

## Original Research Article

# Protective role of cannabinoids against diabetic nephropathy induced in rats by streptozotocin

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Sent for review: 26 November 2020

Revised accepted: 20 June 2021

### Abstract

**Purpose:** To investigate the effects of cannabinoid ligand in diabetic nephropathy mediated by streptozotocin (STZ) injection in rats.

**Methods:** Rats deprived of food were injected streptozotocin (STZ, 70 mg/kg) followed by confirmation of diabetes by checking the blood glucose level for three consecutive days. The kidney tissue was exposed to periodic acid Schiff reagent (PASR) for the investigation of glomerular sclerotic injury under microscope.

**Results:** Cannabinoid treatment decreased mesangial expansion, glomerular volume, proteinuria, reactive oxygen species and apoptosis in STZ rats. The cells with 40 mM of glucose for 8 h in which the podocytes responded with 2.9-fold increase in dihydroethidium fluorescence signal, compared to the podocytes cells cultured in low glucose (10 mM). However, cannabinoid treatment decreased ROS production in podocytes as indicated by dihydroethidium relative fluorescence. Further, the effect of ROS production by glucose on podocytes was inhibited by NADPH oxidase inhibitor, DPI (10 mM). Moreover, cannabinoid treatment reduced the expression of Sgk1, NADPH oxidase activity which was elevated by high glucose.

**Conclusion:** Treatment with cannabinoid decreases mesangial expansion, glomerular volume, proteinuria, and reactive oxygen species production in STZ rats. Thus, cannabinoid may be a protective agent against diabetic nephropathy in humans.

**Keywords:** Diabetic nephropathy, Cannabinoids, Streptozotocin, Podocytes, ROS

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## INTRODUCTION

The most common reason for end stage renal disease (ESRD) is diabetic nephropathy (DN). Reports have revealed that albuminuria and proteinuria are the two major risk markers

associated with diabetic nephropathy [1]. Podocytes are terminally differentiated into glomerular epithelial cells, which form end barrier to loss of proteins through urine and injury which are involved in many proteinuria renal disease [2,3]. Patient with normotensive type 1 diabetes

and glomerulopathy showed decreased number of podocytes which results in the increase of proteinuria which is the main step towards the progression of diabetic nephropathy [4]. It was shown that in chronic renal disease, mineralocorticoid receptor (MR) inhibition turn down albuminuria in chronic renal disease patients which indicates the relationship between MR and podocyte injury [5]. Literature suggests that aldosterone mediated damaged podocyte in rat was reversed by inhibiting MR receptor thus preventing proteinuria [6]. Renal injury causing agents like n-nitro-l-arginines methyl ester (*l*-NAME) that leads to proteinuria which is linked with high levels of aldosterone in plasma and its effect of renal injury, these processes were prevented with treatment of spironolactone (SPL) [5]. Our data reveals that aldosterone enhances podocyte injury and protein loss by activating MR receptor and inhibition protects the loss of podocytes.

Diabetic mellitus patients usually have unbalanced renin-angiotensin-aldosterone system. Aldosterone is generally dysregulated in diabetes and thus suppression of MR is effective in diabetic nephropathy [7]. Diabetic patients showed decrease in albuminuria upon MR inhibition even when aldosterone level is low [8]. Furthermore, in diabetes patients the high glucose level is responsible for excessive ROS generation through MR which causes podocyte injury or initiate podocyte apoptosis (9). In both type 1 and 2 diabetes models, podocyte apoptosis leads to podocyte depletion, aggravation of meningeal expansion and excretion of albumin through urine depicting podocyte loss represent the earlier on set of renal pathology that culminate into diabetic nephropathy (DN) [7]. Various natural products have potential to cure renal associated diseases and this study was an attempt to find an effective therapy modality for diabetic nephropathy treatment. In this study, our aim was to find the relationship between cannabinoid as a MR blockers and decrease in proteinuria as well as podocyte injury [7]. Cannabinoid (Canb) is naturally occurring compound with wide spectrum biological and pharmacological implications mostly for central nervous system related problems. Recent work on cannabinoid has widen the therapeutic spectrum for other affected organs. Cannabinoid role in renal diseases like DN is less explored.

