004). The mean level of the relative expression (μ^2) was calculated using the following formula:

μ^2 = Multiples of light microscope × Mean positive intensity × area of the positive / 260000 (among which 260000 was pixel).

Detection of the IGF-1 levels in serum by ELISA method

The ELISA experiment was performed following the instructions of ELISA kit, and the OD values were measured by automatic microplate reader at 450 nm. Standard curve was drawn using the density of standard products and OD values as abscissa and vertical axis, respectively. Then, the density of the sample was found through the OD value in the standard curve.

RESULTS

The effects of IFN-γ on IGF-1 mRNA levels in ovary and uterus of pregnant rats

Expression of IGF-1 mRNA was detected throughout the rat ovary in each experimental group. IGF-1 mRNA expression in the ovary of normal saline group was the highest, while it was the lowest in the 300 IU/g IFN-γ group ($P < 0.01$), but there was no statistical difference between the control group and the 100 IU/g IFN-γ group ($P > 0.05$). The IGF-1 mRNA in uterus displayed the same pattern of response among the groups similarly to the ovary. The expression level of IGF-1 mRNA was also lower in 300 IU/g IFN-γ group than normal saline group ($P < 0.01$) (Table 1, Figures 1 to 2).

Table 1. Expression of IGF-1 mRNA in ovary and uterus from each experimental group.

Parameter	Normal saline group	100 IU/g IFN-γ group	300 IU/g IFN-γ group
Ovary	2.60 ± 0.031	1.96 ± 0.091	0.62 ± 0.024**
Uterus	2.26 ± 0.047	1.98 ± 0.015	0.54 ± 0.007**

