

# Saxagliptin Attenuates Diabetic Nephropathy with Suppressing Oxidative Stress by Inhibiting AGEs-RAGE Axis in Streptozotocin-Induced Diabetic Rats

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## To cite this article:

Ye Feng, Chengjiang Li, Yuehuan Liu, Long Zhang, Zhe Zhang. Saxagliptin Attenuates Diabetic Nephropathy with Suppressing Oxidative Stress by Inhibiting AGEs-RAGE Axis in Streptozotocin-Induced Diabetic Rats. *American Journal of Internal Medicine*. Vol. 6, No. 6, 2018, pp. 161-169. doi: 10.11648/j.ajim.20180606.13

Received: September 16, 2018; Accepted: October 11, 2018; Published: November 5, 2018

**Abstract:** As a dipeptidyl peptidase-4 (DPP-4) inhibitor used in diabetes mellitus (DM) therapy, saxagliptin (Saxa) has been reported an additional protective benefit of diabetic nephropathy (DN), which might be independent of its glucose-lowering effect. However, the mechanism is not fully understood. In this study, STZ-induced DM rat model received a placebo or Saxa (10mg or 20mg/kg, 8-10 rats in each group). Blood glucose, serum lipid, creatinine, blood urea nitrogen, as well as urine protein and albumin concentration, were examined. Gene expression and protein level of advanced glycation end products (AGEs) and their receptor (RAGE) were also tested. Moreover, markers for oxidative stress and antioxidant ability were determined. The results showed moderate albuminuria in diabetic rats was attenuated after Saxa treatment, consistent with morphological improvement supported by histological analysis. Both AGEs and RAGE levels were elevated in DM group but reduced after Saxa administration. Furthermore, the level of malondialdehyde (MAD), Caspase 3, and 8-hydroxy-2'-deoxyguanosine (8-OHdG) in kidney were much lower in Saxa group compared with DM group, indicating the oxidation and apoptosis in DM were ameliorated by Saxa. On the other hand, markers of antioxidation such as total antioxidation capability (TAOC), glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD), had a relevant increase, suggesting enhanced antioxidation in the kidney. In conclusion, these findings show that Saxa possesses anti-oxidative activity to ameliorate diabetic renal damage, which is related to the regulation of the AGEs-RAGE axis.

**Keywords:** Dipeptidyl Peptidase-4 Inhibitor, Diabetes Mellitus, Advanced Glycation End Products, AGE Receptor, Oxidative Stress

## 1. Introduction

Diabetic nephropathy (DN) is one of the most common chronic complications in diabetes mellitus (DM) patients and affects one-third of them, disregarding the type of diabetes [1]. It may have fatal consequences due to kidney dysfunction and cardiovascular disease [2]. However, therapy preventing DN progression is rather modest.

As characterized by albuminuria and a reduced glomerular

filtration rate (GFR) [3], typical histological changes of DN include thickening of glomerular basement membrane (GBM), glomerulosclerosis, and expansion of the mesangial cells that leads to kidney fibrosis [4]. The pathogenesis of DN involves many potential mechanisms. Both clinical and experimental studies have suggested the imbalance between oxidative and anti-oxidative reactions playing a critical role in DN [5]. Recently, it has been shown that hyperglycemia itself induces the production of advanced glycation end products (AGEs) that may trigger reactive oxygen species (ROS) production

and mitochondrial dysfunction by binding to their receptor RAGE [6]. The accumulation of AGEs and increased binding to their receptor are related to the alteration in the redox state, which affects the renin-angiotensin system and the signaling of the transforming growth factor-beta (TGF- $\beta$ ), producing chronic inflammation and glomerular and tubular hypertrophy [7]. Thus, the AGEs-RAGE axis is considered as a promising therapeutic target for DN.

As a new antidiabetic medication, incretin-based drugs are widely used in clinical therapy due to their definitely antidiabetic effect. Unlike glucagon-like peptide-1 (GLP-1) receptor agonist directly stimulating GLP-1 secretion, dipeptidyl peptidase-4 (DPP-4) inhibitor, which delays GLP-1 degradation, seems to be safer and more convenient for patients because of its lower risk of hypoglycemia and oral administration. Based on the results from SAVOR-TIMI 53 trials focusing on renal outcomes of 16,492 patients with type 2 DM following for 2.1 years, Saxa could improve albumin to creatinine ratio (ACR) even in the patients with normal urine albumin (<30mg/day) without affecting estimated GFR. This benefit cannot be explained by its effect on glycemic control, as there is no significant correlation between ACR and HbA1c [8]. The probable reason for this renal improvement might be related to attenuated oxidative stress, as another DPP-4 inhibitor sitagliptin has been reported to diminish the oxidative marker in experimental DM animal model [9]. However, the mechanism for Saxa underlying this potential nephroprotection is not completely understood. In this study, the effects of Saxa in DM was evaluated and the probable mechanism was investigated as well.

