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Rac1 inhibition protect against platelet induced organ injury in Diabetes Mellitus

Helen Jawdat Sabri (BSc)[1] , Rundk Ahmad Hwaiz (PhD)

[1] Department of Clinical Biochemistry, College of Health Sciences, Hawler Medical University, Erbil ,Iraq

[2] Department of Medical Biochemical analysis, Cihan University, Erbil , Iraq

Abstract

Background: Diabetes mellitus is one of the common causes for activation of platelet. Inflammation-induced abnormal platelet function contributes to chronic complications, which are consider the leading causes of death and morbidity among diabetics.

Objective: Rac1, a 21kD G-protein has been shown to regulate a variety of platelet functions; we predicted that Rac1 could regulate platelet release of CXCL4 and CCL5, which may leads to organ injury in Diabetes Mellitus.

Patients and Methods: Diabetes Mellitus' effect on Rac1 activation implicated in platelet activation, was investigated as platelet-induced inflammation and organ injuries. Swiss albino male mice were pretreated with 5 mg/kg of a specific Rac1 inhibitor NSC23766 and injected with (45 mg/kg body wt.) streptozotocin, twice for five days. Moreover, the concentration of serum chemokines CXCL4 and CCL5 were assayed using ELISA, and histology scores for kidney and pancreas was examined.

Results: Our results show that Diabetes Mellitus was induced in mice by streptozotocin. In addition, platelet chemokines (CXCL4, CCL5) were markedly higher in diabetic mice when compared to the sham group. Moreover, pretreatment of diabetic mice induced by STZ, with NSC23766 decreased kidney and pancreatic injuries assessed by histology score, P-value <0.05.

Conclusion: Our study reveals that Rac1 has a critical role in platelet chemokines secretion due to diabetes-induced inflammation in the kidneys and pancreas, and targeting Rac1 could be a target for innovative treatment to control inflammation in a diabetic individual. Targeting platelets involved in inflammatory pathways could be part of a strategy in order to control and manage diabetes consequences

Keywords: Rac1, Platelet, CXCL4, Diabetes Mellitus

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Correspondence Address: Rundk Ahmad Hwaiz
Department of Clinical Biochemistry, College of Health Sciences, Hawler Medical University, Erbil , Iraq
Email: Rundk.hwaiz@hmu.edu.krd

Website: https://djm.uodiyala.edu.iq/index.php/djm

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Introduction

Diabetes Mellitus (DM) is a state in which set to be the failure of the Langerhans islet of the pancreas to produce sufficient insulin, or for the body to resist insulin [1]. The figures of people with diabetes are expected to soar by 550 million by the end of the year 2035 [2]. Diabetes has adverse consequences that are mainly because of its micro and macroangiopathy which lead to several debilitating neurological complications and

