

Oleanolic acid prevents progression of streptozotocin induced diabetic nephropathy and protects renal microstructures in Sprague Dawley rats

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ABSTRACT

Objective: To study the effect of oleanolic acid (OA) on streptozotocin induced diabetic nephropathy in Sprague Dawley rats. **Materials and Methods:** Four weeks after intra-peritoneal injection of streptozotocin (STZ; 55 mg/kg), the rats with proteinuria were grouped as: Control (non-diabetic, treated orally with vehicle), diabetic control (treated orally with vehicle) and three diabetic groups receiving 20, 40 and 60 mg/kg/day oral doses of OA. At the end of 8 weeks, urine and serum samples from the rats were processed for determination of creatinine, BUN and GFR. The kidney samples were processed for determination of weight changes, oxidative stress related parameters like catalase, superoxide dismutase and reduced glutathione levels. A part of one kidney from each rat was used for transmission electron microscopy (TEM). **Result:** As evident in TEM, OA inhibited the nephropathy induced alterations in podocyte integrity, basement membrane thickness and spacing between the podocytes at 60 mg/kg dose. It increased GFR and reduced oxidative stress in the kidneys in a dose dependent manner. These findings conclusively demonstrate the efficacy of OA in diabetic nephropathy. Significant decrease in the oxidative stress in kidneys indicates the role of anti-oxidant mechanisms in the effects of OA. However, OA is known to act through multiple mechanisms like inhibition of the generation of advanced glycation end products and improving the insulin secretion. These mechanisms might have contributed to its efficacy. **Conclusion:** These results conclusively demonstrate the efficacy of OA in diabetic nephropathy through its possible antioxidant activity.

Key words: Electron microscopy, glomerular basement membrane, pentacyclic triterpenoid, podocytes

INTRODUCTION

Pentacyclic triterpenoids have emerged as a class of multifunctional drugs targeting multiple steps in the signal

transduction pathways.^[1] The plants containing such triterpenoids have been consumed by human beings and animals as food and medicine across history without apparent toxic effects.^[2] Such a prolonged exposure to the triterpenoids has resulted in the evolution of a favored molecular fit between the triterpenoids and the proteins found in animal cells.^[3] Obviously, pentacyclic triterpenoids are being widely investigated for their efficacy in many disease models including diabetes and renal injury.^[4]

Oleanolic acid, a pentacyclic triterpenoid, is vastly reported in the scientific literature for its multiple biological potentials. Several preclinical studies report its antidiabetic effects^[5-7] and multiple mechanisms through which it affects the

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pathophysiology and consequences of diabetes. OA inhibits the generation of advanced glycation end products in diabetic conditions^[6] and favors insulin secretion.^[8,9] Further, OA is reported to protect kidneys of experimental animals from toxicants such as gentamicin and paracetamol.^[10-12] It also exerts antiglycative effect in the STZ-induced diabetic mice kidneys.^[13] Such multifunctional nature and reported antidiabetic potentials of OA emphasize the need to further establish whether it restores normal histological and ultramicroscopic architecture of diabetic kidneys along with improvements in the renal function.

Ultramicroscopic alterations in the diabetic kidneys such as thickening of the glomerular basement membrane, destruction of podocytes and reduced intracellular cell spaces are well established.^[14-16] These changes in the kidney architecture confirm the experimentally induced DN and can be applied in the evaluation of efficacy of drugs. The present study was aimed to find out the weather the orally administered OA restores the functions and architecture of STZ-induced nephropathic kidneys of diabetic rats.

MATERIALS AND METHODS

Healthy male Sprague Dawley rats weighing 150-200 g were procured from National Institute Nutrition (NIN), Hyderabad, India. They were housed in polypropylene cages and maintained under standard conditions (12 h light and dark cycles, at $22 \pm 1^\circ\text{C}$ and 35-60% humidity). Standard palletized feed (Amrut Pellets for rats, Pune, India) and tap water were provided *ad libitum*. All the experimental procedures were approved by Institutional Animal Ethics Committee (Regd. No. 651/02/C/CPCSEA) constituted under 'Prevention of Cruelty to the Animals Act- 1960, Government of India'. Oleanolic acid was isolated and purified from *Viscumarticulatum* Burm. f. (Viscaceae) according to a method previously reported.^[10]