## 2. Materials and Methods

### 2.1. Animal Models

7 weeks old male Sprague-Dawley rats (Experimental Animal Center, Zhejiang Academy of Medicine Sciences, Hangzhou, China, Certificate NO.: SCXK (Zhe) 2008-0033) weighing 180-200g, were housed in a 12-hour light/dark altered room at a controlled temperature (20-24°C), allowing free access to food and water with exception of preoperative fasting. The bedding was changed every day. These efforts were made to minimize the number of animals used and their suffering. The experimental protocols were approved by the Ethics Committee of the Laboratory of Animal Care and Welfare, School of Medicine, Zhejiang University. All animal procedures adhered to the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

10 rats were grouped as a control group. Diabetes was induced by a single intraperitoneal injection of streptozotocin (STZ, purchased from Sigma-Aldrich, St Louis, MO, S0130, 65mg/kg body weight) dissolved in 0.1 M citrate buffer, pH 4.5. An equal volume of citrate buffer was administered to the control group. The onset of diabetes was confirmed by the blood glucose level  $\geq 16.7$ mmol/L during random measurements made every day for 3 consecutive times. After the diabetic model was established successfully (3 days after

STZ injection), diabetic rats were divided into three groups (8-10 rats per group), daily gavaged with saline or Saxa (provided by AstraZeneca) at different doses (10mg/kg/day as low dose, 20mg/kg/day as high dose). All animals were monitored daily for their well-being and checked on a weekly basis for the body weight and appearance. All rats were kept in metabolic cages for 24 hours to collect urine before sacrificed. At the end of the 10-week treatment, the rats were sacrificed after overnight fasting and tissue were harvested for further detection.

### 2.2. Biochemical Analysis

The serum and urine were separated by centrifugation at 8000 rpm for 5 min and the supernatant was collected and stored in -80°C for measurement. The level of fasting blood glucose (FBG), serum and urine creatinine (SCr and UCr), triglyceride (TG), total cholesterol (TC), blood urea nitrogen (BUN), 24-hour urine total protein and albumin were determined by commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instructions.

### 2.3. Histological and Immunohistochemical (IHC) Analysis

Paraffin-embedded kidney sections were deparaffined, rehydrated, and stained by hematoxylin and eosin (H&E). Periodic acid-Schiff (PAS) staining was used to evaluate glomerular mesangial expansion, sclerosis, and tubular injury. The slides were stained with periodic acid-silver methenamine (PASM) to assess GBM and mesangial expansion. Masson trichrome staining was used for evaluation of kidney fibrosis. Photomicrographs were captured with a Carl Zeiss Axiokop 2 plus microscope (Carl Zeiss GmbH, Jena, Germany) with final magnifications of 400 times for glomeruli, and 100 to 150 random glomeruli were counted in a blinded fashion by two independent investigators for each sample.

For IHC staining, sections with deparaffinization and rehydration were incubated with monoclonal anti-AGEs (dilution 1:100, Sigma-Aldrich, MABN 1837) or anti-RAGE antibody (dilution 1:100, Santa Cruz Biotechnology, OAA00343) overnight. The slides were further incubated with peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology, OARA04989) for 30min. DAB substrate (Santa Cruz Biotechnology, OOSA09529) was used to enhance staining and nuclei were stained by hematoxylin before mounting.

### 2.4. Oxidative and Anti-Oxidative Evaluation

Serum total anti-oxidative capability (TAOC, Abcam, Cambridge, UK, ab65329) and malondialdehyde (MDA, Abcam, ab118970) were tested by colorimetry. To quantitatively access oxidative and anti-oxidative level in kidney, markers including malondialdehyde (MDA), Caspase 3, TAOC, glutathione peroxidase (GSH-PX), and superoxide dismutase (SOD) were determined (Abcam, ab118970, ab39401, ab65329, ab102530, and ab65354 respectively) according to the manufacturer's instructions. The result was

normalized by milligram of protein. Additionally, 8-hydroxy-2'-deoxyguanosine (8-OHdG) was tested by ELISA (Abcam, ab201734).

### 2.5. AGEs and RAGE Detection

ELISA was used for the quantitative measurement of serum AGEs. Kidney tissue was homogenized with radioimmunoprecipitation assay buffer and protease inhibitors. An equal amount of protein extract was separated on 12% SDS-PAGE gel running at 100V for 1 hour before transferred onto a polyvinylidene difluoride (PVDF) membrane. After blocked with 5% non-fat milk for 30 minutes at room temperature, the membrane was incubated overnight at 4°C with rabbit antibodies raised against AGEs, RAGE or  $\beta$ -actin (all 1:1000, Abcam, ab23722, ab3511 and ab8227 respectively). Then the membrane was probed with a secondary anti-rabbit IgG-horseradish peroxidase-linked antibody (1:800-1000, Abcam, ab6721) for 1 h at room temperature, and the bands were revealed with enhanced chemiluminescence solution (GE Healthcare, Buckinghamshire, UK), followed by exposure to X-ray film. Quantification of protein bands was performed by the ImageJ program (NIH, Bethesda, MD).

