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Reduction of diabetes-induced renal oxidative stress by a cantaloupe melon extract/gliadin biopolymers, oxykine, in mice

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Abstract. Oxidative stress is implicated as an important mechanism by which diabetes causes nephropathy. Oxykine is the cantaloupe melon extract rich in vegetal superoxide dismutase covered by polymeric films of wheat matrix gliadin. In this study, we examined whether chronic oral administration of oxykine could prevent the progression of diabetic nephropathy induced by oxidative stress using preclinical rodent model of type 2 diabetes. We used female db/db mice and their non-diabetic db/m littermates. The mice were divided into the following three groups: non-diabetic db/m; diabetic db/db, and diabetic db/db treated with oxykine. Blood glucose level, body weight, urinary albumin, and urinary 8-hydroxydeoxyguanosine (8-OHdG) were measured during the experiments. Histological and 8-OHdG immunohistochemical studies were preformed on 12 weeks from the beginning of treatment. After 12 weeks of treatment, the levels of blood glucose and the body weight were not significantly different between the oxykine-treated group and the non-treated db/db group, however both groups kept significantly high levels rather than db/m mice. The relative mesangial area calculated by mesangial area/total glomerular area ratio was significantly ameliorated in the oxykine treated group compared with non-treated db/db group. The increases in urinary albumin and 8-OHdG at 12 weeks of treatment were significantly inhibited by chronic treatment with oxykine. The 8-OHdG immunoreactive cells in the glomeruli of non-treated db/db mice were more numerous than that of oxykine-treated db

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/db mice. In this study, treatment of oxykine ameliorated the progression and acceleration of diabetic nephropathy for rodent model of type 2 diabetes. These results indicated that the oxykine reduced the diabetes-induced oxidative stress and renal mesangial cell injury. In conclusion, oxykine might be a novel approach for the prevention of diabetes nephropathy.

Keywords: Oxykine, diabetic nephropathy, 8-Hydroxydeoxyguanosine, oxidative stress

1. Introduction

Diabetic nephropathy is characterized by the enlargement of glomerular mesangium due to the accumulation of extra-cellular matrix proteins, and is a leading cause of end-stage renal disease [1,2]. Recent clinical studies clearly demonstrated that hyperglycemia is an important causal factor in mediating the development and progression of diabetic kidney disease [3,4]. We postulated that increased oxidative stress by high glucose is important in the pathogenesis of diabetic nephropathy. Studies that have used natural and synthetic antioxidants have provided convincing evidence that glomerular hypertrophy and accumulation of collagen and transforming growth factor (TGF)-β by high glucose is largely mediated by reactive oxygen species (ROS) [5–8].

Under diabetic conditions, ROS are produced by non-enzymatic glycation reaction of proteins, mitochondria, and protein kinase C-dependent activation of NAD(P)H oxidase in mesangial cells, infiltrated inflammatory cells and endothelial cells [9,10]. In addition, the persistence of hyperglycemia has been reported to increase the production of ROS through glucose auto-oxidation, abnormal metabolism of prostaglandins, and high polyol pathway flux. Recent study using a suppression-subtractive hybridization has demonstrated that high glucose induces actin cytoskeleton regulatory genes in mesangial cells, and that the induction is dependent on mitochondria-induced ROS, is independent of protein kinase C and TGF-β [11]. Therefore, reduction of oxidative stress provides a new therapeutic strategy for inhibiting the progression diabetic nephropathy.

Oxykine is the cantaloupe melon extract (CME) selected for its high in vitro superoxide dismutase (SOD) activity, which is covered by polymeric films of wheat matrix gliadin [12]. It has been demonstrated that gliadin biopolymers are able to protect active molecules such as SOD against the digestive process [13,14]. A recent study clearly showed that when the SOD activity is preserved during the digestive process by its combination with wheat gliadin it was possible to elicit *in vivo* the pharmacological effects of this antioxidant enzyme [15]. Oxykine's pharmacological effects in animals are extensive, ranging from protection against liver fibrosis and the protection from neurotoxicity by radiation [12]. However, the effects of oxykine on glucose toxicity and development of diabetic complication in a model of diabetes have not been investigated.

