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Exploration of the preventive effect of ursolic acid on retinopathy in diabetic mice and its mechanism

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ABSTRACT

Objective: To study the preventive effect of ursolic acid on retinopathy in diabetic mice through adjusting insulin sensitivity, glucose transport, angiogenesis and inflammation. **Methods:** Male C57BL/6 mice were selected as experimental animals and randomly divided into control group (N group), model group (D group) and intervention group (D+UA group), D group and D+UA group established diabetes models through intraperitoneal injection of STZ, D+UA group received intragastric administration of ursolic acid, and then insulin sensitivity, glucose metabolism in retina as well as the expression levels of GLUTs, HIF-1 α /VEGF/ VEGFR2 pathway and IKK β /IKB /NF- κ B pathway in retina tissue of three groups were detected. **Results:** AUC of D group was significantly lower than that of N group, and HOMA-IR, sugar content in retina tissue as well as GLUT-1, GLUT-3, HIF-1 α , VEGF, VEGFR2, IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels were significantly higher than those of N group; AUC of D+UA group was significantly higher than that of D group, and HOMA-IR, sugar content in retina tissue as well as GLUT-1, GLUT-3, HIF-1 α , VEGF, VEGFR2, IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels were significantly lower than those of D group. **Conclusion:** Ursolic acid can increase insulin sensitivity, reduce sugar content in retina tissue and inhibit angiogenesis and inflammation degree in retina tissue, and has preventive effect on retinopathy in diabetic mice.

1. Introduction

Diabetic retinopathy (DR) is the most common microvascular complication of diabetes, and macular edema, vitreous hemorrhage and retinal detachment often cause vision loss and even blindness of patients. At present, the pathogenesis of DR has not been fully elucidated, and clinical effective means for the treatment of DR is also needed. Existing research believes that the important pathological characteristics of DR are local insulin resistance as well as excessive retina angiogenesis and excessive activation of local inflammation caused by high glucose environment[1,2], the key to DR treatment is improving insulin resistance, changing local high glucose environment, inhibiting angiogenesis and reducing inflammation. Ursolic acid is a kind of herbal extract with extensive biological effects, and can adjust insulin sensitivity, glucose transport, angiogenesis and inflammation[3,4]. There are already animal researches about ursolic acid for DR treatment, but the pathological changes at molecular levels in retina tissue after ursolic acid treatment have not yet been elucidated. In the following research, the preventive effect of ursolic acid on retinopathy in diabetic mice was analyzed from four aspects: insulin sensitivity, glucose transport, angiogenesis and inflammation.

2. Materials and methods

2.1 Materials

After the approval of the hospital ethics committee, male C57BL/6 mice were chosen as experimental animals, there were a total of 45 mice with body mass 27-30 g, and they were bought and fed by Hebei University animal center; ursolic acid powder was purchased

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from Sigma Company.

2.2 Animal grouping and model establishment methods

After adaptable feeding for a week, C57BL/6 mice were randomly divided into control group (N group), model group (D group) and intervention group (D+UA group), 15 in each group. D group and D+UA group were fasting for 12 h and then received one-time intraperitoneal injection of STZ, the dose was 60 mg/kg, solvent was citric acid buffer with pH=4.2, and N group received intraperitoneal injection of same dose of citric acid buffer. At 72 h, tail vein blood was collected, and blood glucose >16.7 mmol/L indicated successful establishment of diabetes models, which were used for the subsequent intervention and detection.

2.3. Ursolic acid intervention methods

D+UA group received intragastric administration of ursolic acid, the dose was 50 mg/kg/d, the solvent was normal saline, and N group and D group received intragastric administration of same dose of normal saline. Mice received normal diet and free drinking during intervention, tail venous blood was collected every 2 weeks to determine blood glucose, and mice with blood glucose >30 mmol/L received subcutaneous injections of insulin zinc protamine 1 IU. After 4 consecutive weeks of intervention, OGTT experiment was carried out at first, the dose of glucose powder was 2 g/kg, and before glucose load as well as 30 min, 60 min and 120 min after glucose load, peripheral blood was collected and centrifuged to get serum specimens; after OGTT test, mice were executed, eye balls were collected and dissected to get the retinal tissue. All specimens were numbered and kept at -80 ℃ refrigerator.

2.4 Serum index detection and assessment methods

Serum specimens before glucose load were taken, and automatic biochemical analyzer was used to determine fasting blood glucose (FBG) and fasting blood insulin (FBI) levels; serum specimens after glucose load were taken, automatic biochemical analyzer was used to determine blood glucose levels at 30 min, 60 min and 120 min, which were BG30, BG60 and BG120 respectively. The following method was followed to calculate the area under curve of blood glucose (AUC)= (FBG+BG30) 15/60+(B30+BG60) 15/60+(B60+BG120) 30/60, and the unit was mg•h/dL; the following method was followed to calculate the degree of insulin resistance: HOMA-IR=FBG FBI/22.5.