### 2.6. In Vitro ROS Induction and Treatment

Human proximal tubular (HK-2) cells (ATCC, Manassas, VA) were cultured in Dulbecco's modified Eagle medium (DMEM, Thermo Fisher Scientific, Cleveland, OH) with 10% (vol/vol) fetal bovine serum (FBS) and 1% penicillin/streptomycin until 80-90% confluency. After overnight incubation, the cells were treated with 100  $\mu$ g/ml

AGEs (glycoaldehyde AGE-modified BSA, purchased from BioVision, USA, 2221) or vehicle in the presence of different concentration of Saxa (0.1, 0.5, 5  $\mu$ M). 4 hours later, cells were incubated with 4'-6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific, D1306) and CellRox Deep Red Reagent (Thermo Fisher Scientific, C10422) at 37°C for 30min and washed with PBS.

The images were captured with a confocal laser-scanning microscope (ten randomly selected fields for each sample). The intensity was quantified by ImageJ and normalized with cell number. This procedure was repeated three times.

### 2.7. RNA Extraction and Real-Time PCR

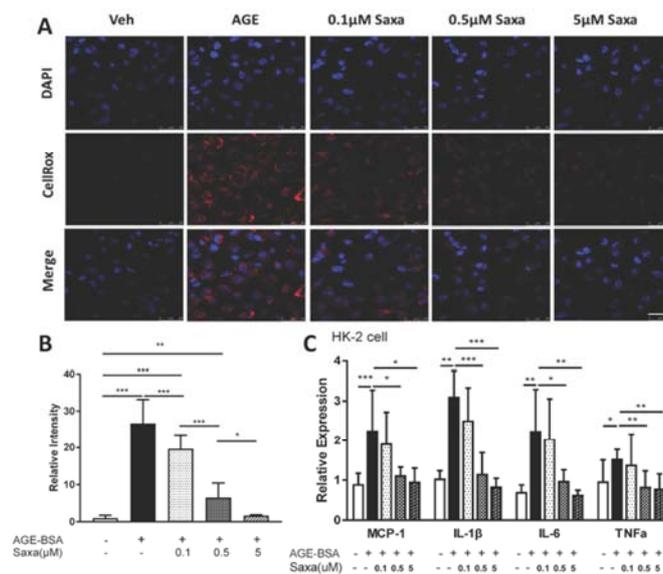
Total RNA was isolated from renal cortex and HK-2 cells using TRIzol reagent (Invitrogen, Carlsbad, CA, 15596026). SYBR green-based Real-time PCR was carried out with the ABI 7500 Real-Time PCR system (Applied Biosystems, Carlsbad, CA) to detect the gene expression of monocyte chemoattractant protein-1 (MCP-1), interleukin-1 $\beta$  (IL-1 $\beta$ ), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF $\alpha$ ). Data were normalized against GAPDH.

### 2.8. Statistical Analysis

GraphPad Prism software (GraphPad, Inc., San Diego, CA) was used for statistical analysis. One-way ANOVA followed by Tukey's comparison tests was performed to compare the differences among multiple groups. The statistical significance threshold was set at  $P < 0.05$ . Data were represented as mean  $\pm$  standard deviation.

## 3. Results

### 3.1. Saxa Protects HK-2 Cells from AGEs-Induced Oxidative Stress in Vitro



**Figure 1.** Saxa treatment reduced AGE-BSA-induced ROS and inflammation in HK-2 cells.

A. HK-2 cells were treated with AGEs-BSA in the presence of an indicated concentration of Saxa for 4 hours. The nucleus was blue stained, and ROS were red stained. Representative images represented three repeated experiments. B. Quantification of ROS level in each group. C. Gene expression of the inflammatory markers in HK-2 cells. \*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$ . Values were presented as mean  $\pm$  SD. Bars, 50  $\mu$ m.

Significant oxidative stress was induced in HK-2 cells in the presence of AGEs (Figure 1A). Quantification analysis showed AGEs group had the dramatically increased level of ROS relative intensity in comparison with control, while the intensity in groups with Saxa administration was much lower (Figure 1B). Genes involved with oxidative stress and inflammation, including MCP-1, IL-1 $\beta$ , IL-6 and TNF- $\alpha$ , were largely elevated in DM group and reduced in 0.5 and 5 $\mu$ M Saxa treated groups (Figure 1C), suggesting Saxa protected kidneys from AGEs-induced oxidative stress in a dose-dependent manner.

### 3.2. Saxa Ameliorated Metabolic Abnormality and Renal Dysfunction in Vivo

Compared with control group, diabetic rats had the higher level of serum glucose and TG, as well as kidney/body weight ratio, but lower bodyweight. Saxa rescued the enlarged kidney and albuminuria, but not hyperglycemia. Creatinine clearance rate (CCr, ml/min.kg) was calculated as follow: UCr ( $\mu$ mol/L) $\times$ urine volume (ml/day) / [SCr ( $\mu$ mol/L) $\times$ 1440 (min/day) $\times$ BW (kg)]. CCr in DM rats was notably lower in comparison to the control group, and Saxa administration seemed to reverse this trend but had no statistical significance (Table 1).

