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Effect of atorvastatin on diabetic rat endothelial cells and retinal lesions

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ABSTRACT. We investigated the effect of atorvastatin on vascular endothelial growth inhibitor (VEGI) expression in rats with diabetic retinopathy. Wistar rats were divided into a blank group and diabetic model group, which was further randomly divided into treatment and control groups. Rats in the treatment group received 10 mg/kg atorvastatin daily, while rats in the blank and control groups received normal saline. Rats were randomly euthanized at 3 or 6 months. Immunohistochemical staining was used to determine changes in VEGI and vascular endothelial growth factor, interleukin-4, and tumor necrosis factor α levels in rats with diabetic retinopathy. Survival rate in the treatment group was 84% (63/75) after 6 months, which was significantly higher than that in the control group ($P < 0.05$); rats in the control group showed the lowest survival rate. Survival in the treatment group was higher than that in the control group but not significant compared with the blank group after 3 months. VEGI, vascular endothelial growth factor, tumor necrosis factor α, and interluekin-4 expression was lower than that in the control group, but higher than the blank group after 3 months. The

expression of each factor decreased to the blank group level in the treatment group and was significantly lower than that in the control group after 6 months ($P < 0.05$). Expression in control and blank groups was similar at 3 and 6 months. Atorvastatin can inhibit VEGI and vascular endothelial growth factor expression to protect rats from diabetic retinopathy.

Key words: Atorvastatin; Diabetic retinopathy; Wistar rats; Vascular endothelial growth inhibitor; Vascular endothelial growth factor

INTRODUCTION

The incidence of diabetic retinopathy (DR), a major and serious microvascular lesion of diabetes mellitus (DM), is continuously increasing in population under the age of 40. The incidence of DR has increased with the development of DM to 44.4% in 5 years and 56% in 7 years (Antonetti et al., 2012; Tsagkataki et al., 2012). DR is one of the most common causes of blindness in diabetic patients; this disease is characterized by retinal microvascular progressive damage, increased vascular permeability, neovascularization, and loss of vision (Lim et al., 2009; Valery et al., 2014). The occurrence and development of DR is associated with multiple growth factors and cytokines, including vascular endothelial growth inhibitor (VEGI), vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumor necrosis factor (TNF), and C-reactive protein (Thomas et al., 2012). VEGF and basic fibroblast growth factor can both specifically promote vascular endothelial cell growth and play an important role in new vessel formation in retina ischemia anoxic lesions. VEGI can interact with and influence other factors to restrain new vessel formation. Atorvastatin is a drug commonly used to treat cardiovascular diseases. It is typically used clinically for cardiovascular complications in patients with long-term DM. However, its effects on the retinal microvasculature have not been thoroughly examined (Suzuma et al., 2001). In this study, we investigated the effect of atorvastatin on VEGI in diabetic retinopathic rats and examined the expression levels of other relevant factors to evaluate the advantages and disadvantages of atorvastatin treatment.

MATERIAL AND METHODS

General information

Rats were used for all experiments, and all procedures were approved by the Animal Ethics Committee of The First Hospital of Jilin University (Jilin, China). A total of 220 specific pathogen-free-grade adult Wistar rats (health, unlimited gender, and weighted 200-250 g) were obtained from the Jilin University Laboratory Animal Center. The rats were fed in a barrier environment, which was maintained at a temperature of 20°-26°C, relative humidity of 40-70%, and ammonia concentration of no more than 14 mg/m^3. Fifty rats were randomly selected for the blank group, while the remaining rats were prepared for the diabetes model (model group). Model group preparation was successful with 150 rats. Rats were randomly divided into 2 equal groups: treatment group and control group.

Modeling method

After fasting for 12 h, the rats were injected with 2% solution containing streptozotocin and 0.1 M citric acid buffer, pH 4.5, at a concentration of 65 mg/kg. The rats were caged with 1 rat in each cage after successful injection and detection of urine glucose and blood glucose after 48 h. The following modeling success standard was used: urine glucose > + + + and blood glucose over 16.7 mM.

Therapeutic method

Rats in the model group were treated with 10 mg/kg atorvastatin 14 days after successful model preparation, while rats in the blank and control groups were given equal quantity of normal saline. The rats were randomly euthanized at 3 or 6 months, and the eyeballs were fixed. VEGI and VEGF expression was observed after fixing of the eyeball.

Sampling method

After treatment for 3 or 6 months, 2 mL blood was collected from the femoral artery, centrifuged for 10 min (1000 *g*), and the serum was refrigerated. The left eyeball was removed under anesthesia, and the vitreous fluid was removed after opening the corneal limbus. The right eyeball was fixed in 4% paraformaldehyde for 24 h.