2.5. Detection methods of protein contents of molecules in retina tissue

Retina tissue was taken, added to appropriate amount of PBS solution and then homogenized, homogenate suspension was collected and centrifuged, the sedimentation after centrifuge was discarded, supernatant was kept, and enzyme-linked immunosorbent kit and matched microplate reader were used to detect GLUT-1, GLUT-3, HIF-1 α , VEGF, VEGFR2, IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels.

2.6 Statistical methods

SPSS 20.0 software was used to input and process data, analysis among three groups was by variance analysis and $P<0.05$ indicated statistical significance in differences.

3. Results

3.1. Insulin sensitivity

AUC and HOMA-IR of three groups were different; comparison between D group and N group was as follows: AUC of D group was significantly lower than that of N group, and HOMA-IR was significantly higher than that of N group; comparison between D+UA group and D group was as follows: AUC of D+UA group was significantly higher than that of D group, and HOMA-IR was significantly lower than that of D group.

Table 1.
In sulin sensitivity of three groups.

Group	*n*	AUC (mg/h/dL)	HOMA-IR
N	15	7.38±1.04	2.89±0.57
D	15	3.35±0.75$^{\triangle\triangle}$	4.92±0.67$^{\triangle\triangle}$
D+UA	15	5.92±0.93*	3.44±0.49**

Note: AUC: area under curve of blood glucose; HOMA-IR: insulin resistance index; $^{\triangle}$: compared with N group, $P<0.05$; $^{\triangle\triangle}$: compared with N group, $P<0.01$; *: compared with D group, $P<0.05$; **: compared with D group, $P<0.01$.

3.2. Glucose metabolism in retina

Sugar content in retina tissue as well as GLUT-1 and GLUT-3 levels of three groups were different; comparison between D group and N group was as follows: sugar content in retina tissue as well as GLUT-1 and GLUT-3 levels of D group were significantly higher than those of N group; comparison between D+UA group and D group was as follows: sugar content in retina tissue as well as GLUT-1 and GLUT-3 levels of D+UA group were significantly lower than those of D group.

Table 2.
Glucose metabolism in retina of three groups.

Group	*n*	Sugar content in retina (nmol/mg protein)	GLUT-1 content (ng/mg protein)	GLUT-3 content (ng/mg protein)
N	15	38.95±5.59	13.53±2.52	5.45±0.71
D	15	189.33±32.54$^{\triangle\triangle}$	45.61±7.82$^{\triangle}$	17.58±2.86$^{\triangle\triangle}$
D+UA	15	87.21±12.15*	33.29±4.49**	11.35±2.16*

Note: GLUT-1: glucose transporter-1; GLUT-3: glucose transporter-3; $^{\triangle}$: compared with N group, $P<0.05$; $^{\triangle\triangle}$: compared with N group, $P<0.01$; *: compared with D group, $P<0.05$; **: compared with D group, $P<0.01$.

3.3 HIF-1 α /VEGF/VEGFR2 levels in retina

HIF-1 α , VEGF and VEGFR2 levels in retina tissue of three groups were different; comparison between D group and N group was as follows: HIF-1 α , VEGF and VEGFR2 levels in retina tissue of D

group were significantly higher than those of N group; comparison between D+UA group and D group was as follows: HIF-1 α , VEGF and VEGFR2 levels in retina tissue of D+UA group were significantly lower than those of D group.

Table 3.
HIF-1 α /VEGF/VEGFR2 levels in retina of three groups.

Group	*n*	HIF-1 α (ng/mg protein)	VEGF (ng/mg protein)	VEGFR2 (ng/mg protein)
N	15	35.86±6.55	127.76±20.34	73.62±10.35
D	15	103.28±19.14$^{\triangle\triangle}$	293.45±52.68$^{\triangle}$	193.41±26.74$^{\triangle}$
D+UA	15	67.81±9.31*	182.44±29.16*	113.52±21.35**

Note: HIF-1 α : hypoxia inducible factor-1 α ; VEGF: vascular endothelial growth factor; VEGFR2: vascular endothelial growth factor 2; $^{\triangle}$: compared with N group, $P<0.05$; $^{\triangle\triangle}$: compared with N group, $P<0.01$; *: compared with D group, $P<0.05$; **: compared with D group, $P<0.01$.

3.4 IKK β /IKB α /NF- κ B levels in retina

IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels in retina tissue of three groups were different; comparison between D group and N group was as follows: IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels in retina tissue of D group were significantly higher than those of N group; comparison between D+UA group and D group was as follows: IKK β , IKB , NF- κ B, TNF- α , ICAM-1, VCAM-1 and E-selectin levels in retina tissue of D+UA group were significantly lower than those of D group.

4. Discussion

Diabetic retinopathy is a common microvascular complication in patients with type 2 diabetes, its pathogenesis is complex and has not yet been fully clear, and it can cause patients' vision loss and even blindness. Insulin resistance is a common pathological feature of patients with type 2 diabetes, and also the common pathological physiological basis of a variety of complications in various diabetic patients[7,8]. In the research, diabetic animal models were established through intraperitoneal injection of STZ, then the insulin sensitivity of control group and model group were compared, and the results showed that AUC of D group decreased significantly and HOMA-IR significantly increased. It indicated that the diabetic mouse models in the study were with obvious insulin resistance. These animal models were used as the research objects to deeply explore the preventive effect of ursolic acid on diabetic retinopathy.