**Table 1.** 10-week-Saxa treatment attenuated biochemical markers in STZ-induced diabetic rats.

|                              | Control           | DM                   | 10mg Saxa              | 20mg Saxa              |
|------------------------------|-------------------|----------------------|------------------------|------------------------|
| Bodyweight (g)               | 411.2 $\pm$ 22.6  | 170.4 $\pm$ 29.2 **  | 279.6 $\pm$ 22.9 **##  | 243.0 $\pm$ 23.6 **##  |
| Kidney weight (mg)           | 1469 $\pm$ 113.4  | 1301 $\pm$ 205.3     | 1459 $\pm$ 177.1       | 1228 $\pm$ 189.7*      |
| Kidney/Bodyweight ratio      | 3.575 $\pm$ 0.245 | 7.838 $\pm$ 1.750 ** | 5.211 $\pm$ 0.327 **## | 5.042 $\pm$ 0.453 **## |
| BG (mmol/L)                  | 6.44 $\pm$ 1.59   | 49.13 $\pm$ 5.98**   | 48.25 $\pm$ 15.19**    | 47.95 $\pm$ 8.03**     |
| TG (mmol/L)                  | 0.475 $\pm$ 0.271 | 4.564 $\pm$ 1.390**  | 2.274 $\pm$ 1.717 *##  | 1.586 $\pm$ 1.210##    |
| TC (mmol/L)                  | 1.180 $\pm$ 0.089 | 1.425 $\pm$ 0.180    | 1.604 $\pm$ 1.165      | 1.392 $\pm$ 1.023      |
| SCr ( $\mu$ mol/L)           | 19.7 $\pm$ 3.1    | 38.9 $\pm$ 4.5 **    | 27.0 $\pm$ 9.0##       | 24.6 $\pm$ 6.7##       |
| BUN (mmol/L)                 | 6.06 $\pm$ 0.62   | 43.26 $\pm$ 16.96 ** | 22.95 $\pm$ 13.63 #    | 32.55 $\pm$ 12.04**    |
| BUN/SCr                      | 3300 $\pm$ 752    | 1034 $\pm$ 415**     | 1610 $\pm$ 123**       | 847 $\pm$ 329**        |
| Urine protein (mg/day)       | 7.54 $\pm$ 1.77   | 34.39 $\pm$ 12.56**  | 15.54 $\pm$ 3.15 ##    | 12.98 $\pm$ 3.25 ##    |
| Urine albumin ( $\mu$ g/day) | 146.6 $\pm$ 6.2   | 1120.0 $\pm$ 10.8**  | 523.5 $\pm$ 8.2**##    | 561.8 $\pm$ 8.3 **##   |
| CCr (ml/min.kg)              | 32.05 $\pm$ 19.98 | 15.45 $\pm$ 7.54 **  | 19.98 $\pm$ 14.42      | 21.36 $\pm$ 6.92       |

\*p<0.05 compared with the control group; \*\*p<0.01 compared with the control group.

#p<0.05 compared with the DM group; ##p<0.01 compared with the DM group.

### 3.3. Saxa Improved Diabetic Nephropathy in Histological Analysis

As shown in H&E staining, in comparison with control rats, diabetic rats had moderate glomerular hypertrophy with an increased number of inner cells without glomerulosclerosis (Figure 2B). Two Saxa treatment groups had a similar morphological appearance to the non-diabetic group. Quantitative analysis indicated that glomerulus was expanded in diabetic rats with increased diameter and area, but contracted after Saxa administration. Although duct diameter had a slight decrease in 20mg Saxa group, there was no difference compared with the other three groups (Figure 2C). Diffused mesangial matrix expansion was observed in rats with diabetes supported by PAS staining, which was attenuated after 10 weeks of Saxa treatment (both in 10mg and 20mg group). Local hyperplasia of Bowman's capsule and GBM thickening were showed in diabetic rats evidenced by PASM, attenuated by Saxa therapy. Masson trichrome revealed that diabetic rats had more green materials between vascular loops,

suggesting fiber deposition in this area. This deterioration was moderately weakened by Saxa (Figure 2D).

### 3.4. Saxa Attenuated Local and Systemic AGEs and RAGE Accumulation

AGEs and RAGE expression were determined by IHC in glomeruli and tubule. The 10-week period of hyperglycemia resulted in high expression of AGEs and RAGE, which were reversed by Saxa treatment (Figure 3A and 3B). Protein level was consistent with this observation examined by Western blot (Figure 4A and 4B). Elevated AGEs production in DM rats had a large reduction after Saxa therapy, while RAGE protein expression presented a modest decrease in DM rats receiving high dose Saxa treatment. A similar change was also demonstrated by RAGE gene expression in kidney (Figure 4C). Additionally, AGEs was not only accumulated locally in kidney but also on a systemic level in the context of DM, supported by the evidence that diabetic rats had increased serum AGEs levels, which was reduced by Saxa (Figure 4D).