** P < 0.01 different from normal saline group. Results are expressed as Mean ± SD.

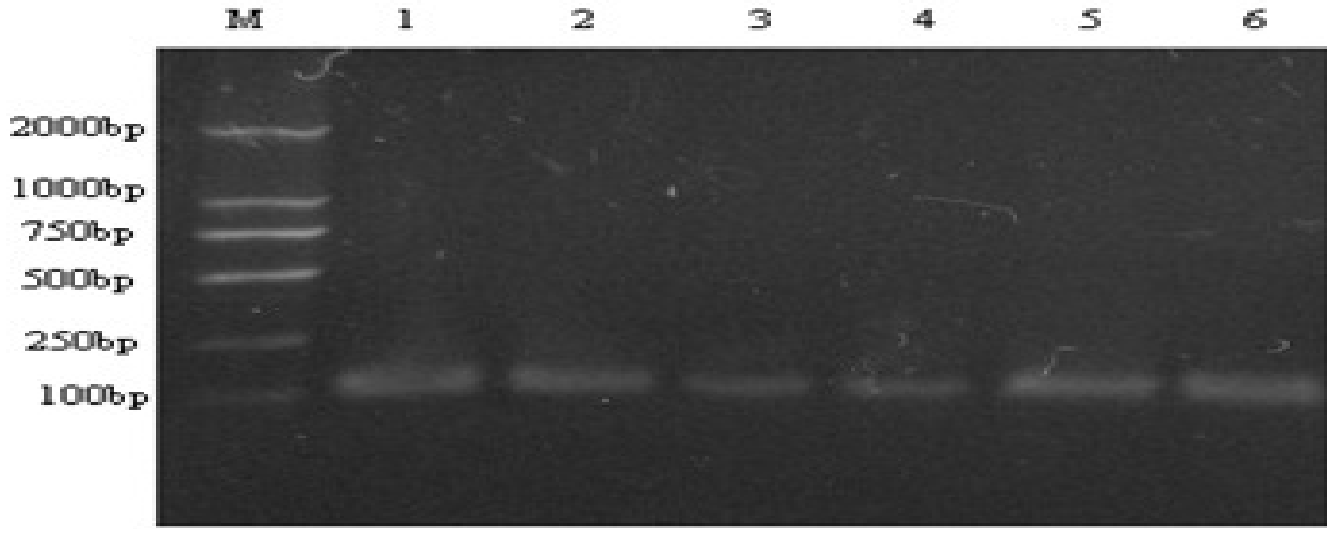

Figure 1. Effect of IFN-γ on the expression of IGF-1 mRNA in ovary and uterus of D9 rats from different experimental groups. M, Marker; Lane 1, the expression of IGF-1 mRNA in uterus of normal saline group; lane 2, the expression of IGF-1 mRNA in uterus of 100IU IFN-γ group; lane 3, the expression of IGF-1 mRNA in uterus of 300IU IFN-γ group; lane 4, the expression of IGF-1 mRNA in ovary of 300IU IFN-γ group; lane 5, the expression of IGF-1 mRNA in ovary of 100IU IFN-γ group; lane 6, the expression of IGF-1 mRNA in ovary of normal saline group.

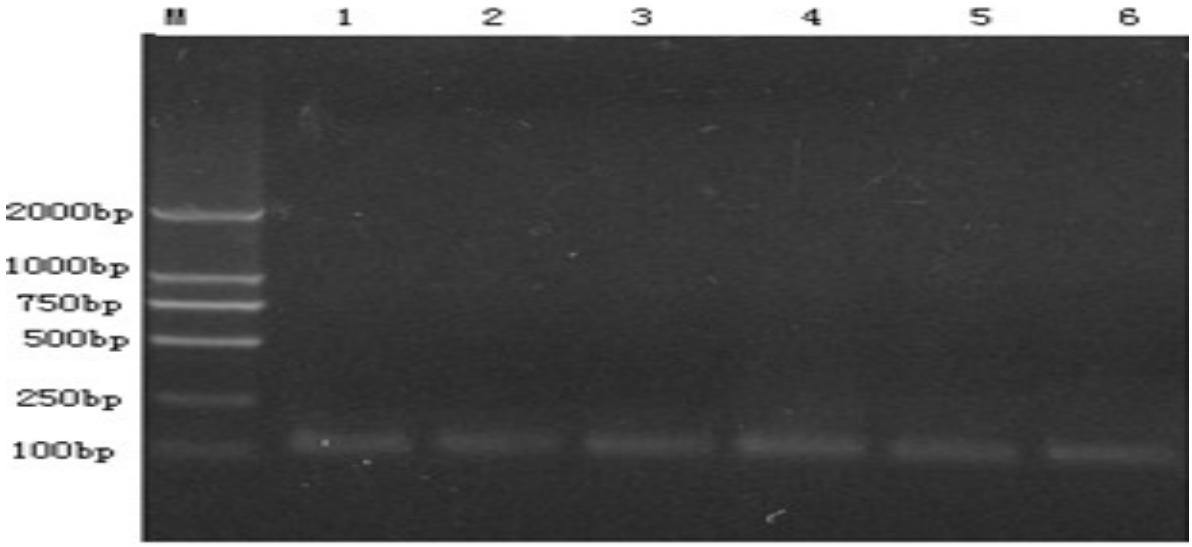

Figure 2. Effect of IFN-γ on the expression of β-actin mRNA in ovary and uterus of D9 rats from different experimental groups Lane M, Marker; Lane 1, The expression of β-actin mRNA in uterus of normal saline group; lane 2, The expression of β-actin mRNA in uterus of 100IU IFN-γ group; lane 3, The expression of β-actin mRNA in uterus of 300IU IFN-γ group; lane 4, The expression of β-actin mRNA in ovary of 300IU IFN-γ group; lane 5, The expression of β-actin mRNA in ovary of 100IU IFN-γ group; lane 6, The expression of β-actin mRNA in ovary of normal saline group.