Therefore, the aim of our study was to investigate the role and mechanism of the endocannabinoid system (ECS) in the recovery of podocyte injury.

## EXPERIMENTAL

### Animals

Sprague–Dawley (SD) rats (4 weeks old, 60 - 70 g) were obtained from Medical University and were allowed to adopt for a week in new conditions. The rats were deprived of food for 12 h and were injected intraperitoneal with streptozotocin (STZ, 70 mg/kg) or vehicle alone. Injection was followed by confirmation of diabetes by monitoring the blood glucose level for three consecutive days which was above 300 mg/dL blood glucose. The study was performed in accordance with the guidelines issued by National Institutes of Health, US [10]. The protocol described in this study was reviewed and approved by Medical University, Animal Care Committee Yunnan, China [approval no. MUY/2019/008]. Rats were categorized into three groups streptozotocine (STZ) injected diabetic rat, STZ rats treated with cannabinoids (STZ + CB) and vehicle injected control rats, all the three groups contain 22 animals each (n = 22). Cannabinoids were given orally to achieve a dosage of 35 mg/kg body weight of a rat (n = 6) for 8, 16, 24 weeks for each time points and systolic blood pressure were monitored every 8 weeks by tail cuff method. Before terminating the experiment, rats were kept in metabolic cages for 24 h per week to collect urine and blood for glucose level analysis. Rats were scarified by using standard anesthetizing agent pentobarbital (50 mg/kg), for either blood collection or for tissue harvesting (e.g., kidney) at 8, 16, 24 weeks after STZ or vehicle injection.

### Culture of cells

Podocytes (MPC5) from mouse were cultured in RPMI-1640 medium initially at 33°C and differentiated as previously described [11]. The differentiated cells were allowed to grow at 37°C without interferon- $\gamma$  containing either normal D-glucose (5 mM) and high glucose (40 mM) in presence or absence of cannabinoids or NADPH oxidase inhibitor diphenyleneiodonium (DPI, 10  $\mu$ M). Cannabinoid or DPI was treated 1 h before glucose was added into the cells.

### Histopathological examination

The kidney tissue was fixed with 4% paraformaldehyde embedded in paraffin and 2  $\mu$ m thickness sections were exposed to periodic acid Schiff reagent (PASR) for the investigation of glomerular sclerotic injury under the microscope. Semiquantative scale (1 - 4) was used to grade the mesangial matrix expansion as earlier described [7]. Glomerular volume was

measured by using formula  $GV = 4\pi r^3/3$  4 (where  $\pi = 3.14$ ,  $r =$  glomerulus radius).

### Western blotting

Tissue of kidney cortex or cultured cells were homogenized and lysates were prepared by using lysis buffer (RIPA + proteinase inhibitors). Protein estimation were done by using Bradford method. The proteins were resolved by SDS-PAGE for 2 h at 100 V and transferred into activated PVDF membrane for 2 h at 100 V at 4 °C. The protein membranes were probed with primary antibodies like BCL-2, Bax, PARP,  $\beta$ -actin at 4 °C overnight. The HRP-conjugated secondary antibodies were added followed by ECL and the signal was captured on X-Ray.

### Assessment of caspase-3 activity

Kit based colorimetric assay was used to measure the caspase-3 activity as per manufacturer protocol. The treated or untreated cultured podocyte or kidney cortex were homogenized, prepared with equal volume of protein (25  $\mu$ g) and incubated with 20 ng Ac-DEVD-pNA for 2 h at 37 °C into 96 well plate. Activity of caspase-3 was determined by measuring the absorbance of pNA, a cleaved fragment of Ac-DEVD-pNA, at 405 nm.