In this study, we used the BKS.cg-m + $\mathrm{Lepr^{db}}$/$\mathrm{Lepr^{db}}$ (db/db) mouse which exhibits clinical and histological features of diabetic nephropathy that track the human disease [16]. This animal exhibits hyperglycemia and renal insufficiency by 16 weeks of age. The kidneys show the characteristic histological lesions of diabetic nephropathy, including mesangial matrix expansion and glomerular basement membrane thickening [16]. Recently we have demonstrated that oxidative stress increased in renal mesangial cells determined by urinary excretion level and immunoreactive expression of 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA oxidation, and that it correlated with the development of renal damage in db/db mice [17]. In addition, DeRubertis et al. [18] have showed that overexpression of Zn, Cu-SOD activity attenuates biochemical and structural changes induced in kidney in db/db mice, indicating a crucial role for superoxide *in vivo* in the pathogenesis of diabetes-induced nephropathy.

In the present study, we examined whether chronic administration of oxykine, orally effective vegetal SOD, could prevent the glomerular mesangial expansion in a preclinical model of diabetes, and whether oxykine could ameliorate oxidative injury in renal mesangial cells.

2. Materials and methods

2.1. Animals and experimental design

Female db/db mice, a rodent model of type 2 diabetes, and their nondiabetic db/m littermates, purchased from the Clea Japan Co. Ltd. (Tokyo, Japan), were randomly divided into the following three groups; nondiabetic db/m; diabetic db/db; diabetic db/db treated with oxykine. The db/db mice were confirmed as being diabetic by measuring blood glucose levels, which exceeded 200 mg/dl at the age of 6 weeks. They were kept under controlled conditions with a 12-h light:dark cycle and at 21–25 °C. All mice were fed commercial CE-2 (Clea Japan, Tokyo, Japan) with free access to water for 1 week to adapt to the new environment. The control diet, CE-2, contained (g/100 g): moisture 8.9, protein 25.4, fat 4.4, fiber 4.1, ash 6.9 and carbohydrate 50.3, and sufficient vitamins and minerals to maintain the health of the mice. Each of the groups contained 10 mice. The diet for the oxykine supplementation group was prepared by mixing CE-2 powder with oxykine (Combi Co., Tokyo, Japan) at 0.08 %. The food intake was measured daily for 12 weeks before dissection. Body weight of the mice and non-fasting blood glucose level was measured every 4 weeks. Maintenance of animals and experimental procedures were carried out in accordance with the US National Institutes of Health Guidelines for Use of Experimental Animals. All procedures were approved by the Animal Care Committee of the Kyoto Prefectural University of Medicine (Kyoto, Japan).

2.2. Preparation of oxykine

In this study, we used the hydrophobic gliadin/CME biopolymers, oxykine (Combi Co., Tokyo, Japan), to preserve the SOD activity present in the CME during the digestive process. Briefly, CME (100 IU/mg) preparations were mixed with gliadin in a 40% hydro-alcoholic solution, in such a ratio that the SOD activity in the final product is 1 IU/mg, determined by the reduction of nitroblue tetrazolium.

2.3. Intraperitoneal glucose tolerance test

Intraperitoneal glucose tolerance test (IPGTT) was performed at 18 weeks of age as follows: a 20% glucose solution (1.0 g/kg body weight) was injected intraperitoneally into the animals in the fasting state. Blood glucose concentrations were measured at each time point.

2.4. Urinary albumin and 8-OHdG analysis

Twenty-four-h urine samples were collected from mice, mixed well, and centrifuged at 3,000 g for 10 min, and then 1 ml of supernatant was stored frozen at −80 °C until use. 8-OHdG concentrations in urine were determined by a competitive enzyme-linked immunosorbent assay (ELISA) kit (8-OHdG Check; Japan Institute for the Control of Aging, Fukuroi, Japan) according to the manufacturer's instructions. The absorbance of each well was read at 450 nm by a microplate reader (MPR-A4i; Tosoh, Tokyo, Japan). The determination range was 0.5–200 ng/ml. The urinary 8-OHdG was expressed as the total amount excreted in 24 h. Urinary albumin levels were measured by a competitive ELISA (Albuwell M, Exocell Inc., Philadelphia, PA) according to the manufacturer's instructions.