Detection method

An enzyme-linked immunosorbent assay was used to determine the cytokine TNF-α and interleukin (IL)-4 expression levels according to the manual. The kit for these experiments was purchased from Tianjin JunYao LianYing Biological Co., Ltd. (Tianjin, China). The fixed right eyeball was routinely dehydrated and embedded using paraffin. An enzyme-linked immunosorbent assay kit for VEGI and VEGF (R&D Systems, Minneapolis, MN, USA) was used for detection under a microscope, and positive cells were counted.

Statistical analysis

The SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA) was used for data analyses. A χ^{2-} test was applied for enumerate data, while the *t*-test was applied for measurement data, with statistically significant differences defined as $P < 0.05$.

RESULTS

Rat survival rate in each group

The survival rate of rats in the treatment group was 84% (63/75) after 6 months, which was significantly higher than that in the control group, blank group, and treatment group after 3 months ($P < 0.05$). Rats in the control group presented the lowest survival rate. The survival rate in the treatment group was better than that in the control group after 3 months, while the difference was not significant compared with the blank group (Table 1).

Table 1. Comparison of rat survival rate among the groups.

Group	N	3 months	6 months
Blank group (a)	50	37 (74.0)	34 (68.0)
Control group (b)	75	46 (61.3)	35 (46.7)
Treatment group (c)	75	54 (72.0)	63 (84.0)
χ^2		χ^2 (a *vs* b) = 2.344; χ^2 (a *vs* c) = 0.664; χ^2 (b *vs* c) = 2.022	χ^2 (a *vs* b) = 2.664; χ^2 (a *vs* c) = 3.344; χ^2 (b *vs* c) = 4.522
P value		P < 0.05; P > 0.05; P < 0.05	P < 0.05; P < 0.05; P < 0.05

Changes in VEGI, VEGF, TNF-α, and IL-4 expression levels in each group

VEGI, VEGF, TNF-α, IL-4 expression levels were 33.4 ± 2.7 mg/L, 25.9 ± 3.1 mg/L, 84.3 ± 4.5 pg/mL, and 24.5 ± 9.7 ng/L in the treatment group after 3 months, respectively. These values were lower than that in the control group, but higher than the blank group (Table 2). The expression of each factor decreased to the blank group level in the treatment group after 6 months, which was significantly lower than that in the control group (P < 0.05). The expression of factors in the control and blank group was very similar at 3 and 6 months (Table 3).

Table 2. Changes in VEGI, VEGF, TNF-α, and IL-4 expression levels after 3 months.

Group	TNF-α (pg/mL)	VEGI (mg/L)	IL-4 (ng/L)	VEGF (mg/L)
Treatment group (a)	84.3 ± 4.5	33.4 ± 2.7	24.5 ± 9.7	25.9 ± 3.1
Control group (b)	97.2 ± 6.4	47.6 ± 2.5	36.2 ± 9.8	32.2 ± 2.8
Blank group (c)	76.5 ± 5.6	13.6 ± 2.6	16.5 ± 9.4	12.7 ± 2.6
T value	T value (a *vs* b) = 2.665; T value (a *vs* c) = 2.342; T value (b *vs* c) = 2.784	T value (a *vs* b) = 2.245; T value (a *vs* c) = 2.532; T value (b *vs* c) = 2.774	T value (a *vs* b) = 2.325; T value (a *vs* c) = 2.542; T value (a *vs* c) = 2.777	T value (a *vs* b) = 2.335; T value (a *vs* c) = 2.654; T value (a *vs* c) = 2.875
P value	P < 0.05; P < 0.05; P < 0.05	P < 0.05; P < 0.05; P < 0.05	P < 0.05; P < 0.05; P < 0.05	P < 0.05; P < 0.05; P < 0.05

Table 3. Changes in VEGI, VEGF, TNF-α, and IL-4 expression levels after 6 months.