### Determination of reactive oxygen species

Generation of ROS in podocytes were detected using dihydroethidium dye (DHE). Treated or untreated podocytes were harvested and incubated with 5  $\mu$ M DHE at 37 °C for 30 min in incomplete media. ROS levels of treated or untreated freshly frozen kidney tissue samples were measured by chemifluorescent based assay kit as per manufacturer protocol. Olympus fluorescent microscope were used to capture images.

### NADH oxidase activity

Leucigenin enhanced chemiluminescence was used to measure the production of NADPH oxidase dependent  $O_2^{\cdot-}$  production as reported previously [12]. The signal of chemiluminescence was measured at 10 s interval for 10 min and average value of reading was taken.

### Evaluation of SOD and MDA

Cultured podocytes and cortex homogenate of kidney were processed for detection of markers of oxidative stress which included superoxide dismutase activity and malondialdehyde (MDA) content. The mentioned parameters were

measured by using assay kit and according to manufacturer's protocol.

### Reverse transcription polymerase chain reaction (RT-PCR)

The mRNA expression of various genes-  $\beta$ -actin (ACTB), serum and glucocorticoid-regulated kinase 1 (Sgk1), NADPH oxidase 4 (Nox4) was done by quantitative real time PCR. It is a two-step SYBER-RT-PCR process and was done according to manufacturer instructions. The primer sequence used in this experiment are as follows: Sgk1 sense 5'-AAACTCGGCAAGGCACCTC-3' and antisense 5'-CGGGATCAAAGTGCCGAAG-3'; rat NOX4 sense 'ACTGCCTCCATCAAGCCAAGA-3' and antisense 5'-CTTCCAAATGGGCCATCAATGTA-3'  $\beta$ -actin sense 5' AGCCATGTACGTAGCCATCC-3' and antisense'-CTCTCAGCTGTGGTGGTCAA-3'.

### Staining with dihydroethidium

Dihydroethidium dye was used to measure the intracellular  $O_2$  levels. Differentially podocytes were pre-incubated with cannaboid (10  $\mu$ M) and DPI (10  $\mu$ M) for 1 h at 37 °C followed by the induction of high glucose. The cells were washed with PBS and incubated with dihydroethidium (10  $\mu$ M) for 30 min. These samples were washed 3 times with PBS and were taken for microscopic observation. The average intensity of fluorescence were taken from 30-35 cells with 8 different fields and measured by using the software scion cooperation.

### Statistical analysis

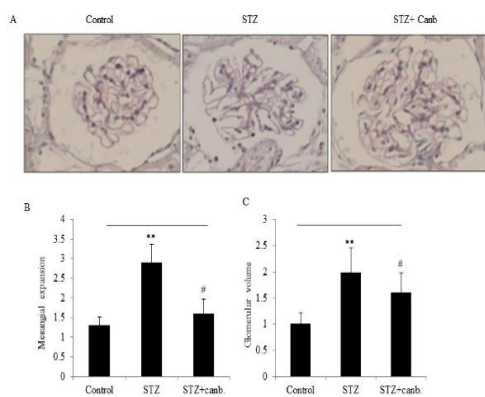
Data presented here are the mean  $\pm$  SEM of ( $n = 3$ ). Significance between groups was demonstrated using ANOVA followed by student's  $t$ -test.  $P < 0.05$  was considered significant statistically. GraphPad Prism software was used to analyse the data.

## RESULTS

### Cannabinoids enhances mesangial expansion and glomeruli volume in STZ rats

Histopathological results showed mesangial expansion and glomeruli volume status in STZ and STZ + Canb treated mice groups. Both these parameters were increased in STZ rats as compared to control or STZ + Canb treated groups (Figure 1 A, B and C). Blood parameters of control, STZ and STZ + canb were also done as shown in Table 1. No change was found in systolic blood pressure measured at 8, 6, 24 weeks after injection. Instead, plasma glucose

level was deranged in STZ and STZ + canb treated rats compared with normal saline injected rats. Whereas, creatinine level was decreased in STZ as well as STZ + Canb treated rats, but there was no significant change in creatinine clearance (Ccr) in the above-mentioned groups. However, aldosterone level was low in STZ treated rats compared with control groups but the protein excretion through urine was upregulated in STZ rats which was controlled following Canb treatment (Table 1).

