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RESEARCH ARTICLE

The Expression of Visfatin in Mouse Ovary and its Regulatory Effect on IFN-γ

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The expression of visfatin in ovary and the changes of IFN-γ in peripheral blood were detected by the immunohistochemical method and the double antibody sandwich ELISA test method to investigate the distribution situation of visfatin in the ovary and its regulatory effect on the development of mouse ovarian follicles. Our research showed that visfatin positive cells were mainly located in the follicle cell layer of ovary in different developmental stages. The strongly positive staining were seen in the cells of the granular layer of growing follicle, the positive cells were numerous and compressed, and the cells of the granular layer of mature follicle also showed significantly positive reaction though their amount was fewer. Visfatin positive cells were rarely expressed and were weakly positive in oocytes, and it lacked in germinal epithelium, theca folliculi, follicular matrix and atretic follicle. Statistical results showed that the visfatin level of the control group was not significantly different from that of the protein group or the antibody group in the peripheral blood, while the protein group was significantly different from antibody group ($P<0.05$). The above results suggested that visfatin positive cells are widely expressed in the grained layer of ovarian follicle in different development stages, which shows its links with the development of follicular. The effect of visfatin on the level of IFN-γ in periphery blood tips the role of visfatin in regulating the development of follicles.

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INTRODUCTION

Visfatin promotes the synthesis and storage of fat, regulates glucose and lipid metabolism in vivo, and it is widely expressed in bone marrow stromal cell, activated lymphocyte, macrophage, liver, uterus, islet, pancreas, muscle tissues and embryolemma besides visceral adipose tissue (Samal*et al*., 1994; Ognjanovic *et al*., 2005; Ons *et al*., 2010; Kover *et al*., 2013; Jung *et al*., 2013), meanwhile. It directly correlates with the fetal growth in uterus, gestational diabetes and polycystic ovary syndrome (Shang *et al*., 2009; Ma *et al*., 2010; Dıkmen *et al*., 2011; Güdücü *et al*., 2012; Rafraf *et al*., 2012; Kaygusuz *et al*., 2013). The relationship between the expression of visfatin in uterus and embryolemma and intrauterine fetal growth, gestational diabetes and polycystic ovary syndrome suggests that visfatin might functionally relate with the reproductive system.

The formation of follicles is a highly coordinated physiological process, and the growth and development of follicle are controlled by a number of factors. Studies have shown that there are many other hormones, growth factor and cytokines in the ovary which play essential roles in the development of follicles. The follicles grow and develop orderly under the control of endocrine hormone (Britt *et al*., 2004; Lu and Zhu, 2008; Karavan and Pepling, 2012; Qin *et al*., 2013; Szafarowska and Jerzak, 2013), growth factor accurately expresses at different time points and spaces (Wang and Roy, 2004; Berisha *et al*., 2004; Liu and Ge, 2007), and the cytokines which associate with the follicular development include TNF-α, IL-1and IFN-γ (Zolti *et al*., 1991; Dobrzanski *et al*., 2012; Jackson *et al*., 2012). The clarification of the relationship between the follicular development related factors and visfatin which acts as a peptide hormone is essential for understanding the relationship between visfatin and the reproductive system as well as the mechanisms of the role of visfatin in reproduction, and furthermore discoursing the pathogenesis of related reproductive system diseases.

MATERIALS AND METHODS

Sample collection and treatments: Twenty-four eight-week old clean female Kunming White mice were

purchased from Hubei Center for Disease Control and Prevention. The experimental mice were randomly divided into 3 equal groups, the two test groups were separately injected with visfatin recombinant protein and visfatin antibody through caudal vein with a dosage of 200 μL/d/each mouse, while the mice in control group were injected with equal amount of PBS, 7 days later these mice were executed, the blood samples were collected serum was separated and stored. Bilateral ovarians were removed and placed in fixation fluid, embedded by paraffin, continuously sliced with the thickness, sections were stained by H.E. and immunohistochemistry method, observing and photographing by OLYMPUS microscopy.