Ursolic acid is a class of weakly acidic pentacyclic triterpenoid compound extracted from Chinese herbal medicine, is with a variety of biological activities, and can adjust glucolipid metabolism and insulin sensitivity[9]. After ursolic acid intervention in the research, the changes of insulin sensitivity in diabetic mice were analyzed, and the results show that AUC of D+UA group was significantly higher than that of D group, and HOMA-IR was significantly lower than that of D group. This meant that ursolic acid could increase the insulin sensitivity in diabetic mice, thus preventing diabetic complications. The most basic pathological change of diabetic retinal complications is persistent high glucose in local retina caused by insulin resistance, then activating oxidative stress and inflammation, forming new blood vessels and causing retinal damage. In the study, sugar content significantly increased in retinal tissue of diabetic model mice, and after ursolic acid intervention, the sugar content in retina tissue significantly decreased. This meant that ursolic acid could not only regulate the insulin sensitivity in diabetic model mice, but could also directly reduce the sugar content in retinal tissue of the diabetic model mice. Therefore, it was believed that ursolic acid had preventive value for diabetic retinopathy.

In recent years, more and more researches elucidate the possible pathogenesis of diabetic retinopathy at the molecular level. Glucose transporters (GLUTs) are the important proteins involved in glucose metabolism and transport in the body, among which GLUT-1 and GLUT-3 are expressed in the retina, and are the important carriers that mediate blood glucose crossing through the blood-retina barrier. Study has confirmed that in diabetic retinopathy tissue, GLUT-1 and GLUT-3 expression levels significantly increase[10], and targeted injection of GLUT-1 siRNA can reduce the sugar content in the retinal tissue of diabetic animal models[11]. In order to further clarify whether ursolic acid adjusted the expression of GLUT-1 and GLUT-3 so as to reduce the sugar content in retinal tissue of the diabetic mice, the GLUT-1 and GLUT-3 expression levels in retinal tissue were analyzed in the research, and the results proved that GLUT-1 and GLUT-3 expression levels significantly increased in retinal tissue of diabetic model mice, and ursolic acid could inhibit the expression of GLUT-1 and GLUT-3 in retinal tissue.

In the occurrence and development of diabetic retinopathy, the high glucose environment in retina caused by increased expression of GLUTs will further cause retinopathy through a variety of downstream signaling pathways. According to the existing research, high glucose environment can cause local angiogenesis through HIF-1 α /VEGF/VEGFR2 pathway, and can also increase the secretion of TNF- α , ICAM-1, VCAM-1, E-selectin and other cytokines through IKK β /IKB /NF- κ B pathway. Angiogenesis is the basic pathological characteristic of IV-VI phase diabetic retinopathy, and at this time, retinal microcirculation has been unable to compensate the local tissue hypoxia, and massive angiogenesis

Table 4.
IKK β /IKB /NF-kB levels in retina of three groups.

Group	IKK β (ng/mg protein)	IKB (ng/mg protein)	NF- κ B (ng/mg protein)	TNF- α (ng/mg protein)	ICAM-1 (ng/mg protein)	VCAM-1 (ng/mg protein)	E-selectin (ng/mg protein)
N	23.9±4.1	11.5±2.8	58.4±9.3	9.3±1.1	18.4±2.8	13.6±2.3	33.2±5.6
D	83.4±11.3$^{\triangle\triangle}$	56.8±9.2$^{\triangle\triangle}$	134.4±18.7$^{\triangle\triangle}$	24.6±5.7$^{\triangle\triangle}$	55.3±8.7$^{\triangle\triangle}$	30.5±5.4$^{\triangle}$	89.3±12.7$^{\triangle}$
D+UA	45.7±8.2*	30.1±5.5 **	80.6±11.7*	13.9±2.8*	30.2±5.2**	21.4±3.9*	50.2±7.8**

Note: TNF- α : tumor necrosis factor- ; ICAM-1: intercellular adhesion molecule-1; VCAM-1: vascular cell adhesion molecule-1; E-selectin; $^{\triangle}$: compared with N group, $P<0.05$; $^{\triangle\triangle}$: compared with N group, $P<0.01$; *: compared with D group, $P<0.05$; **: compared with D group, $P<0.01$.

will increase local exudation and cause retinal damage[12,13]. IKK β /IKB /NF- κ B, through the mediated inflammatory cytokines, adhesion molecules and chemokines, can activate inflammatory response so as to destroy blood-retina barrier, increase local hyperosmolar hyperglycemic remission and cause retinal damage[14-16]. In the research, analysis of the levels of above signal pathway molecules in the retinal tissue showed that ursolic acid could inhibit the activation of HIF-1 α /VEGF/VEGFR2 pathway and IKK β / IKB /NF- κ B pathway in retina tissue.

To sup up, ursolic acid has preventive effect on retinopathy in diabetic mice, and the molecular mechanisms for the drug to exert preventive effect include increasing insulin sensitivity, reducing sugar content in retina tissue and inhibiting angiogenesis and inflammation degree in retina tissue.

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