Group	TNF-α (pg/mL)	VEGI (mg/L)	IL-4 (ng/L)	VEGF (mg/L)
Treatment group (a)	74.7 ± 2.1	13.1 ± 1.1	17.1 ± 7.8	13.7 ± 4.2
Control group (b)	95.4 ± 2.2	45.4 ± 1.9	33.2 ± 8.1	36.4 ± 4.7
Blank group (c)	75.7 ± 2.4	12.6 ± 1.7	16.8 ± 8.2	13.7 ± 4.5
T value	T value (a *vs* b) = 2.665; T value (a *vs* c) = 0.622; T value (b *vs* c) = 2.784	T value (a *vs* b) = 2.575; T value (a *vs* c) = 0.542; T value (b *vs* c) = 2.664	T value (a *vs* b) = 2.735; T value (a *vs* c) = 0.732; T value (a *vs* c) = 2.684	T value (a *vs* b) = 2.655; T value (a *vs* c) = 0.632; T value (a *vs* c) = 2.754
P value	P < 0.05; P > 0.05; P < 0.05	P < 0.05; P > 0.05; P < 0.05	P < 0.05; P > 0.05; P < 0.05	P < 0.05; P > 0.05; P < 0.05

DISCUSSION

Most diabetic patients focus on blood glucose level, while diabetic vascular complications are largely ignored (Mottaghi et al., 2013; de Korte et al., 2014). The pathogenesis of DR is unclear. Traditionally, DR was thought to be caused by numerous factors, while modern research suggests that DR is caused by changes in multiple factors, such as blood glucose, blood fat, glycosylated hemoglobin, retinal blood dynamics, and hemorheology, leading to blood-retinal barrier damage and retinal neovascularization (Chen et al., 1989; He et al., 2013).

Atorvastatin is commonly used to treat vascular lesions because it promotes microvascular formation. Whether it exacerbates DR development still remains unclear (Fong et al., 1999; Sundling et al., 2013). Microvascular formation is affected by a variety of factors that commonly function in regulation and interference.

VEGF is a polypeptide growth factor that is closely related to vascular proliferation. It is a potent molecule involved in each step of neovascularization growth. In a previous study, researchers injected VEGF into the primate vitreous cavity and observed induction of capillary occlusion, microaneurysm, hemorrhage, and neovascularization similar to the effects in DR (Knappe et al., 2013; Looker et al., 2013). Chronic hyperglycemia can cause VEGF overexpression, while DR severity is correlated with VEGF level. *In vitro* experiments showed that VEGF can induce endothelial cells to produce specific factors that destroy the basement membrane and promote capillary endothelial cell migration to form the collagen matrix and capillary microtubules (Tsagkataki et al., 2012). Additionally, ILs and TNF are involved in DR lesion development, as they can directly or indirectly increase VEGF levels to promote vascular endothelial cell proliferation and are positively correlated with DR degree. In this study, VEGF, TNF-α, and IL-4 increased in the control group, which is consistent with the results of a previous study (Tanaka et al., 2013).

VEGI is an autocrine molecule expressed in endothelial cells. Its main role is to restrict new vessel formation, and it is the main factor involved in autoimmune disease (Chew et al., 2013; Scanlon et al., 2013). Numerous studies have suggested that VEGI concentration was increased in the serum of patients with DR (Valery et al., 2014) and is excessively overexpressed in patients with hyperplasia DR. It also significantly increased in the vitreous humor, indicating its role in DR development. After surgical treatment for DR, symptoms were significantly alleviated, confirming the results of previous studies (Laursen et al., 2013). In the present study, VEGI concentration was significantly higher in the control group DR rats than the blank group rats, which is consistent with the other results of this study. VEGI primarily antagonizes VEGF function; VEGI and VEGF expression were found to be positively correlated in previous studies (Vujosevic et al., 2013; Guo et al., 2014).

Atorvastatin treatment was applied to treat DR rats in our experiments, and VEGI and VEGF levels in the eyeball were determined. Our results showed that the survival rate in the treatment group rats was higher than that in the control, indicating that atorvastatin has a therapeutic effect in diabetic rats. The VEGI, VEGF, TNF-α, and IL-4 expression levels were lower in the treatment group than that in the control group after 3 months, but higher than that in the blank group. The expression of each factor decreased to the blank group level in the treatment group after 6 months, while these values were lower than the control group and 3-month group. As the treatment time extended to 6 months, the level of the measured factors gradually decreased in the treatment group and became close to the values in the blank group. These results not only confirmed the role of atorvastatin for the treatment of diabetic rats, but also revealed that atorvastatin did not promote retinal microvascular formation. The drug also played a protective role in the retina by lowering the levels of microvascular formation-promoting factors. A previous study suggested that different doses of atorvastatin showed different effects on diabetic rats (Lim et al., 2009). Low-dose atorvastatin showed a strong inhibitory effect on microvascular formation. In our study, we used low-dose 10 mg/kg atorvastatin therapy and found that it clearly inhibited the DR progress. This illustrated that atorvastatin application may be dose-dependent. Further studies are needed to explore the effects of different doses of atorvastatin on DR.

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