Immunohistochemical staining: The immunohistochemistry staining was performed after the intact tissue morphology has been determined by H.E. staining. The paraffin sections were generally dewaxed, and immersed in PBS buffer. Then the sections were placed in plastic containers containing citrate buffer to retrieve the antigen. The sections were then incubated with 0.3% H_2O_2 to inactivate endogenous peroxides, washed with PBS and blocked by 5% normal goat serum at room temperature before incubating with the diluted primary antibody (1:100 dilution) in a humid chamber, meanwhile negative control was incubated with PBS. Following washing in PBS, a mixture of biotinylated sheep anti-rabbit (SP-0023, Bioss, Beijing, China) secondary antibodies was applied, then dropped SABC and developed dyeing by DAB. Finally, the sections were washed by distilled water, dehydrated, transparentized and mounted.

Double anti-sandwich ELISA method: The Kit (Chemclin Biotech, Beijing, China) was balanced to room temperature. The standards and samples were respectively added into the reaction plates. Then the liquid was removed and the reaction plates were washed with washing liquid. The anti-mouse IFN-γ Biotin was added into each well. Then the prepared 1xHRP was added into each well. After removing the liquid and washing the reaction plates, the TMB chromogenic reagent was added into each well. Stop solution was added into each well. After drawing the standard curve with OD value as the Y-coordinate, and standard concentrations as the X-coordinate, the concentration of sample determined by the standard curve according to the OD value of the sample.

Data processing and result judgment: Immunohistochemistry staining: Ten view fields were taken randomly on each section to observe the expression of immunoreactive cells. Result judgment standards: The staining in control group sections was negative (-), no positive staining within the cells. The cytoplasm or cytoplasmic membrane of positive cells showed brownish yellow (Song *et al*., 2012).

ELISA method: The data was analyzed by SPSS 11.5 software, the statistical results were indicated by mean±SD and the differences between each group were compared by t test, $P<0.05$ meant significantly different, which was expressed as a-b.

RESULTS

Immunohistochemical staining results and analysis: Round or oval visfatin immunoreactive cells were observed under microscope in those sections dyed by immunohistochemical method, which showed brownish yellow of different levels.

Ovarian follicles in different stages, such as the growing follicles (primary and secondary follicles), mature follicles and atretic follicles could be observed in the ovary of mice. The visfatin immunoreactive cells were more commonly seen in ovarian cortex, visfatin widely expressed in the follicle cells in different developmental stages, visfatin positive cells mainly located in the follicle cell layer of ovary in different developmental stages (Fig.1A), and strongly positive visfatin staining could be seen in the cells of the granular layer of growing follicle, the positive cells were numerous and compressed (Fig.1A, 1C and 1D), and the cells of the granular layer of mature follicle also showed significantly positive reaction though their amount was fewer (Fig.1A). Visfatin positive cells were rarely or weakly expressed in oocytes (Fig.1C), while it was lacked in germinal epithelium, theca folliculin, ovarian stromal and follicular atresia. Thus we concluded that visfatin positive products were mainly distributed in the cells of stratum granulosum in growing follicles. The result in control group was negative (Fig.1B).

ELISA test results and analysis: The levels of IFN-γ in serum in control group, visfatin antibody group, visfatin protein group were respectively 26.766±24.09, 25.869±13.44 and 45.179±20.77 pg/ml. The statistical results showed that the level of IFN-γ in visfatin antibody group and visfatin protein group were different with that in control group. The level of IFN-γ was increased in visfatin protein group as compared to control group, but the difference was not significant ($P=0.146$), while it was decreased in visfatin antibody group as compared to control group, and the difference was not significant too ($P=0.934$). While the difference between visfatin protein group and visfatin antibody group on the level of IFN-γ was significant ($P<0.05$) (Fig. 2).

DISCUSSION

The location expression of visfatin in the ovary: Visfatin is a kind of protein cytokine secreted by adipose tissue, it specifically and highly expresses in visceral adipose tissue. Researchers have shown that visfatin is widely expressed in bone marrow stromal cell, activated lymphocyte, macrophage, liver, uterus, pancreas, islet, muscle tissues and embryolemma besides visceral adipose tissue (Samal *et al*., 1994; Ognjanovic *et al*., 2005; Ons *et al*., 2010; Kover *et al*., 2013; Jung *et al*., 2013). The finding of visfatin promotes the research of fetal growth in uterus, gestational diabetes and polycystic ovary syndrome, etc., and the mechanism of its action has been payedmore and more attention. However, many issues such as the distribution of visfatin in generative organ and the correlation between visfatin and the cells in generative organ still needs to be clarified. The relationship between the expression of visfatin in uterus and embryolemma and