2.5. Histological and morphometric analysis of kidney

For morphometric analysis of the glomeruli, sections were stained with periodic acid-Schiff (PAS). To quantify mesangial expansion, sections were coded and read by an observer unaware of the experimental protocol applied. For each animal of the three experimental groups, 20 glomeruli cut at the vascular pole were analyzed morphometrically. The extent of increase in mesangial matrix was determined by the presence of PAS-positive and nuclei-free area in the mesangium; the glomerular area was also traced along the outline of the capillary loop using NIH Image Software and Photoshop (Adobe systems, San Jose, CA).

2.6. Immunohistochemical analysis

Serial 8-μm transverse sections made with a cryostat (Bright 5030 Microtome; Bright Instrument, Huntingdon Cambridgeshire, UK) were mounted on silianized slides (Dako Japan, Tokyo, Japan). All subsequent steps were as described previously [19]. Briefly, the sections were incubated overnight at 4 °C with a primary antibody against 8-OHdG (Japan Institute for the Control of Aging, Fukuroi, Japan) diluted in phosphate-buffered saline (PBS). Sections were subsequently rinsed well with PBS, and incubated with biotinylated anti-mouse IgG (1:500 dilution; Vector Laboratories, Burlingame, CA) for 30 min at RT. After extensive rinsing, sections were incubated for 30 min with peroxidase streptavidin conjugate (Vector), and visualized with DAB and H_2O_2. The sections were mounted in a glycerol-based medium containing p-phenylenediamine. As a negative control, the immunostaining procedure was performed without the primary antibody. To determine the number of 8-OHdG-positive cells per glomeruli, sections were corded and read by an observer unaware of the experimental protocol applied. For each animal of the three experimental groups, the average of 20 glomeruli was used for analysis.

2.7. Statistics

All values in the figure and text are expressed as mean+SE. The data were compared by two-way analysis of variance (ANOVA), and differences were analyzed by Scheffe's multiple-comparison test. Simple regression analysis was used to test the correlations between albumin level and 8-OHdG concentration in urine. Differences between the groups were considered significant if the p value was less than 0.05. All analyses were performed using the Stat View 5.0-J program (Abacus Concepts, Berkeley, CA) with a Macintosh computer.

3. Results

3.1. Effect of oxykine on body weight and blood glucose levels

The db/db mice exhibited a significantly higher incidence of hyperglycemia associated with obesity than their non-diabetic db/m littermates throughout the experiments (6–18 weeks of age) (Tables 1, 2). No differences in food intake were observed between the oxykine-treated db/db mice and the non-treated db/db mice. The blood glucose levels and body weights of diabetic db/db mice were not influenced by the oxykine treatment during the experimental period.

Table 1
Body weight before treatment, and 4, 8, and 12 weeks after treatment

Group	db/m	db/db	
		Control	Oxykine
6-week	21.1 ± 0.3	28.0 ± 0.9	26.5 ± 0.3
10-week	22.3 ± 0.5	42.8 ± 1.5	38.6 ± 0.9
14-week	23.5 ± 0.6	47.0 ± 1.9	42.9 ± 1.2
18-week	24.2 ± 0.6	49.4 ± 2.1	47.4 ± 1.1

The db/db mice were confirmed as being diabetic by measuring blood glucose levels at the age of 6 week, and the treatment with oxykine was started. The body weight of the mice of each group was measured every 4 weeks. Values of body weight (g) are mean $\pm$ SEM of 5 animals.

Table 2
Non-fasting blood glucose level before treatment, and 4, 8, and 12 weeks after treatment

Group	db/m	db/db	
		Control	Oxykine
6-week	127 ± 5	341 ± 40	304 ± 51
10-week	123 ± 7	509 ± 41	520 ± 30
14-week	116 ± 7	558 ± 25	559 ± 20
18-week	101 ± 4	466 ± 47	444 ± 23

The db/db mice were confirmed as being diabetic by measuring blood glucose levels at the age of 6 week, and the treatment with oxykine was started. Non-fasting blood glucose level of mice of each group was measured every 4 weeks. Values of non-fasting blood glucose level (mg/dl) are mean $\pm$ SEM of 5 animals.