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nephropathies and serious glomerular complications develop leading to dropping the level of GFR and eventually renal failure [3], [4]. The overall prevalence of diabetic kidney disease among US people with diabetes did not change significantly between 1988 and 2014, whereas albuminuria prevalence decreased and lowered eGFR prevalence rose [5]. Pre-diabetic individuals or impaired glucose tolerance subjects have demonstrated lower levels of mean platelet volume (MPV) than diabetic people in several studies [6]. Higher platelet activity is frequently found to be more in diabetic patients, platelet glycation proteins promotion is directly link to platelet reactivity [7]. Diabetic patients are doomed to develop higher platelet reactivity, as both insulin resistance and deficiency levels up platelet reactivity [8]. Chronic diabetic patients in general, are not an exclusion of the vicious circle in which hyperglycemia leads to high platelet activation: the latter is thought to be contributing to the production of the reactive oxygen species (ROS) in endothelium since this has been shown in several studies. Other than this ROS production in endothelium happens directly due to glucose metabolism and autooxidation indirect ROS production may also occur via the development of pro-inflammatory cytokine receptors for advanced glycation end products (RAGE) [9]. The cytosol of platelets embed proinflammatory derivatives like CXCL4 which is sent to be important and mainly produced from mRNA and the fate is to be spilled on platelet activations. This proinflammatory derivative is released upon platelet activation during CLP mice which in its turn leads to the expression of CXCL4 and CCL5, which may cause aggregation of other immune cells and exaggerate inflammation during abdominal sepsis [10], [11]. The previous study showed that Rac1 plays crucial role in diabetes mellitus as a signaling molecule [12]. Furthermore, platelet secretion of CXCL4 and CCL5 in sepsis is also controlled by Rac1 [10], [11], [13]. Numerous theses have emphasized the principal contribution of Rac1 in sepsis, lamellipodia formation, and the activation of phospholipase, granule secretion and clot retraction in platelets. So far, the molecular and cellular mechanisms that contribute to the inflammation and complication effects of diabetes are only partially recognized. Rac1 is a small GTPase protein playing a potential role in diabetes mellitus, hence the present study aimed to investigate the Rac1 role in releasing CXCL4 and CCL5 from platelets in diabetes mellitus and the inhibition of Rac1 to protect against kidney damage due to diabetes mellitus.

Patients and Methods

Animals

Swiss albino male mice were used in all experiments 8–9 weeks of age (weight:20–25 g). The Department of Pharmacy Hawler Medical University, Iraq, by welfare standards legislation, and the Regional Animal Experimentation Ethical Committee has given its approval. The animals were housed in a pathogen-free environment with a 12-to-12-hour light–dark cycle. Water and food were provided twice daily. Clean and fresh drinking water was provided specific nipple was used to access ad libitum. The trials were conducted after a 7-day acclimatization period. Animals were sub-grouped into three groups sham (injected

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with saline only), vehicle (injected with streptozotocin) and treatment group which was pretreated with NSC23366 and streptozotocin (NSC+STZ), each group containing five mice. With environment enrichment, the mice were maintained in cages with no more than five mice per cage in each group.

Materials

Streptozotocin (STZ; Glentham Life Science. Ltd., U.K.), is the chemical used to induce diabetes mellitus [14]. NaOH the buffer that uses for preparation of Streptozotocin, was made by dissolving (10.7 g) of sodium citrate in (200 ml) of distilled water. And (9.6 g) of citric acid was added and the volume completed to l000 ml with distilled water. By adding (NaOH) to the solvent, the pH of the solution was adjusted to (4.5). NSC23766 (N6-[2-[[4-(Diethylamino) -1-methylbutyl] amino] -6-methyl- 4-pyrimidinyl] -2 methyl-4, 6-quinolinediamine trihydrochloride, Chem Cruz, Santa Cruz Biotechnology, California). The Accu-Chek Active blood glucose meter.

Animal experiments

5 mg/kg of Rac1 inhibitor (NSC23766) was administered intraperitoneally to the animals. Based on past research, this dose of NSC23766 was chosen [13], [15], [16]. After 30 minutes, the animals were treated using multiple (i.p.) STZ (45 mg/kg body weight) was injected intraperitoneally into the experimental mice to produce diabetes mellitus. STZ was dissolved in a buffer of 0.01 M sodium citrate (pH = 4.5) and given to mice for five days. This dose of STZ is selected based on previous study [17]. To minimize hypoglycemia produced by STZ, After the injection, the animals were given a glucose solution (5 percent w/v) to drink overnight. Sham mice were given an equivalent dose of vehicle (citrate buffer) only. For 5 days, STZ and NSC+STZ-treated mice were housed in normal settings after dosing was completed. After this time mice developed diabetes by measuring fasting blood glucose levels were approximately 11.1 mmol/l. Blood samples from the tail vein of NSC+STZ and STZ-treated mice were taken after a 12-hour fast to assess blood glucose levels. Diabetes was defined as fasting blood glucose levels higher than 11.1 mmol/l in diabetic mice [17]. And hence they were selected for further studies. Intravenously, Sedation was achieved by administering 75 mg ketamine hydrochloride (Hoffman-La Roche, Basel, Switzerland) and 25 mg xylazine (Janssen Pharmaceutica, Beerse, Belgium) kg-1 body weight. The Animals were harvested and blood took from Vena Cava. The serum was allowed to coagulate at room temperature for 10-20 minutes. before being centrifuged for 20 minutes at 2000-3000 RPM. The serum was extracted from the supernatant and kept at 80°C for use in an ELISA test later. For histopathology, the kidney was preserved in formaldehyde.