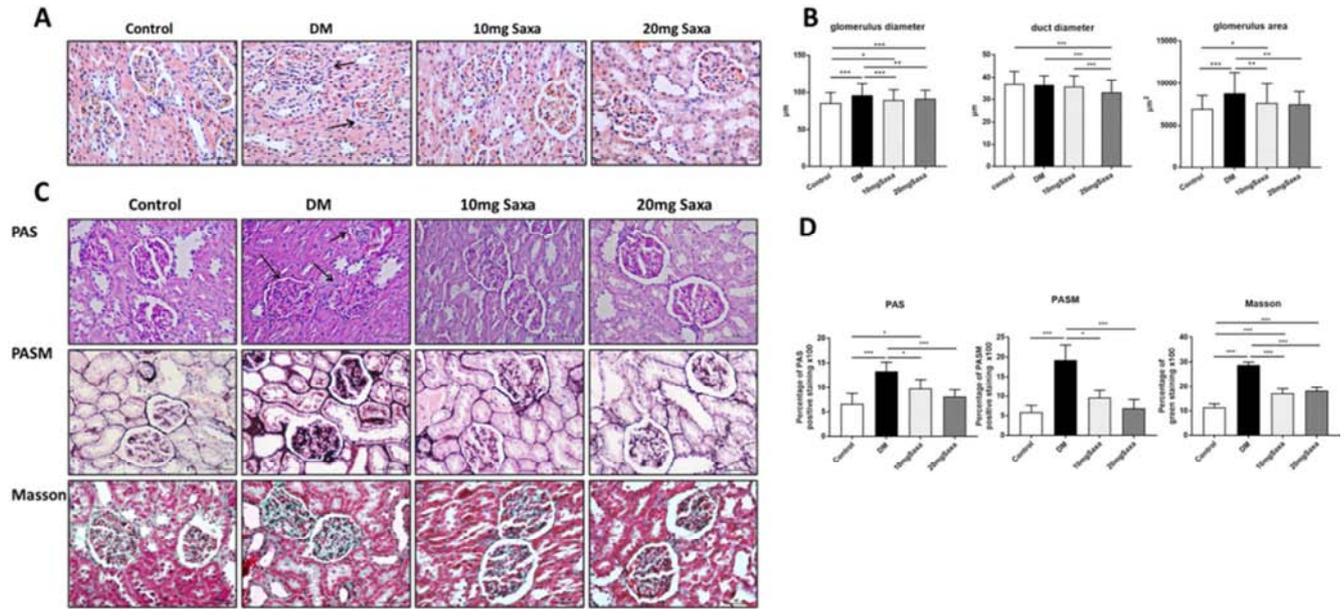


Figure 2. Saxa-attenuated diabetic nephropathy is detected by histological analysis.

A. Representative micrographs of H&E staining showed a moderately increased number of cells inside glomerulus (indicated with black arrows). B. Quantitative analysis showed that glomerulus was expanded in diabetic rats with increased diameter and area, while contracted after Saxa administration. C. Representative renal section of PAS, Masson and PASM staining. DM group showed diffused mesangial matrix expansion in PAS staining (indicated with black arrows), more green materials between vascular loops (indicated in Masson staining), and local hyperplasia of Bowman’s capsule and GBM (stained black in PASM). D. Quantification for the intensity of PAS-positive staining, PASM-positive staining and green materials stained in Masson respectively. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001. Values are presented as mean±SD. Bars, 50 μm.