3.2. Effect of oxykine on blood glucose levels on IPGTT

To determine the effect of oxykine on the function of the pancreatic β-cells in db/db mice, we carried out IPGTT by administration of glucose (Fig. 1). The oxykine-treated db/db group showed no significant changes in blood glucose levels compared to non-treated db/db mice.

3.3. Effect of oxykine on diabetic nephropathy

Albumin levels in urine of db/db mice significantly ($p < 0.05$) increased compared with those of db/m mice at 10 weeks of age (Fig. 2). The increase in urinary albumin at 14 weeks of age tended to be reduced, and at 18 weeks of age was significantly inhibited by chronic treatment with oxykine ($p < 0.01$). Kidney weights were slightly, but not significantly, increased in db/db mice as compared to db/m mice. The glomerular appearance in db/db mice showed accelerated mesangial expansion characterized by an increase in PAS-positive mesangial matrix area relative with that observed in db/m mice at 18 weeks of age (Fig. 3). On the other hand, therapy with oxykine for 12 weeks partially reversed the mesangial matrix accumulation that had been established by 18 weeks of age. The glomerulus contains less PAS-positive matrix material and the capillary loops are more widely open. Mesangial expansion was further quantitated by a morphometrical analysis. The relative mesangial area calculated by mesangial area/total

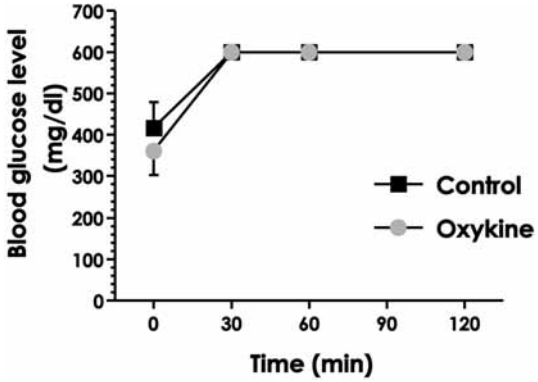

Fig. 1. Effect of oxykine on IPGTT. IPGTT was performed in db/db mice in each group at 18 weeks of age. After an overnight fast, glucose was injected intraperitoneally at a dose of 1 g/kg, and blood glucose levels were measured.

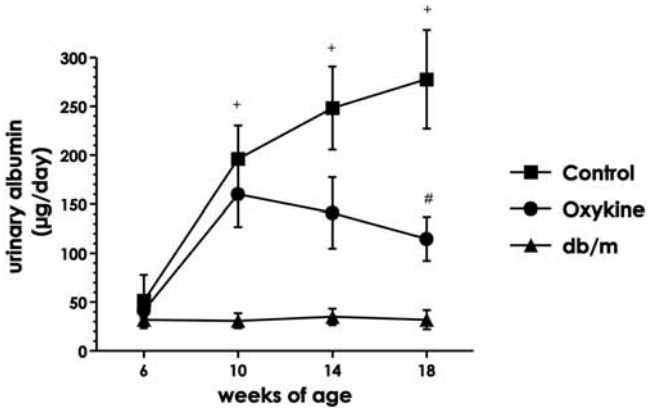

Fig. 2. Effect of oxykine on the urinary albumin excretion rate of diabetic db/db mice. A 24 h urine sample for each mouse was collected in metabolic cages 12 weeks after the start of this experiment. Urine samples were processed to measure urinary albumin concentration using a competitive ELISA. Data were shown as mean $\pm$ SE from 5 mice. $^{+}p < 0.01$ vs. db/m mice and $^{\#}p < 0.05$ vs. db/db mice.

glomerular area ratio was increased by 300% ($p < 0.01$) in db/db mice as compared with db/m mice. Administration of oxykine significantly ameliorated the increase in the relative masangial area in db/db mice (Fig. 4).

3.4. Effects of oxykine on urinary and renal levels of 8-OHdG

The urinary 8-OHdG level in db/db mice was significantly higher than that in db/m mice during the experiments (Fig. 5a). In non-treated db/db mice, the increase in urinary 8-OHdG levels was significantly enhanced at 18 weeks of age (Fig. 5a), however, the enhancement of urinary 8-OHdG levels was significantly reduced by the treatment with oxykine. Figure 5(b) shows the correlation between the levels of 8-OHdG and albumin in urine of db/db mice at 18 weeks of age. 8-OHdG levels closely paralleled the increase in albumin levels in urine ($r = 0.83$, $p < 0.05$ by the simple regression analysis).