Biochemical determination

Before the start of the procedure, all experimental animals' blood glucose levels were monitored. Fasting blood glucose levels were checked regularly until diabetes was confirmed. Mice with a fasting blood glucose level of 11.11 mmol/l or above were classified as diabetic. Blood was collected from the tail veins of all experimental animals (2–3 µl). An Accu-Chek active blood

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glucose meter was used to monitor blood glucose levels.

ELISA

Serum CXCL4 and CCL5 levels were measured successfully in all groups of mice and samples were assessed by enzyme-linked immunosorbent assay (ELISA) using the (Mouse Platelet Factor 4 ELISA Kit, BT LAB Cat. No E0686Mo) and (Bender MedSystems, Vienna, Austria kit.). Following the manufacturer's directions, at 450 nm absorbance was measured; and standard curve had been set on each microplate by diluting a known concentration standard. Using a logistic curve-fitting technique, the mean absorbance for the wells was used to calculate the chemokine concentration for each sample. The linear section of the standard curve contained all of the absorbance values. The data were represented as ng/ml and pg/ml respectively.

Histology

Kidney and pancreatic tissue were fixed overnight in a 10% formaldehyde phosphate buffer before being dried and paraffin-embedded. Hematoxylin and eosin were used to stain four-micrometer sections. A modified scoring system was used to quantify kidney injury in a blinded manner [18]. including mixed inflammatory cells, necrosis (irreversible injury), apoptosis, fibrosis, vascular congestion and edema, and degeneration (irreversible injury) Infiltration is evaluated on a scale of 0 to 4, zero represents (absence) and four represents (extensive). The mean value was calculated after assessing five random locations in each tissue sample. The sum of all six criteria determines the histology score.

Statistical Analysis

The data was provided as mean values with standard error (SEM). Nonparametric tests were used to do statistical analyses (Mann-Whitney). n is the total number of mice in each group, and P <0.05 was considered significant. Statistical analysis was performed using SPSS (IBM Corp., Armonk, N.Y., USA).

Results

Streptozotocin induced diabetes mellitus in mice

Injection of (45 mg/kg body wt.) of STZ into mice significantly increased the fasting blood sugar of mice compared to the sham group, P-value <0.05. However, the group delete treated with Rac1 inhibitor markedly reduced the high blood sugar induced by streptozotocin. Pretreatment of mice with 5 mg·kg−1 of the Rac1 inhibitor (NSC23766) reduced the fasting blood sugar from 556.20±26.65 to 375.20±20.7 with P-value <0.05 Figure (1). So, attenuating Rac1 activation by NSC23766 prevents high blood glucose which might induce platelet activation.

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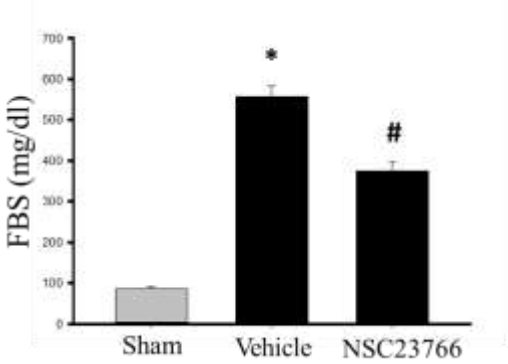