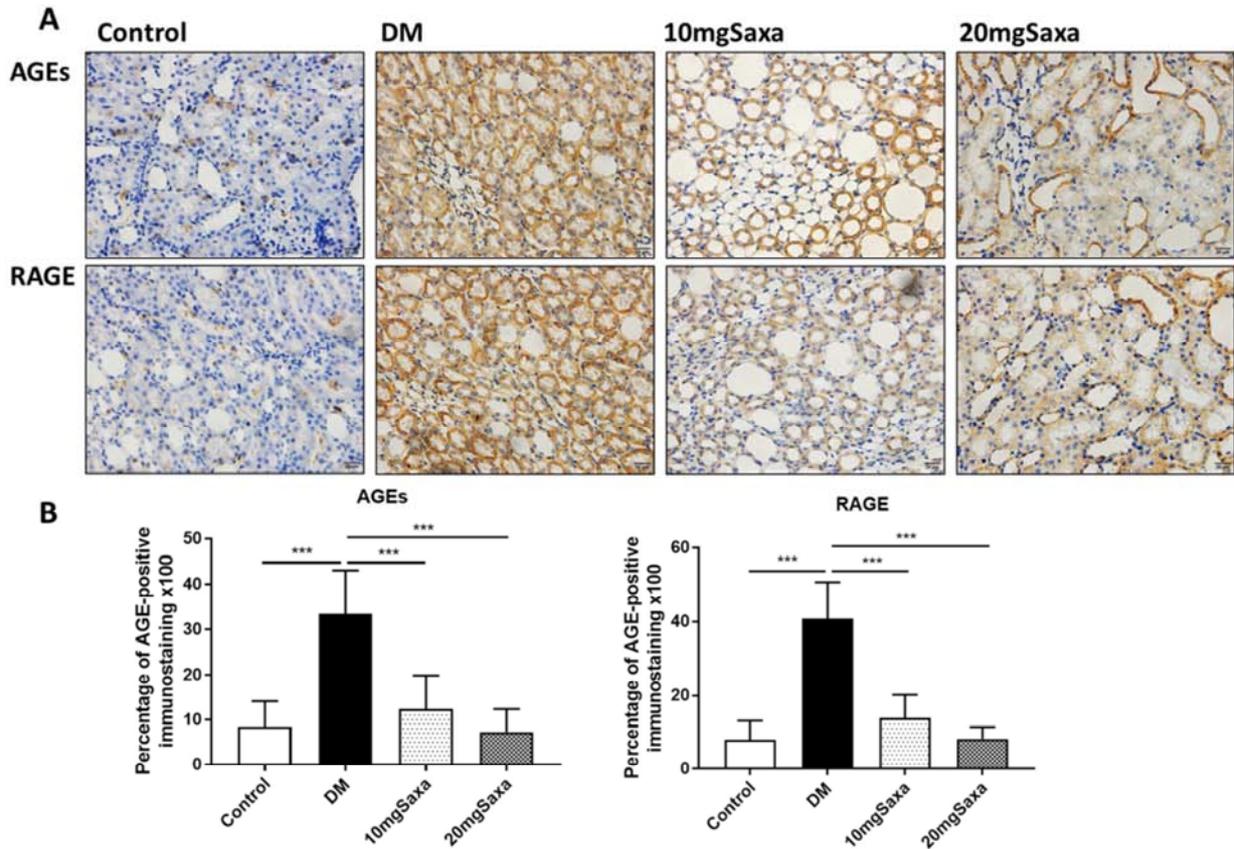
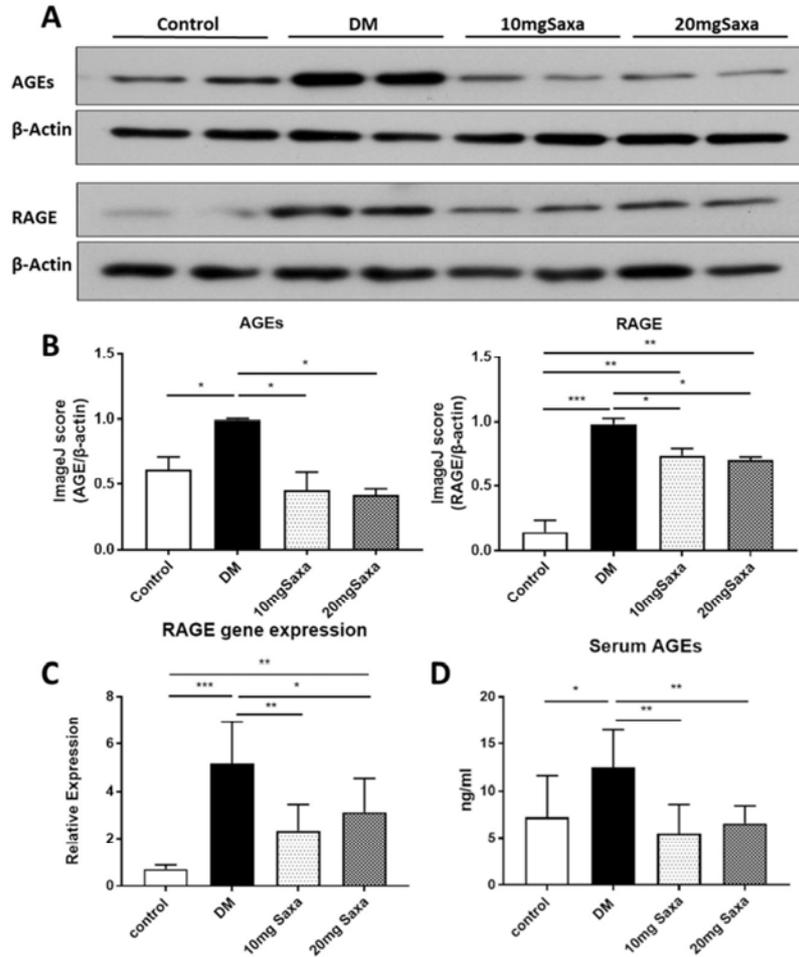