To evaluate the damage of oxidative stress, we performed 8-OHdG immunostaining for kidney of each group. The results revealed that the 8-OHdG immunoreactive cells in glomeruli of non-treated db/db mice were more numerous than that of oxykine-treated db/db mice (Fig. 6). The number of 8-OHdG

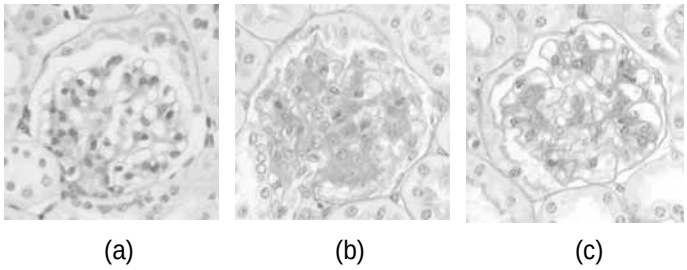

Fig. 3. Effect of oxykine on accelerated mesangial expansion in db/db mice. Paraffin-embedded sections of the renal cortex were stained with periodic acid-Schiff (PAS). Representative light micrographs (magnification: 400 x) from each of the mouse groups are shown. (a) Normal glomerulus from a non-diabetic db/m mouse at 18 weeks. (b) Glomerulus from an untreated db/db mouse at 18 weeks of age, showing mesangial matrix expansion characterized by an increase in PAS-positive mesangial matrix area. (c) Glomerulus from a db/db mouse treated with 12 weeks of oxykine until 18 weeks of age, depicting partial reversal of mesangial matrix expansion.

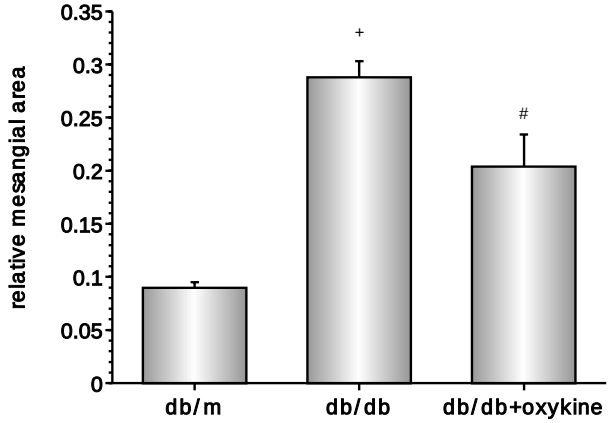

Fig. 4. Effect of oxykine on relative mesangial area shown as the ratio of mesangial area/glomerular area. Data were shown as mean $\pm$ SE from 5 mice. $^{+}p < 0.01$ vs. db/m mice and $^{\#}p < 0.05$ vs. db/db mice.

positive cells in db/db mice was significantly increased compared with that of db/m mice. The increase in the number of 8-OHdG-positive cells in the db/db mice was significantly inhibited by the treatment with oxykine (Fig. 7).

4. Discussion

In the present study, we clearly demonstrated that long-term oral treatment of oxykine, gliadin/SOD polymers, reduced not only an increase in albuminuria, but also glomerular histological changes in diabetic db/db mice without affecting blood glucose levels or pancreatic β-cell function determined by IPGTT. There were no significant differences in body weight and food intake between the oxykine-treated and non-treated db/db mice. These data indicate that the renal protection observed in db/db mice treated with oxykine was not attributable to effects on either the magnitude of hyperglycemia or weight gain. It is also unlikely that the renal effects of oxykine treatment were related to modification of hyperinsulinemia judging from the data obtained by IPGTT.