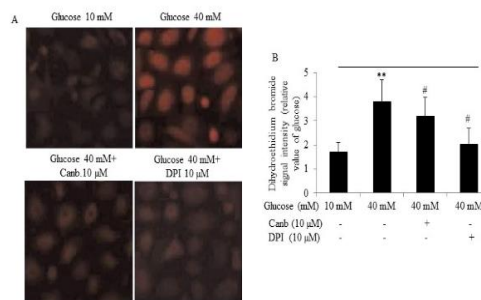


**Figure 1:** Effect of cannabinoid on mesangial expansion and glomerular volume. (a) Microscopic image demonstration of acid-schiff staining of glomerular of control, STZ and STZ + Canb treated rats at 24 weeks. Quantitative analysis of mesangial expansion and glomerular volume, (n = 8). \**P* < 0.05 vs control, #*p* < 0.05 vs STZ

**Cannabinoids decreased the glucose level-mediated production of ROS in podocytes**

Effect of cannabinoids on glucose mediated ROS production was determined in cultured podocytes. Fluorochrome dihydroethidium (DHF) was used to measure the intracellular ROS like superoxide ion (O<sup>2-</sup>) levels. The cells were cultured with 40 mM of glucose for 8 h in which the podocytes responded 2.9-fold increase in dihydroethidium fluorescence signal compared to

the podocytes cells cultured in low glucose 10 mM (Figure 2 A and B). On the contrary, when podocytes treated with cannabinoids there was decreased ROS production as depicted by calculating relative fluorescence of dihydroethidium. Further the effect ROS production by glucose on podocytes was inhibited by NADPH oxidase inhibitor DPI (10 mM). The above data indicated that cannabinoids decreased glucose mediated ROS production in podocytes.



**Figure 2:** Effect of cannabinoid on dihydroethidium fluorescence in podocytes under high glucose (a) Fluorescence microscopy showed the intracellular superoxide anion (O<sub>2</sub><sup>-</sup>) was increased in high glucose (40 mM) which was lower in cannabinoid treatment (10 μM) or DPI as detected by dihydroethidium fluorescence. \**P* < 0.05 vs NG; #*p* < 0.05 vs HG

**Cannabinoids decreased the glucose level-mediated production of ROS in podocytes**

Induction of ROS generation plays an important role in initiation of apoptosis or injury to podocytes. So we examined ROS, SOD and MDA levels in normal or high glucose treated podocytes. Podocytes were treated with or without cannabinoid at a concentration of 10 μM for 1 h and allowed to grow in media with high glucose (HG, 50 mM) or without high glucose for 8, 16, 24 h. HG is a potentially strong inducer of ROS production in time dependent manner.

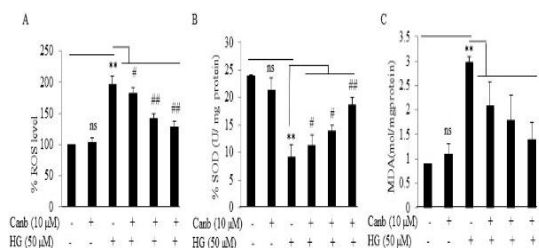
**Table 1:** Clinical parameters assessed at 24 weeks

Blood parameter	Control n=6	STZ (n=6)	STZ Canb (n= 6)
Body weight (g)	563 ± 18	543 ± 16*	543 ± 16*
Blood pressure (mmHg)			
8 weeks	137 ± 1.2	149 ± 3.3	141 ± 8
16 weeks	132 ± 2.4	143 ± 2.5	133 ± 2.96
24 weeks	136 ± 2.1	135 ± 3.6	134 ± 4.6
Plasma aldosterone (pg/ml)	435.7 ± 23.4	260 ± 21.5*	641 ± 31.36#
Blood glucose (mg/dL)	107.5 ± 3.2	589 ± 12*	522 ± 16.7*
Serum creatinine (mg/dL)	0.44 ± 0.016	0.35 ± 0.046*	0.28 ± 0.0316*
Creatinine clearance (mL/min)	3.1 ± 0.12	3.23 ± 1.001	4.7 ± 1.1