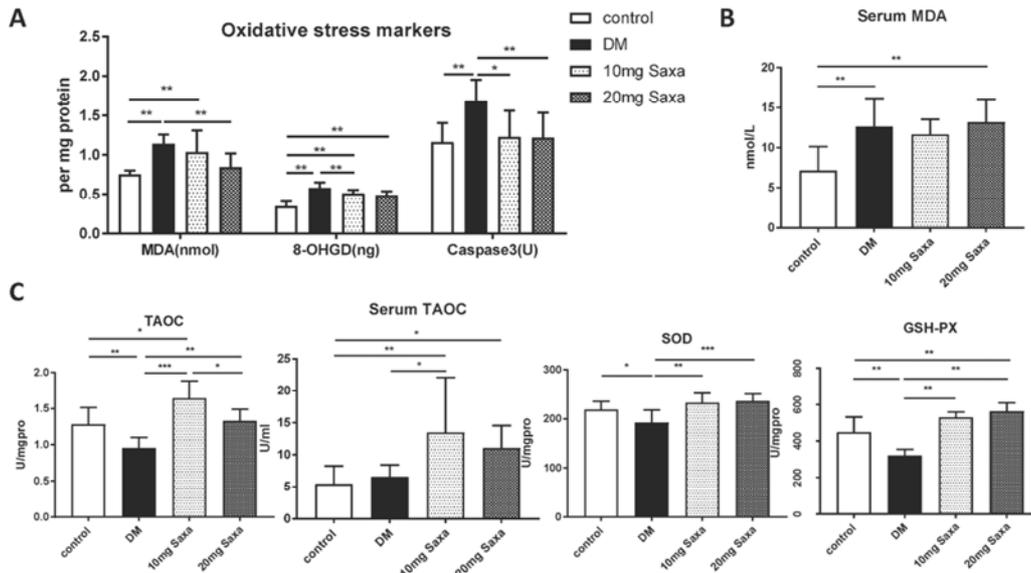
Figure 3. Saxa decreased AGEs and RAGE accumulation in kidney confirmed by IHC.

A. Representative IHC staining of AGEs and RAGE in the kidney. B. The protein level of AGEs and RAGE determined by Western Blot.



**Figure 4.** Saxa decreased AGEs and RAGE accumulation in kidney and circulation.

A. The protein level of AGEs and RAGE determined by Western Blot. B. Densitometric quantification of the blots calculated by ImageJ. C. Gene expression of RAGE in the kidney. D. AGEs' level in serum. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001. Values are presented as mean±SD.



**Figure 5.** Saxa treatment decreased oxidative level and restored anti-oxidative ability.

A. Levels of oxidative markers in kidney, normalized per mg of protein. B. The concentration of MDA in serum. C. The concentration of anti-inflammatory markers in serum and kidney. \* p<0.05, \*\* p<0.01, \*\*\* p<0.001. Values are presented as mean±SD.

### 3.5. Saxa Inhibited Oxidative Stress and Enhanced Anti-Oxidative Level

Markers for oxidative stress, such as MDA, 8-OHGD, and caspase-3, were elevated in diabetic rats but attenuated by Saxa administration (Figure 5A). The change in serum MDA level was not consistent with the result in kidney (Figure 5B), meaning a protective effect in the target organ of Saxa. As expected, anti-oxidative markers including TAOC, GSH-PX, and SOD were largely decreased in diabetic groups (Figure 5C), while increased in groups treated with 10mg and 20mg Saxa, indicating that the anti-oxidative capability was restored by Saxa. However, the same result was not found in serum, as TAOC level was similar in control and DM groups but increased after Saxa treatment (Figure 5C).

## 4. Discussion

As a chronic complication affecting a large population and causing great medical expenditure, DN has been intensively investigated. Various mechanisms have been proposed, including the effect of high glucose, AGEs accumulation, ROS production, upregulation of the protein kinase C (PKC) pathway, and glomerular hyperfiltration. These changes could result in various cellular responses, such as excessive expression of secretory factors and extracellular matrices, ultimately damaging the glomerular filtration barrier, shown as mesangial expansion, nodular glomerular sclerosis, and tubulointerstitial fibrosis in histological analysis [10].

Tons of evidence indicate ROS plays a crucial role in high glucose-induced renal injury [11]. Numerous macromolecules are considered to be involved in ROS generation, such as NAD(P)H oxidase, AGEs, uncoupled nitric oxide synthase (NOS), and mitochondrial respiratory chain mediated via oxidative phosphorylation. An excessive amount of ROS modulates activation of PKC as well as various cytokines and transcription factors, leading to increased expression of extracellular matrix genes with progression to renal fibrosis and even renal failure [12]. It is common for DM patients to have ubiquitously and irreversibly non-enzymatic glycated proteins and AGEs [13]. Therefore, a hypothesis is proposed that AGEs play a causal role in the development of DN [14]. One of the mechanisms suggests AGEs are involved in RAGE-dependent renal complication due to the expression of this receptor on the surface of a variety of cell types, including endothelial cells [15]. RAGE-overexpressed diabetic mice have been shown to exhibit progressive renal dysfunction compared with diabetic littermates without RAGE transgene, while RAGE null mice with diabetes displayed reduced albuminuria and glomerulosclerosis and failed to develop mesangial matrix expansion or thickening of the GBM [16]. In C57BLKS/J db/db mice, blocking AGEs formation or their binding to RAGE by different chemicals can decrease albuminuria, GBM thickening, and expression of profibrogenic factor, eventually reducing urine excretion of albumin as well as improving renal function [17].