Figure (1): Blood Glucose concentration. Fasting blood glucose levels were measured on day of harvest after the mice were induced with STZ (Vehicle) for 5 days and pretreated with Rac1 inhibitor (NSC23766). Data represent mean ± SEM (sham=5, vehicle=5). *P <0.05 vs. sham

Rac1 regulates platelet secretion of CXCL4 and CCL5 in DM

Diabetes mellitus increased plasma levels of CXCL4 from 6.40±0.4 ng/ml in sham mice up to 13.60±1.32ng/ml, corresponding to a 2.12-fold increase Figure (2A). Moreover, DM increased the level of CCL5 from 21.800±1.908 pg/ml in sham mice to 67.400±7.332 pg/ml, a 3-fold increase in vehicle group induced with STZ Figure (2B). We found the induction of diabetes mellitus by injecting (45 mg/kg body wt.) SZT in plasma Figure (2) suggests that DM induces the platelet chemokine secretion of CXCL4 and CCL5 in Diabetic mice. Notably, (NSC23766) a Rac1 inhibitor, significantly reduced DM-induced platelet aggregation and chemokines secretion in platelets Figure (2 A and B), showing that NSC23766 which is an effective inhibitor of Rac1 activation, also inhibits the increased level of platelet chemokines. Pretreatment with NSC23766 attenuates serum levels of CXCL4 and CCL5 in diabetic mice from 13.60±1.32ng/ml to 7.20±0.8 ng/ml and from 67.4±7.332 pg/ml to 30±2.236 pg/ml respectively, equating to a drop of more than 50% Figure (2A and B).

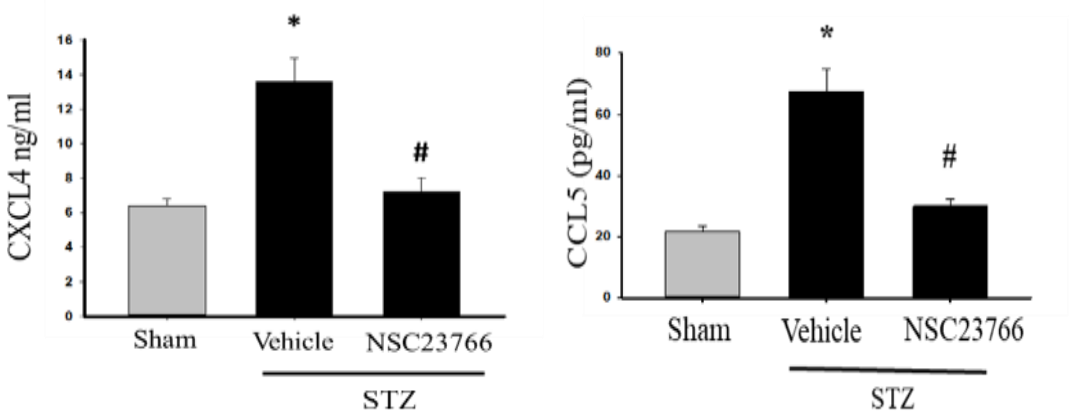

Figure (2): Activated platelets secrete chemokines in diabetic mice. A) ELISA was used to quantify the levels of CXCL 4 in the diabetic mice plasma. B) ELISA was used to quantify the levels of CCL5 4 in the diabetic mice plasma. Data represent mean ± SEM (sham=5, vehicle=5). *P <0.05 vs. sham

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Histological alterations of kidney and pancreas in diabetic mice