Pre-treatment with cannabinoid to HG treated podocytes decreased the production of ROS

compared with high glucose (50 mM) treated podocytes at a time dependent manner (Figure

3A). Furthermore, high glucose (50 mM) treated podocytes decreased the SOD activity and increased the MDA expression which was reversed by cannabinoid treated podocytes at 10  $\mu$ M (Figure 3B, C). Thus, cannabinoid significantly decreased high glucose induced ROS production and thus downregulated the oxidative stress in podocytes.



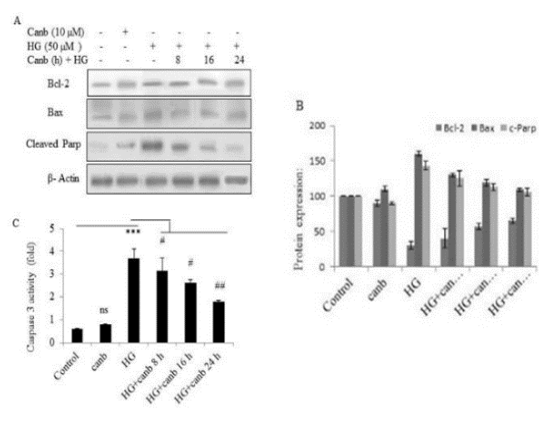
**Figure 3:** Cannabinoids decreases high glucose induced ROS production. (A) Representative quantification of ROS production by podocytes when exposed to high glucose (40 mM) or normal glucose (10 mM) in presence or absence of cannabinoid. (B, C) SOD and MDA levels in presence or absence cannabinoid as mentioned in the figure. \* $P < 0.05$ , \*\* $p < 0.01$  vs control group, \* $p < 0.05$ , \*\* $p < 0.01$  vs HG group

**Cannabinoids reduced apoptosis induced by high glucose in podocyte**

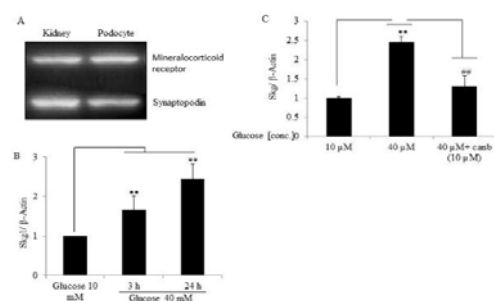
Podocyte cell death by apoptosis leads to podocyte injury in diabetic nephropathy. The expression of apoptotic proteins like Bax, PARP and activation of caspase-3 was upregulated while as, Bcl2 was downregulated by high glucose for 8, 16 and 24 h was reversed by cannabinoid at a concentration of 10  $\mu$ M in podocytes as determined by western blot (Figure 4 A). Furthermore, the activity of caspase-3 was decreased by cannabinoids which was activated by high glucose in podocytes (Figure 4 B).

**High glucose level in STZ-treated podocytes activates mineralocorticoid receptor**

The mRNA expression of mineralocorticoid receptor in podocytes treated with high glucose was measured by RT-PCR. The level of mineralocorticoid receptor and synaptopodin was upregulated in high glucose treated kidney as well as cultured podocytes as compared with low glucose podocytes (Figure 5 A). However, the mineralocorticoid receptor which was upregulated by high glucose was reversed with cannabinoid administration. Moreover, the expression of Sgk1 mRNA was upregulated in high glucose treated samples and were significantly decreased in cannabinoid treated podocytes (Figure 5 B and C).