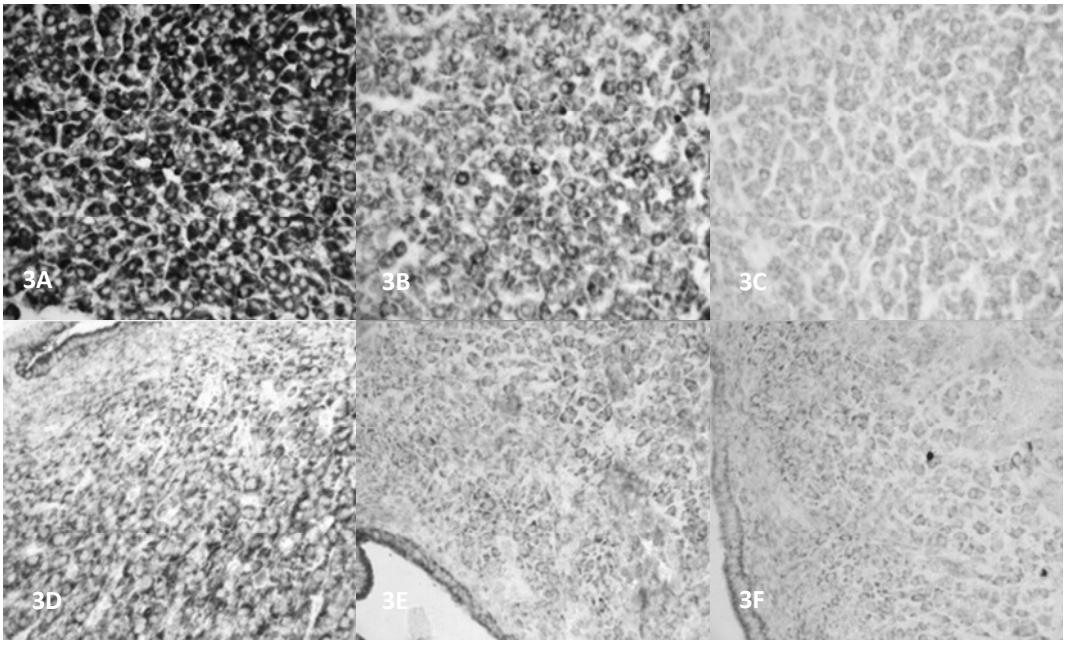

Figure 3. Immunolocalization of IGF-1 protein in ovary and uterus from each experimental group3A; The expression of IGF-1 in the ovary granulosa luteal cells of normal saline group, (400) 3B; The expression of IGF-1 in the ovary granulosa luteal cells of 100IU IFN-γ group, (400×) 3C; The expression of IGF-1 in the ovary granulosa luteal cells of 300IU IFN-γ group, (400×) 3D; The expression of IGF-1 in the uterus decidua cells of normal saline group, (400×) 3E; The expression of IGF-1 in the uterus decidua cells of 100IU IFN-γ group, (400×) 3F; The expression of IGF-1 in the uterus decidua cells of 300IU IFN-γ group, (400×).

Table 2. Expression of IGF-1 protein in ovary and uterus from each experimental group.

Treatment	Relative expression in endometrium or deciduas (μ^2)	Relative expression in uterus (μ^2)
Normal saline group	40.668 ± 3.479	23.708 ± 3.695
100IU IFN-γ group	33.091 ± 2.865*	18.803 ± 5.143*
300IU IFN-γ group	26.379 ± 4.223**	13.647 ± 3.288**

* P < 0.05, ** P < 0.01 different from normal saline group. Results are expressed as Mean ± SD.