Histological study based on a deposit of Inflammatory cells, necrosis, apoptosis, fibrosis, degeneration, vascular congestion, and edema. STZ exposure resulted in pathological alterations in the kidneys, degeneration (irreversible injury) (arrow 1 on vehicle picture), and infiltration of inflammatory cells (arrow 2 on vehicle picture), according to histopathology Figure (3B). Moreover, STZ exposure resulted in pathological alterations in the pancreas, degeneration (irreversible injury) (arrow 1 on the vehicle picture), and infiltration of inflammatory cells (arrow 2 on the vehicle picture), according to histopathology Figure (3B). Our result showed a high degree of kidney injury. The induction of DM by injection of streptozotocin, significantly induced kidney and pancreatic injuries in diabetic mice compared to the sham group (1.13±0.08) and (0.83±0.14) with P-value <0.05 Figure (3). However, administration of NSC23766 to the mice reduced the histological score in kidney and pancreas to 0.26±0.21 and 0.32 ±0.16 respectively with p-value <0.05 Figure (3 A, B, C, D).

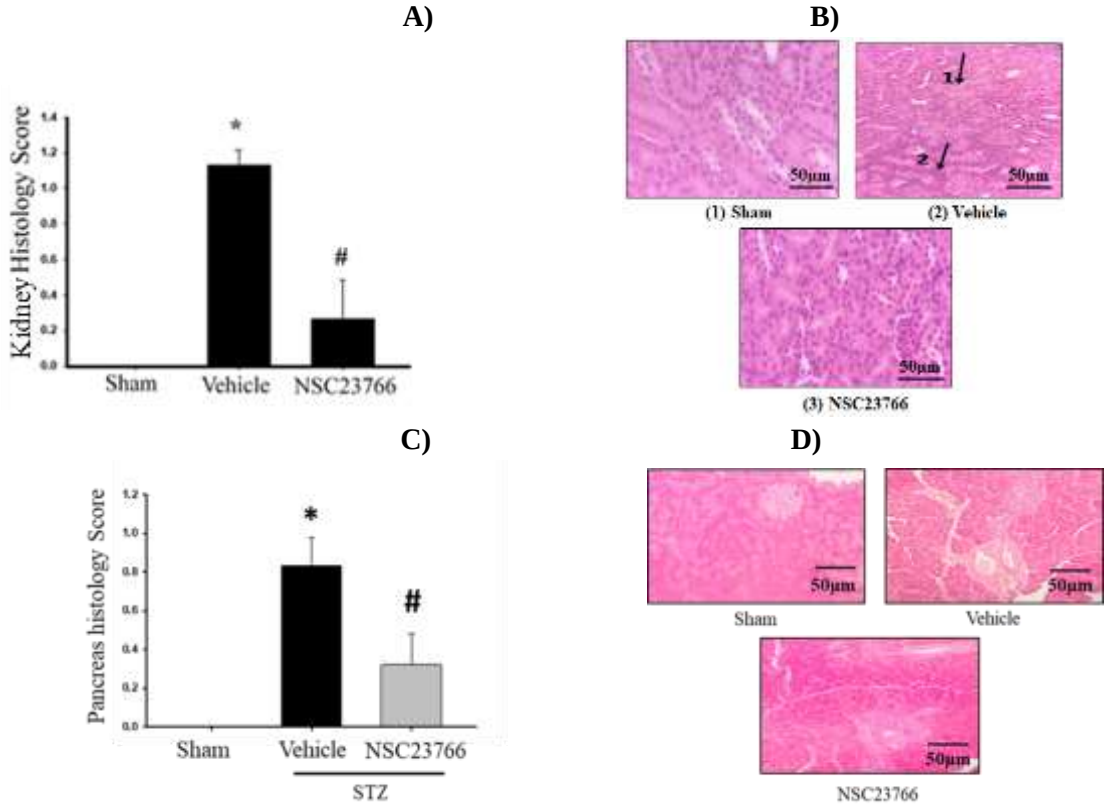

Figure (3): Rac1 regulates kidney and pancreatic damage in DM. (A) Histology score in the kidney. (B) Representative H&E sections of the kidney are shown. (C) Histology score in the pancreas. (D) Representative H&E sections of the pancreas are shown. Animals were treated with streptozotocin (vehicle), or an Rac1 inhibitor (NSC23766) before DM induced by streptozotocin. Kidney and pancreas injuries scores, as described in Materials and Methods, 5 days after DM induction. Sham animals served as negative controls. Data represent mean±SEM, and n = 5. *P< 0.05 versus Sham; #P< 0.05 versus NSC23766 + STZ