**Figure 4:** High glucose mediated apoptosis was rescued by cannabinoid. (A) Expression of apoptotic mediated proteins Bax, Bcl-2 and cleaved PARP was analyzed by western blotting in high glucose induced podocytes. (B) Graphical representation of Caspase-3 activity in presence or absence of cannabinoid in high glucose treated podocytes in a time dependent manner. \* $P < 0.05$ , \*\* $p < 0.01$  vs control group; \* $p < 0.05$ , \*\* $p < 0.01$  vs HG group

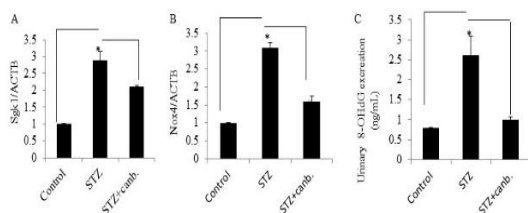


**Figure 5:** Effect of cannabinoid on expression of Sgk1. (A) Mineralocorticoid receptor and synaptopodin expression by RT-PCR. (B) Demonstration of Sgk1 expression in podocytes, in presence or absence of high glucose (40 mM) for 3 and 24 h or normal glucose (10 mM) for 24 h. (C) Expression of Sgk1 in podocytes grown in elevated glucose (40 mM) with or without cannabinoid treatment (10  $\mu$ M). \* $P < 0.05$ , \*\* $p < 0.01$  vs control group; \* $p < 0.05$ , \*\* $p < 0.01$  vs high glucose group

**Cannabinoids inhibits oxidative stress**

Oxidative stress injury usually involved at various cellular levels and its role in podocyte injury in STZ rats was investigated. The expression of Sgk1 is transcriptionally upregulated to mineralocorticoid receptor in renal cortex of STZ rats as compared with control which was further reversed by cannabinoid treated STZ rats (Figure 6 A). Moreover, the expression of diabetic oxidative stress marker NOX4 (NADPH oxidase isoform) was calculated in STZ treated rats, which was high compared with control group. Whereas, e, in cannabinoid-treated STZ rats, the level of NOX4 was significantly decreased near

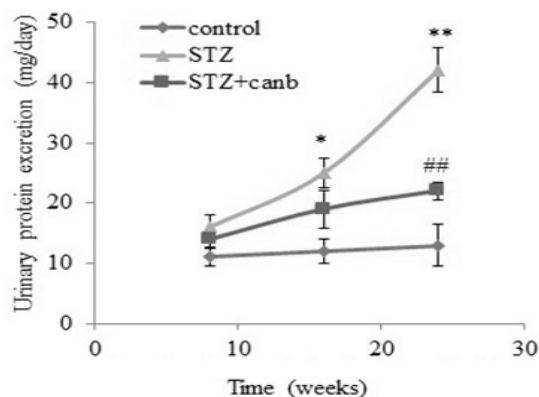
to control level (Figure 6 B). Furthermore, 8-OHdG level was upregulated in STZ rats and this effect was also downregulated by treating STZ rats with cannabinoids (Figure 6 C).



**Figure 6:** Cannabinoid affect the expression of Mineralocorticoid receptor and oxidative stress generation. (A) Sgk1 (B) Nox4 (C) Urinary 8-OHdG expression in renal cortex tissues of rat's post STZ injection and combinatory effect of cannabinoid with STZ treated rats. \* $P < 0.05$  vs control group; # $p < 0.05$  vs STZ

### Effect of cannabinoid on protein loss through podocytes injured by STZ treatment

To determine the role of unhealthy podocytes in the process of proteins loss (proteinuria), podocyte number per glomerulus was compared between control and STZ rats. The protein excretion through urine is upregulated in STZ rats which was decreased significantly by cannabinoid treated STZ rats (Figure 7).