Induction and assessment of diabetic nephropathy

Diabetes was induced in the S.D. rats by intra peritoneal administration of STZ (Sigma) at 55 mg/kg dose as a solution in 0.1 mol/L citrate buffer (pH 4.4).^[17] To avoid STZ induced hypoglycemia, the rats were provided with 5% glucose solution instead of drinking water for the first 24 h after STZ challenge. Two days after STZ injection, blood samples were collected and serum glucose levels were estimated using the GOD-PAP diagnostic kit (Span Diagnostic, India). After maintenance for four weeks, the blood and urine samples from these rats were again tested for hyperglycemia and proteinuria. The urine protein was estimated by Biuret's method using a commercial kit (Aspen laboratories, India). Rats with hyperglycemia (≥ 250 mg/dl) and proteinuria (≥ 8.0 mg/dl) at the end of four weeks post STZ injection were selected for further study.

The diabetic rats were divided into four groups ($n=8$) and assigned to treatments as follows: control group (non-diabetic vehicle treated), diabetic control group (vehicle treated), OA (20 mg/kg/day *p. o.*) group, OA (40 mg/kg/day *p. o.*) group, OA (60 mg/kg/day *p. o.*) group. The treatments continued for four weeks.

At the end of eight weeks after STZ injection, urine and serum samples were collected from all the rats. For urine collection, the rats were individually kept in the metabolic cages for 24 h. The rats were sacrificed by cervical dislocation and one of the excised kidneys was homogenized and used for determination of lipid peroxidation, levels of reduced glutathione, catalase and superoxide dismutase. The remaining kidney from each rat was processed for histopathological and transmission electron microscopic examination.

Lipid peroxidation was estimated as malondialdehyde (MDA) equivalent according to a method described by Ohkawa *et al.*^[18] and level of reduced glutathione. The reduced glutathione was determined by 5-5-dithio bis-(2-nitrobenzoic acid (DTNB) method.^[19] The post mitochondrial supernatant prepared as per a report by Casalino *et al.*^[20] was used to determine the levels of catalase^[21] and superoxide dismutase.^[22] The remaining kidney from each rat was processed for histopathological and transmission electron microscopy examination.

Assessment of renal function

At the end of the eight weeks, blood samples (under light ether anesthesia through retro-orbital puncture) and 24 h urinesamples were collected from each rat. The renal function was assessed by measuring serum and urine levels of creatinine, urea and albumin. Creatinine clearance and urea clearance were determined to further calculate the glomerular filtration rate (GFR) as reported earlier.^[10]

Histological examinations

One kidney excised from each of the sacrificed rats was rinsed in saline, blotted dry on a tissue paper, weighed and half of the kidney sectioned longitudinally was immediately transferred to 10% v/v formaldehyde in saline in separate containers. These sections were processed for microscopic examinations after staining with hematoxylin Eosin stain.

Transmission electron microscopic examination

The remaining halves of the isolated kidneys were processed for electron microscopy as described by Dasgupta *et al.*^[23] The cortical part of the kidney was cut into blocks having approximately 1 mm³ dimensions. These blocks were dipped into 2.5% v/v glutaraldehyde solution for 24 h. Then the blocks were stored in phosphate buffered saline (PH- 7.4) at 4°C till further processing for TEM examination under Morgagni 268D transmission electron microscopes (Fei Company, The Netherlands). The TEM images were taken at $\times 5600$ magnification.

Assessment of food and water intake

The food and water intake was recorded on previous day of shifting rats to the metabolic cages. The average food intake was measured by providing the rats in individual cages with weighed amount of food and determining the weight of remaining chow in each cage on the next day. Similarly, water intake was measured by determining the average amount of water consumed by the rats.

Statistical analysis

Data are presented as mean $\pm$ SEM. The significance of differences was analyzed by one-way ANOVA followed by Bonferroni's multiple comparison test. $P < 0.05$ was considered significant.

RESULTS

As observed at the end of 8th week, five hours urine output in the diabetic rats was significantly more (5.4 ± 0.3 ml) than the control group rats (1.0 ± 0.1) ($P < 0.001$). OA treatment at all the tested doses reduced the urine output. Statistically significant reduction was seen at 40 mg/kg (2.9 ± 0.3 ml) ($P < 0.01$) and 60 mg/kg doses of OA (2.3 ± 0.4 ml) ($P < 0.001$).