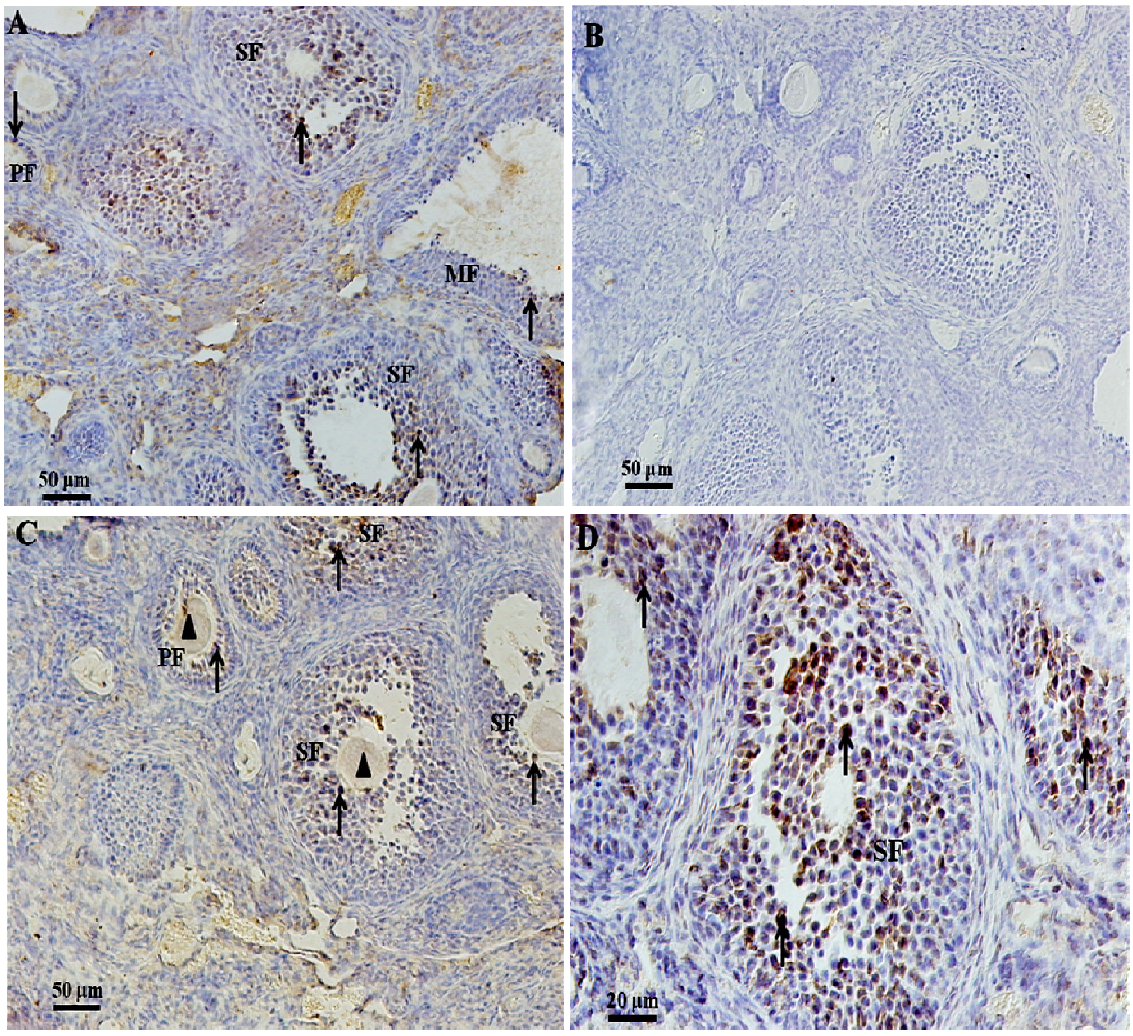

Fig. 1: Distribution of visfatin immunoreactive cells in the ovary. (A) The distribution of visfatin positive cells in the follicle cells (arrow), Primary Follicle (PF), Secondary Follicle (SF) and Mature Follicle (MF) (10×20), (B) Distribution of visfatin positive cells in ovary in negative control (10×20), (C) Distribution of visfatin positive cells in the granular layer of growing follicle(arrow) and the oocytes (triangle) (10×20), (D) Distribution of strongly visfatin positive cells in the granular layer of secondary follicle (SF) (arrow) (10×40).