**Figure 7:** Cannabinoid effect on urinary protein excretion. Protein expression representation of STZ alone or in presence or absence of cannabinoid at different times. \*\* $P < 0.05$  vs control; ### $p < 0.05$  vs STZ

## DISCUSSION

Cannabinoid has wide implications in central nervous system ranging from many pathophysiological events including memory loss and various other neurodegenerative diseases (13,14). Recent reports have shown that cannabinoids are involved in other pathological

conditions like cardiovascular diseases, inflammation, cancer and renal disease (15). The role of cannabinoids in diabetic nephropathy has not been explored. In this study we evaluated the role of cannabinoids in preventing the podocyte injury and severe proteinuria in diabetic nephropathy rats. It has been reported that high level of glucose like in hyperglycaemia or high aldosterone level or both in blood have direct or indirect role in podocyte injury (16).

This study also elucidated the relation between cannabinoid and aldosterone level when glucose levels are high (hyperglycaemia). Present investigation reveals that mineralocorticoid receptor (MR) activation under high glucose leads to podocyte injury while cannabinoid treatment decreased such podocyte abrasion. Other studies also revealed that hyperglycaemia induce the strong ROS generation, activates NADPH oxidase activity and Sgk1 expression (17). Such high level of ROS production by glucose is also responsible for podocyte injury which was strongly reduced by cannabinoids. Moreover, strong podocyte injury and proteinuria induced post-streptozotocin injection mediated hyperglycaemia in rats was substantially reduced by cannabinoid treatment.

Podocytes are connected to each other by slit diaphragms that cover basement membrane externally of glomerulus capillaries maintaining the functional as well as structural integrity of glomerular capillary loops. Podocyte apoptosis has major role in pathogenesis of diabetic nephropathy (18). When the balance between oxidative insult and antioxidant resistant is disturbed it leads to oxidative stress which leads to various pathological conditions like induction of pro-apoptotic pathways (19). Superoxide dismutase enzyme (SOD) scavenge ROS oxidative damage by decreasing its level and finally protects the cellular damage. High glucose level at cellular level showed low SOD activity was increased by cannabinoid treatment. In earlier studies, it was shown that streptozotocin induced podocyte injury and protein loss through urine (20). Therefore, we attempted streptozotocin induced podocyte injury in cannabinoid-treated podocytes. This is in agreement with our hypothesis that treatment with cannabinoids significantly reduced protein loss through urine. In diabetes type 1 patients, podocytes density was very poor and deformed when compared to control (21). This possible mechanism indicates that high glucose level induces activation of NADPH oxidase dependent ROS generation thus initiating the process of podocyte apoptosis (16).

In present study, NADPH oxidase activation by high glucose level generated ROS leads to death of podocytes which was drastically reduced by cannabinoids with significant recovery of podocytes health. Moreover, the induction of apoptosis in podocytes by high glucose level was decreased by cannabinoids. ROS generation is known to cause injury and alter the gene expression of podocytes (22) and various studies suggest high glucose levels like that in diabetes generates the ROS at higher levels (23). We reported that cannabinoids protect podocytes by decreasing the damage caused by presence of higher glucose levels and ROS, in addition to this, cannabinoids also showed the renal recovery effect in STZ rats. Various reports suggest that *Sgk1* are upregulated in diabetic nephropathy (24). *In vitro* studies also suggest that high glucose upregulates the *Sgk1* level in collecting duct cells. It was found that *sgk1* upregulation was MR dependent which was inhibited by cannabinoids. Under high glucose level NADPH-dependent ROS and upregulated expression of *NOX4* was reversed by cannabinoid treated STZ rats and podocytes.

## CONCLUSION

Cannabinoid treatment decreases mesangial expansion, glomerular volume, proteinuria, reactive oxygen species production in STZ rats. Moreover, cannabinoid treatment reduces the expression of *Sgk1* and NADPH oxidase activity which is elevated by high glucose. Therefore, cannabinoids may be of use as new therapeutic agents for preventing podocyte injury and proteinuria under hyperglycaemic condition.

## DECLARATIONS

### Acknowledgement

This work was supported by a grant from Yunnan Health Technical Training Project of Highlevel Talents (Project no. D2018027) and Yunnan Basic Research Projects - General Program (Project no. 2019FB092).

### Conflict of interest

No conflict of interest is associated with this work.

### Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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