In diabetic rats, the average weight of kidneys was significantly lesser as compared to the control rats. In the OA treated animals, the kidney weight was consistently maintained near the average kidney weight as observed in the control group rats [Table 1].

OA improved the creatinine and BUN clearances in the diabetic rats. These parameters were further used in the calculation of GFR. The GFR of diabetic rats (74.5 ± 2.5) was significantly lesser ($P < 0.01$) than that of control group rats (106.9 ± 5.2). OA treatment improved the GFR in the diabetic rats. The average GFR of the group treated with 60 mg/kg OA was similar (106.0 ± 5.0) to that of control group. At 20 mg/kg and 40 mg/kg doses of OA, the GFR was 89.3 ± 5.5 and 94.2 ± 4.6 respectively. Urine albumin, an indicator of renal damage was also reduced by the OA treatment [Table 1].

In diabetic rats, the average weight of kidneys was significantly lesser (0.7 ± 0.05 g) as compared to the control rats (0.9 ± 0.05 g) ($P < 0.5$). In the OA treated animals, the kidney weight was consistently maintained near the average kidney weight as observed in the control group rats [Table 1].

The values of these parameters are given as percentage of the control group values as reported earlier.^[24] The reduced glutathione content in the diabetic rat kidneys was reduced to $61.8 \pm 2.4\%$ as compared to control group rats ($P < 0.001$). Whereas, in the OA (60 mg/kg) treated rats the level of reduced glutathione was $91.0 \pm 6.3\%$. In the lower doses of OA, the reduced glutathione levels were improved in a dose dependent and statistically significant manner but the rise was not statistically significant. The activities of catalase and SOD were reduced in the diabetic control group rats to $58.6 \pm 4.3\%$ and $57.2 \pm 4.5\%$ respectively ($P < 0.001$). For the group treated with OA at 60 mg/kg dose, the enzyme activities were $80.8 \pm 2.5\%$ for catalase and $91.0 \pm 6.3\%$ for SOD. The level of lipid peroxidation was significantly increased in the diabetic rats up to 177.5 ± 13.0 ($P < 0.001$), whereas treatment with OA reduced the lipid peroxidation. This reduction in the lipid peroxidation was dose dependent with maximum effect (reduction up to $116.3 \pm 5.2\%$) in the group treated with 60 mg/kg/day of OA ($P < 0.001$).

The values of these parameters are given as percentage of the control group values as reported earlier.^[24] The reduced glutathione content in the diabetic rat kidneys was lower than the control group. OA treatment improved the levels of reduced glutathione in a dose dependent manner; however this rise was significant only in the group that received 60 mg/kg of OA. The activities of catalase and SOD in the diabetic control group rat kidneys were significantly lower than that in the control group. The level of lipid peroxidation was significantly increased in the diabetic control group rats, whereas OA treatment reduced the lipid peroxidation dose dependently [Table 1].

As shown in [Figure 1], the histology of kidneys of diabetic rats presented characteristic changes like glomerular thickening, interstitial fibrosis, necrosis, arteriopathy

Table 1: Effect of oleanolic acid on streptozotocin induced diabetic nephropathy and kidney functions tests

Group/Parameters (%)	Control	Diabetic control	OA 20 mg/kg	OA 40 mg/kg	OA 60 mg/kg
Weight of Kidney (g)	0.9 ± 0.1	0.7 ± 0.1	0.8 ± 0.03	0.8 ± 0.1	0.8 ± 0.1
Urine albumine (mg/dl)	4.22 ± 0.2	12.0 ± 0.6	9.6 ± 0.4^b	8.3 ± 0.3^c	6.6 ± 0.4^c
GFR	106.9 ± 5.2^b	74.5 ± 2.5	89.3 ± 5.5	94.2 ± 4.6	106.0 ± 5.0^b
Lipid peroxidation	100 ^c	177.5 ± 13.0	165.9 ± 9.6	143.2 ± 7.0	116.3 ± 5.2^c
Catalase	100 ^c	58.6 ± 4.3	67.1 ± 1.9	71.5 ± 2.9	80.8 ± 2.5^c
SOD	100 ^c	57.2 ± 4.5	59.2 ± 2.5	71.6 ± 2.3	91.0 ± 6.3^c
Reduced Glutathione	100 ^c	61.8 ± 2.4	76.2 ± 5.2	78.5 ± 5.5	90.6 ± 5.4^b