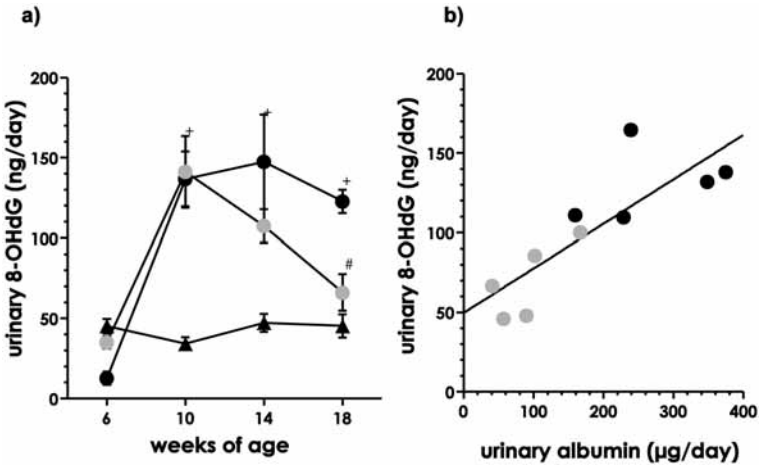

Fig. 5. Effect of oxykine on the urinary 8-hydroxydeoxyguanosine (8-OHdG) excretion rate of db/db mice and db/m mice (a), and the correlation between 8-OHdG levels and albumin level in the urine in db/db mice at 16 weeks of age (b). Black circle indicates the untreated-db/db mice, grey circle shows treated db/db mice, and triangle shows db/m mice. $^{+}p < 0.01$ vs. db/m mice and $^{\#}p < 0.05$ vs. db/db mice. 8-OHdG levels closely paralleled the increase in albumin levels in urine ($r = 0.83$, $p < 0.05$ by the simple regression analysis).

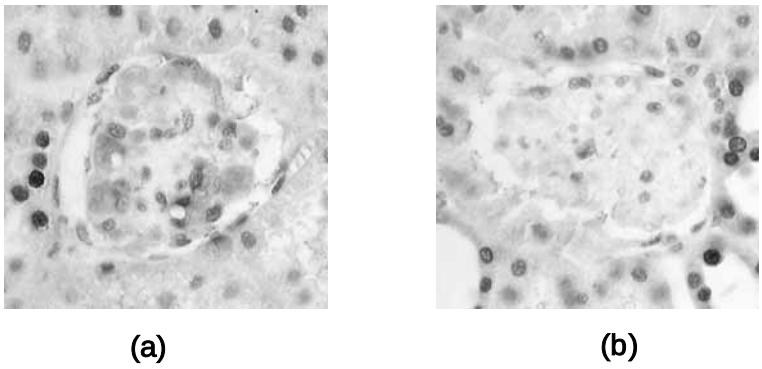

Fig. 6. Effect of oxykine on the expression of 8-hydroxydeoxyguanosine (8-OHdG) in the kidney of db/db mice. A representative result for immunostaining for 8-OHdG performed on glomerulus of diabetic db/db mice (a) and mice treated with oxykine (b).

More importantly, 8-OHdG, an index of oxidative stress, was increased in urine and kidney of diabetic db/db mice, and these increases were significantly inhibited by the treatment with oxykine. Recent data support that 8-OHdG is a good marker to evaluate renal oxidative stress in several models of diseases and ageing. In 1994, Ha et al. [20] first demonstrated that the formation of 8-OHdG is closely related to the process of diabetic nephropathy in experimental diabetic rodents. They showed that the 8-OHdG levels in both rat renal cortex and papilla were significantly increased compared to those of controls after streptozotocin (STZ) administration, and that daily injection of insulin after STZ treatment significantly reduced both urinary albumin excretion and 8-OHdG formation, which suggests that these are associated with the diabetic state induced by STZ rather than a direct nephrotoxic effect of the drug [20]. Recent clinical evaluation indicates that 8-OHdG in urine is a useful clinical marker not only for detecting micro- and macro-vascular complications [21] but also for predicting the development of diabetic nephropathy in diabetic patients [22]. In the present study, the urinary levels of 8-OHdG was increased at 10 weeks of age