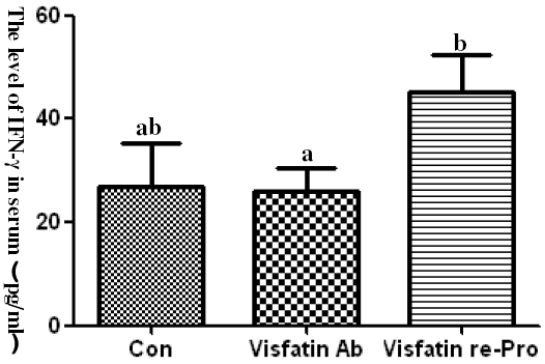

Fig. 2: The changes on the level of IFN-γ in serum after the treatment of visfatin recombinant protein and visfatin antibody (n≥8). Alphabets a-b indicate significant difference (P<0.05), Control group (Con), visfatin antibody group (visfatinAb), visfatin recombinant protein group (visfatin re-Pro).

intrauterine fetal growth, gestational diabetes and polycystic ovary syndrome suggests that visfatin might functionally relate with the reproductive system. Rare reports concerned whether visfatin was expressed ovary so far. Ovarian follicles is one of the normal tissues which grow most rapidly, the differentiation of granular cell which constitutes ovarian follicles with oocytes is the primary cause of follicle growth. Granular cell plays important roles in the initiation of primary follicle and the following follicle development. During to the fact that granular cells synthesize multiple hormones and growth factors and express their receptors, as well as regulate the growth, and differentiation and mature of thecal cells and oocytes through gap connections, the growth and differentiation of granular cells can be regarded as the standard of follicle development (Webb *et al*., 2004; Hirao, 2011; Son *et al*., 2011; Tavasoli *et al*., 2013). This research found that visfatin immunoreactive cells could be seen in different development stages of mouse ovarian follicles (primary follicle and secondary follicle), especially in the granular layer of growing ovarian follicle, these results not only verified the correlation between visfatin and reproduction, but also indicated the action of visfatin in the development and function of ovarian follicle due to the significant expression of

visfatin in granulosa cell, however, the exact mechanism of the action of visfatin on follicle development, whether visfatin receptor expresses in the ovary and whether visfatin is secreted by follicle are still needed to be studied.

Visfatin regulates the expression level of IFN-γ in peripheral blood: Visfatin is a protein hormone secreted by adipose tissue, researches in recent years have shown that visfatin not only regulated energy metabolism balance, but also associated with the reproductive function (Chan *et al*., 2007; Kowalska *et al*., 2007; Rafraf *et al*., 2012; Kaygusuz *et al*., 2013). The ovarian follicular development is regulated by a number of hormones and cytokines, such as TNF-α, IL-1 and IFN-γ (Zolti *et al*., 1991; Dobrzanski *et al*., 2012; Jackson *et al*., 2012). The relationship between visfatin, a peptide hormone, and those factors affecting follicular development is unclear. In this research we found that the level of IFN-γ in serum was altered along with the variation of visfatin, which suggested that visfatin affected the expression of IFN-γ. Statistical analysis of data showed that the levels of IFN-γ in visfatin protein group and visfatin antibody group were different with that in control group, while the difference was not significant, we surmised that due to the fact that the time during injecting visfatin recombinant protein and visfatin antibody through caudal vein lastsshort, which caused that there was no significant difference on the level of visfatin in vivo between the treated and control groups. However, the effect of visfatin on the expression level of IFN-γ in mouse serum was significantly different between visfatin recombinant protein group and antibody group. The fact that the level of IFN-γ was raised when visfatin increased (visfatin recombinant protein group) further indicated that the effect of visfatin on follicular development was exerted mainly through regulating these follicular development related cytokines, and this effect was somewhat dose dependent. The formation of follicles is a highly coordinated physiological process, and the growth and development of follicles were under the control of a number of factors. However, the level of IFN-γ in peripheral blood just is one of the factors which affect the follicular development, and other relevant factors are also needed to be further studied to clarify the action of visfatin in follicular development.

Conclusion: Visfatin positive cells are widely expressed in the grained layer of ovarian follicle in different development stages, and they are linked with the development of follicular. Visfatin regulated the level of IFN-γ in periphery blood, and the plasma IFN-γ increased along with the increase of visfatin, which indicates that visfatin could regulate the development of follicles.

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