OA=Oleanolic acid, GFR=Glomerular filtration rate, SOD=Superoxide dismutase, Data are Mean $\pm$ SEM. The significance of difference between means was determined by One-way ANOVA followed by Bonferroni's posttest, ^b $P < 0.01$, ^c $P < 0.001$ compared to diabetic control, STZ=Streptozotocin

and presence of hyaline casts. Occurrence of such histological changes was less severe in the OA treated rats. In the group treated with OA (60 mg/kg) the kidney histology revealed no other alterations than thickening of the basement membrane that too was less prominent as compared to the diabetic rats.

The important finding of the present study is the effect of OA on the ultramicroscopic changes in the kidney architecture. The characteristic alterations of the diabetic nephropathy appeared in the TEM images of diabetic rat kidneys at the end of 8th week. The TEM images revealed thickening of glomerular basement membrane, decrease in the pore size between the basement cells and decreased number of podocytes [Figure 2]. Treatment of diabetic rats with OA protected the kidneys from such alterations.

OA treatment favorably affected the occurrence of other symptoms of diabetes. It prevented the diabetes induced body weight loss in a dose dependent manner. Diabetes induced polyphagia and polydipsia were also inhibited by OA treatment [Figure 3]. The excretion of urine was found to be 1.03 ± 0.1 ml in 24 hrs in non-diabetic rats. Whereas urine output in diabetic group was around 5.4 ± 0.33 ml in 24 hrs. Food intake in non-diabetic rats was found to be 19.5 ± 0.4 g and in diabetic rat it was found to be 38.33 ± 0.65 g in 24 hrs. OA significantly restore the polyuria and polyphagia in dose dependant manner.

DISCUSSION

In the present investigation, effects of orally administered OA were evaluated in the STZ induced DN model in SD rats. Histological and ultra-microscopic alterations in the rat kidneys pertinent to diabetic nephropathy develop at around eight weeks^[25,26] therefore; present study was extended for eight weeks after induction of diabetes. Treatment with OA was also started after confirmation of the proteinuria, an indicator of the

initiation of the diabetic nephropathy.

OA exerts antidiabetic effects through multiple mechanisms which include alpha glucosidase inhibition,^[7] insulinomimetic activity,^[8] insulin sensitization^[27] and acceleration of glucose

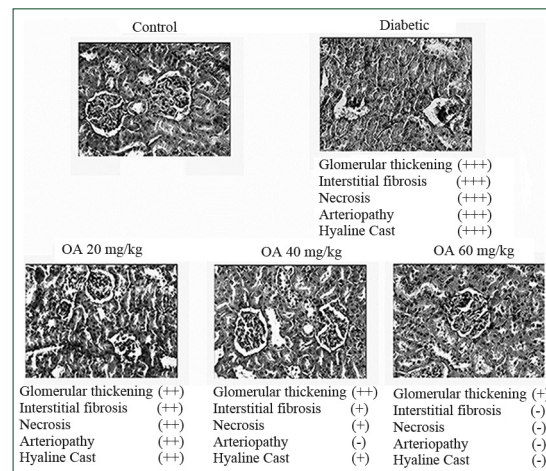


Figure 1: Oleanolic acid protects rats kidneys from histological changes induced during diabetic nephropathy. Magnification: $\times 400$. Arbitrary gradings given in parenthesis by a blinded observer indicate: (+++): Very severe, (++) : severe. (+): Only detectable, (-): Absent