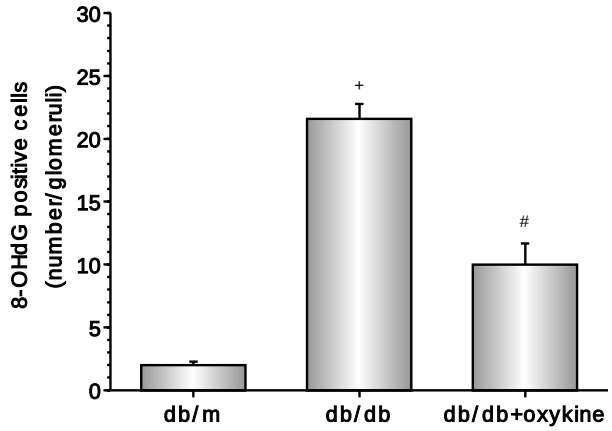

Fig. 7. Effect of oxykine on the number of 8-hydroxydeoxyguanosine (8-OHdG)-positive cells per glomeruli in db/db mice. Data were shown as mean ± SE from 5 mice. $^{+}p < 0.01$ vs. db/m mice and $^{\#}p < 0.05$ vs. db/db mice.

paralleled with the increase in urinary albumin levels, indicating that oxidative stress may play a crucial role in the development of diabetic nephropathy in diabetic mice. In addition, renal immunostaining of 8-OHdG revealed an increase of positive cells in non-treated db/db mice, and increased levels of 8-OHdG in urine and tissue were both attenuated by oral oxykine treatment. These results indicate that the urinary level of 8-OHdG reflects renal oxidative damage in diabetes mellitus and might be a preventive biomarker for diabetic nephropathy. The administration of dietary oxykine improved renal dysfunction in diabetic mice through its antioxidant function, and the urinary and tissue 8-OHdG data support the se results.

Although there is no doubt that hyperglycemia is a major contributor to oxidative stress, there has been debate about the association between the glycemic control and levels of 8-OHdG. First, Leinonen et al. [23] showed a positive association of HbA1c, an index of glycemic control, and urinary 8-OHdG in diabetic patients. Kouda et al. [24] demonstrated the positive correlation between 8-OHdG and pentosidine, a marker of nonenzymatic glycation and oxidation damage, in the urine of patients with hypercholesterolemia and/or hypertension. In this study, urinary and kidney 8-OHdG levels exert marked reduction by the treatment with oxykine in association with reduction of albuminuria, in spite of the high levels of blood glucose during the treatment. These results suggest that oxykine might directly attenuate the diabetic renal oxidative damage induced by hyperglycemia. In addition, our results might provide further insight into therapeutic strategies for diabetic kidney disease.

The protective effect that antioxidants have on certain aspects of nephropathy in diabetic animals has been reported. Recently, it was reported that antioxidant treatment with vitamin E, probucol, α-lipoic acid, or taurine normalized not only diabetes-induced renal dysfunction such as albuminuria and glomerular hypertension but also glomerular pathologies [6]. Ueno et al. [25] have also reported that dietary glutathione can exert beneficial effects on diabetic complications in STZ-induced diabetic rats. Oxykine rich in SOD activity is protected against the digestive process by the biopolymeric wheat gliadin, and can be delivered efficiently to the systemic circulation by the oral route. The diabetes-induced changes observed in the present study were prevented by the potent antioxidant oxykine, which is quite consistent with the effects of this compound in other non-diabetic models of oxidative stress [15]. The unique characteristic of oxykine could contribute the decrease of oxidative stress in these models. In addition to oxygen radical scavenging action of this compound, we should pay attention to immunoregulatory action. It has been reported that the heterologous SOD elicits number of immunoregulatory properties related to their antigenic nature in addition to scavenge superoxide anion [26].

Although the limited data suggest that antioxidants help protect against diabetic nephropathy in human, combined daily treatment with 680 I.U. vitamin E and 1,250 mg vitamin C for 4 weeks reduced the urinary albumin excretion rate by 19% in a crossover study of 30 patients with type 2 diabetes [27]. We have just started to administer oxykine for human. Further studies should help more clearly define the role of oxykine in enhancing the quality of life of individuals with diabetes. In conclusion, our present results reveal for the first time that oxykine can exert beneficial effects on renal mesangial cells in diabetic db/db mice. Thus, oxykine might be a novel approach for the prevention of diabetes nephropathy.

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