AGEs-RAGE interaction could trigger the generation of ROS [18]. NAD(P)H oxidase has been reported to involve in ROS generation during AGEs-RAGE interaction. Engagement of AGEs to RAGE in podocytes can transduce the activation of extracellular signal-regulated kinase and NF- $\kappa$ B dependent pathway, subsequently driving the production of inflammatory markers and generation of ROS [19]. In proximal tubule cells, AGEs treatment and subsequent NF- $\kappa$ B activation increase the pro-inflammatory cytokine IL-6 [20]. Moreover, the interaction between AGEs and RAGE also promotes the generation of intercellular ROS [20].

As a new treatment option for T2DM, DPP-4 inhibitors have some additional benefits besides its hypoglycemic effect. In a rodent model, DPP-4 inhibition improves hyperlipidemia, inflammation and hypertension. It also increases glucose-dependent insulin secretion and  $\beta$  cell mass restoration [21]. Additionally, some studies have disclosed renal injury is ameliorated after DPP-4 inhibitor therapy both in acute and chronic models [22,23]. In our study, rats were administered with different doses of Saxa (10mg/kg/day or 20mg/kg/day) for 10 weeks since the DM model was established. Rats in the DM group showed elevated albumin concentration in urine and reduced CCr compared with the control group. Histological change for DN was only described as mild to moderate GBM thickening and mesangial matrix expansion without typical Kimmelstiel-Wilson lesion. Saxa ameliorated albuminuria but not CCr, while histopathologic lesion was also improved. In parallel with previous studies, our findings revealed that Saxa had similar renal protective effects as other DPP-4 inhibitors.

The possible mechanism of renal protection of DPP-4 inhibitors is reportedly related to ameliorating oxidative stress mediated by AGEs-RAGE interaction. After PKF275-055 (a DPP-4 inhibitor) treatment for 8 weeks, SD rats had less macrophage infiltration and reduced inflammation factors in the cortex of kidney [24]. In another study, sitagliptin was reported to enhance anti-oxidant response in kidney, involving a down-regulation of Nrf2 repressor [9]. In an in vitro study about acute kidney injury induced by indoxyl sulfate in proximal tubular cell, the DPP-4 inhibitor decreased expression of caspase 3 and inhibited NF- $\kappa$ B activation, indicating a suppression in both apoptosis and inflammation [22]. Nakashima et al found AGEs stimulated DPP-4 release from endothelial cells followed by oxidative stress generation and subsequent RAGE increase [25]. In DPP-4-deficient diabetic rats, they observed lower AGEs and RAGE expression in kidney compared with age-matched control rats [26]. In addition, the DPP-4 inhibitor linagliptin blocked the AGEs-RAGE-induced oxidative stress generation in the kidney, as demonstrated by decreased AGEs protein level, less RAGE gene expression and 8-OHdG expression [27].

Saxa is also considered to benefit DN both in clinical and animal studies, especially in the SAVOR study. The SAVOR trial has strongly supported the fact that Saxa has the great benefit of controlling DN-independent glycemia, even in the early stage with normal albuminuric range [8]. Nonetheless,

there has been less evidence to reveal the probable target pathway. In vivo, Saxa limited renal hypertrophy, transforming growth factor-beta-related fibrosis and NF- $\kappa$ B p65-mediated macrophage infiltration [28]. Saxa also attenuated diabetes-induced activation of inflammasome and progression of DN [29]. In the present study, Saxa inhibited AGEs production both in vitro and in vivo, as well as RAGE expression. Furthermore, it was observed that the end product of lipid peroxidation MDA was decreased in kidney but not in serum, and 8-OHdG, a marker of oxidative damage of DNA, also had a slight reduction in kidney. Caspase 3 decreased in both 10mg and 20mg-treated groups, indicating apoptosis in DN was attenuated by Saxa. On the other hand, the anti-oxidative enzymes such as T-AOC, GSH-PX, and SOD, slightly increased in the kidney. These results could not be simply considered to rely on the glycemic improvement, since hyperglycemia remained unchanged in Saxa groups, suggesting the improved kidney function was independent of glucose level. There was no obvious difference between low and high dose of Saxa both in animal and cells model, probably because of the relatively short period of Saxa treatment.

#### 5. Conclusions

In conclusion, these findings provided the evidence that the DPP-4 inhibitor Saxa slowed the progression of diabetic nephropathy by decreasing proteinuria and albuminuria, as well as preventing GBM thickening, diffused mesangial matrix expansion, cell size increase in the glomerulus, and lining local hyperplasia of Bowman's capsule. This protection probably resulted from the suppressed oxidative stress and restored anti-oxidative ability, supported by the reduction of MDA, 8-OHGD, and caspase-3 as well as rescued T-AOC, GSH-PX, and SOD. Saxa therapy also caused an inhibition of the interaction between AGEs and its receptor, which might have a causal association with the weakened oxidative stress. Thus, Saxa may prevent DN progression by the possible mechanism of suppressing AGEs-RAGE axis and oxidative stress, rendering it as a promising therapy for early DN.

## Competing Interest

The authors have declared that no competing interests exist.

## Acknowledgements

This study was funded by AstraZeneca China.

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