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Discussion

The current findings suggest that Rac1-mediated platelet activation and CXCL4 and CCL5 secretion from Platelets play an important role in diabetes mellitus pathogenesis. These data imply that Platelets play a critical role in diabetes, and that reducing Rac1 signaling and/or CXCL4 and CCL5 function could prevent complications associated with platelet activation and DM. Platelets are important for wound healing and thrombosis, but they also contribute to the host's response to bacterial invasion by performing a variety of pro-inflammatory actions [19]. Platelets, for example, govern a variety of features of Responses of leukocytes to severe infections, according to research [10], [11], [13]. Platelets have been demonstrated to play an important role in the development of diabetes mellitus in previous investigations however, in this study we found that the injection of mice with streptozotocin (45 mg/kg body wt.) significantly induce hyperglycemia compared to the sham group P-value <0.05. Similar to our study, previous studies showed the elevation of glucose after induction of streptozotocin [17], [20], [21], [22].

However, the role of Rac1in the regulation of platelet chemokines in the DM has not been studied earlier. Induction of streptozotocin-induced DM, significantly increased platelet chemokine secretion. However, the induction of DM by streptozotocin was abolished by the pre-treatment with Rac1 inhibitor (5 mg·kg−1), by more than 80% reduction Figure (2). This emphasizes the role of platelets and Rac1 in the DM molecular process. Morover, the histological study showed pathological changes in the kidney and pancreas in diabetic mice induced by injection of streptozotocin. Pre-treatment with Rac1 inhibitor (5 mg·kg−1) significantly reduced the kidney damage induced by hyperglycemia.

The role of platelets in renal damage was demonstrated in Previous studies [23], [24], [25], [26]. Talat *et al* showed that platelet count significantly increased in diabetic patients [27]. The glomerulus and tubule's basement membrane thickens and this lead to the recruitment of inflammatory cells. Gene expression and protein production of extracellular matrix components such as collagen IV, laminin, and fibronectin increase when extracellular matrix components accumulate. The weight of the kidneys was reported to increase as a result of these changes [28].

In platelets, CXCL4 and CCL5 are the most prevalent chemokines [10], [11]. Numerous studies have found that patients with metabolic syndrome had higher levels of (IL-6), (TNF), CXCL16, and (CRP) than healthy people. Furthermore, metabolic syndrome has been linked to an increase in the number of immune cells such as leukocytes, monocytes, and platelets [29], [30], [31]. Interestingly, the level of CXCL4 and CCL5 in diabetic mice's serum was higher than in sham animals, according to our findings Figure (2 A, B).

Rac1 has been implicated in the regulation of platelet chemokine production in previous investigations [10], [11], [13]. Furthermore, it is well known that high blood glucose levels assist in the development of reactive oxygen species (ROS) on the endothelium lining of

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arteries [32], [33]. ROS, a signaling molecule produced by NADPH, is vital in advancing of inflammation and vascular damage in diabetes [33]. Rac1, a small G protein, is a key signaling molecule that connects intracellular signaling pathways to NADPH oxidase activity [35].

Conclusions

In the present study, we showed that Rac1 functions in platelet activation and that the chemokines CXCL4 and CCL5 are overexpressed in diabetic mice. In addition, a morphological change associated with DM in mice is a consequences of platelet activation. Rac1 inhibition may thus be a therapeutic drug to regulate diabetes mellitus via attenuation of chemokine, which is a signaling molecule in aggravating inflammation, as well as chemokine production, and organ damage by activated platelets in DM.