The effects of IFN-γ on the IGF-1 immunoreactivity in ovary and uterus of pregnant rats

IGF-1 protein was mainly distributed in the endometrium or decidua of the rats. In normal saline group, the IGF-1 protein was closely arranged and deeply stained in the endometrium or decidua which was predominantly located in the cytoplasm. Higher expression of IGF-1 was also seen in the endometrial glands (Figure 3D). 100IU IFN-γ group had a higher expression of IGF-1 in the endometrial or decidua cells (Figure 3E), but it is lower than the normal saline group (P <0.05). The expression of IGF-1 in the 300IU IFN-γ group (Figure 3F) was significantly lower than those of 100IU IFN-γ group (P < 0.05) and normal saline group (P < 0.01). Details are shown in Table 2 and Figure 3 D to F. IGF-1 protein was mainly distributed in the cytoplasm of granules luteal cells with vacuoles-shaped nonstaining nucleus in ovary. The cytoplasm of the granules luteal cells was deeply stained in normal saline group rats (Figure 3A). The expression level of IGF-1 protein in ovary of 100IU IFN-γ group (Figure 3B) was lower than the normal saline group (P < 0.05), and the expression level of IGF-1 protein in ovary of 300IU IFN-γ group was the lowest (Figure 3C), which also differ significantly from the normal saline group (P < 0.01). Details are shown in Table 2 and Figure 3A to C.

The influence of IFN-γ on the IGF-1 levels in peripheral blood of pregnant rats

IGF-1 levels in peripheral blood of normal saline group was remarkably higher than those of 100IU IFN-γ and 300IU IFN-γ groups, respectively (P < 0.01). The

expression level of IGF-1 in 100IU IFN-γ group was higher than 300IU IFN-γ group ($P < 0.05$).

DISCUSSION

Pregnancy is a complex and fundamental physiological process (Orsi and Tribe, 2008). In early pregnancy, a broad repertoire of regulatory cytokine network helped the fetus to escape from maternal immune rejection. Recent studies indicated that IGF-1 played important roles in the reproductive process (Yasuda et al., 1992; Corleta et al., 2000; Johnson et al., 2001; Onagbesan et al., 2006). IGF-1 may acts on gonadotropin cells of anterior pituitary through the pituitary portal system, stimulating the release of follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH signals to the ovaries and uterus in a dependent or independent way are used to regulate the reproduction. The exogenous injection of IGF-1 to the pregnant mice could increase the substance intake of the placenta, causing the rapid assimilation and anti-catabolism of the placental protein and carbohydrate, so that there is an overall fetal weight gain (Zhou et al., 1999). In this study, IFN-γ regulated the expression of the IGF-1 in the ovary and uterus of the pregnant rat, indicating that IFN-γ was closely related with the regulation function of IGF-1 in the neuroendocrine system and immune systems which was mediated through the hypothalamus - pituitary - gonadal axis feedback pathway in the uterus and ovarian. IFN-γ and IGF-1 also could be coordinated to prevent abortion and other pregnancy complications to maintain a normal pregnancy. IFN-γ is one of the Th1-type cytokines (Lloyd and Hawrylowicz, 2009) that is essential for maintaining pregnancy. IFN-γ secreted from the pregnant rat uterine decidual cells, is involved in the pregnancy decidua reconstruction which is indispensable in maintaining the integrity of the decidua (Ashkar and Croy, 2001). Chen et al. (1994) detected that there are many cells that expressed IFN-γ receptors in the embryo implantation point. Jin et al. (2008) reported that IFN-γ had a protective role in early pregnancy on toxoplasma infection in mice, which could increase the immunity levels in the early pregnancy. Therefore, keeping a normal IFN-γ level was essential on the success of pregnancy. However, Liu et al. (2002) reported that high doses of IFN-γ could inhibit the progesterone secretion, induct the apoptosis of placental cells and caused the expression of major histocompatibility complex class II (MHCII). MHCII was one of the major antigens which could cause the maternal-fetal immune rejection during pregnancy, and were extremely detrimental for the maintenance of pregnancy.

After injection of high doses of exogenous IFN-γ, the IGF-1 expression decreased significantly compared to the normal saline group in the pregnant rat ovary, uterus and peripheral blood, suggesting that high doses of exogenous IFN-γ could regulate pregnancy by decreasing the IGF-1 level in the ovary, uterus and peripheral blood during early pregnancy.

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