Recommendations

This study shows the role of Rac1 in activation of platelet chemokines by measuring the platelet chemokenes (CCL5 and CXCL4) and histology scores of kidney and pancreas. However, it is better to measure the effect of diabetes mellitus on other organ and role of Rac1 in the controlling the complications of diabetes mellitus on other organ like heart, lung and liver in the future studies.

Aknowledgement

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Ethical clearance: Ethical approval was obtained from the College of Medicine / University of Diyala ethical committee for this study.

Conflict of interest: Nil

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تثبيط يحمي من الاضرار الحاصلة للاعضاء بسبب الصفيحات الدموية في مرضى السكري

هيلين جودت صبري [١] ، رنداك احمد حويز [٢]

الملخص

خلفية الدراسة: مرض السكري هو أحد الأسباب الشائعة لتنشيط الصفائح الدموية. تساهم وظيفة الصفائح الدموية غير الطبيعية التي يسببها الالتهاب في حدوث مضاعفات مزمنة ، وهي الأسباب الرئيسية للوفاة والمرض بين مرضى السكري. يمكن أن ينظم إطلاق الصفائح الدموية.

اهداف الدراسة: Rac1 ، وهو بروتين جي ٢١ كيلو دالتون ثبت أنه ينظم مجموعة متنوعة من وظائف الصفائح الدموية. لقد توقعنا أنRac1 مما يؤدي إلى إصابة الأعضاء في مرض السكري.

المرضى والطرائق: تم التحقيق في تأثير داء السكري على تنشيط لـ CXCL4 و CCL5 المتورط في تنشيط الصفائح الدموية وتسبب التهاب الصفائح الدموية وإصابات الأعضاء. عولجت ذكور الفئران البيضاء السويسرية مسبقًا بـ ٥ مجم / كجم من مثبط Rac1 NSC23766 وحُقنت بـ (٤٥ مجم / كجم من وزن الجسم) الستربتوزوتوسين ، مرتين لمدة خمسة أيام. علاوة على ذلك ، تم فحص تركيز المصل الكيميائي CXCL4 و CCL5 باستخدام ELISA وتم فحص درجة الأنسجة للكلى والبنكرياس.

النتائج: أظهرت نتائجنا أن مرض السكري قد تم تحريضه في الفئران بواسطة الستربتوزوتوسين. بالإضافة إلى ذلك ، كانت كيموكينات الصفائح الدموية (CXCL4، CCL5) أعلى بشكل ملحوظ في الفئران المصابة بداء السكري مقارنة بالمجموعة الصورية. علاوة على ذلك ، أدت المعالجة المسبقة للفئران المصابة بداء السكري الناجمة عن STZ ، باستخدام NSC23766 إلى انخفاض إصابات الكلى والبنكرياس التي تم تقييمها من خلال درجة الأنسجة ، وقيمة P <0.05.

الاستنتاجات: كشفت دراستنا أن Rac1 له دور حاسم في إفراز الصفائح الكيماوية بسبب الالتهاب الناجم عن مرض السكري في الكلى والبنكرياس ، ويمكن أن يكون استهداف Rac1 هدفًا للعلاج المبتكر للسيطرة على الالتهاب لدى مرضى السكري. يمكن أن يكون استهداف الصفائح الدموية المشاركة في مسارات الالتهاب جزءًا من استراتيجية للسيطرة على عواقب مرض السكري وإدارتها.

الكلمات المفتاحية: Rac1 ، الصفائح الدموية ، CXCL4 ، السكري

البريد الالكتروني: Rundk.hwaiz@hmu.edu.krd

تاريخ استلام البحث: ٣ آب ٢٠٢٢

تاريخ قبول البحث: ٢ تشرين الاول ٢٠٢٢

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[١] قسم الكيمياء الحيوية السريرية - كلية العلوم الصحية - جامعة هولير الطبية - اربيل - العراق

[٢] قسم التحاليل البيوكيميائية الطبية - جامعة جيهان – اربيل - العراق