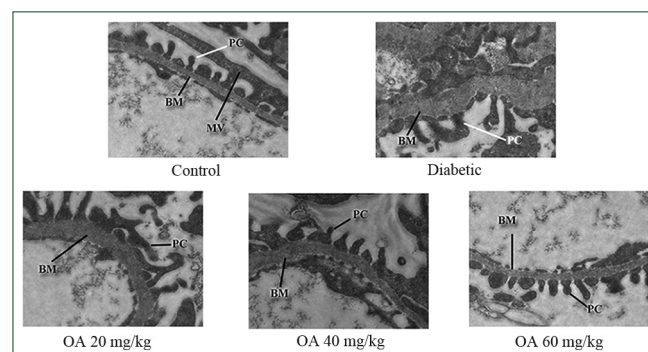


Figure 2: Oleanolic acid protects rats kidneys from ultramicroscopic changes associated with diabetic nephropathy. Magnification: 5600X, BM : Basement membrane, PC : podocyte, MV : Microvasculature

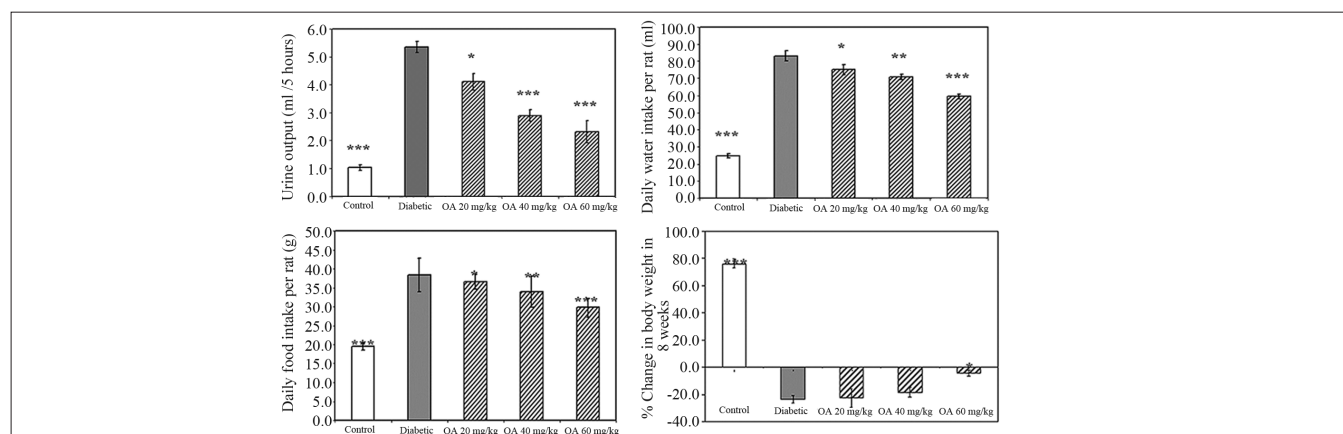


Figure 3: Oleanolic acid ameliorated the symptoms of streptozotocin induced diabetes in rats

metabolism.^[9] The renal damage associated with diabetes results from prolonged hyperglycemia induced oxidative stress that leads to decreased levels of endogenous antioxidants such as catalase, superoxide dismutase, and reduced glutathione. Hyperglycemia generates oxidative stress via formation of reactive oxygen species.^[28] The low level of superoxide dismutase, catalase and reduced glutathione in biological fluids and tissues during the progress of diabetes is a consequence of the oxidative stress.^[24]

The reduction in blood sugar levels in diabetic rats after treatment with OA as observed in the present study is in accordance with earlier reports.^[6,7] This hypoglycemic effect may be attributed to multi-pronged effects of OA like inhibition of α -glucosidase, an enzyme involved in intestinal glucose absorption,^[8] enhancement of insulin secretion^[9] as well as insulinomimetic and insulin sensitizing effects.^[27] Such diverse mechanisms of action are probably unique to OA. Decrease in the food intake observed in present study also indicates inhibition of diabetic polyphagia by OA. Similar effects of OA on food intake in diabetic rats have been reported earlier.^[6,13] OA also inhibited polydipsia in rats which indicates that it has potential to subside the symptoms of diabetes.

It was observed that the diabetic control group rats suffered from polyuria. The major reason for the polyuria in diabetes is osmotic diuresis.^[29] The treatment of diabetic rats with OA reduced polyuria. An interesting aspect about OA is that it is well known to induce diuresis.^[30] However, in diabetic rats it inhibits the polyuria induced by hyperglycemia. In present study it was observed that initially OA (60 mg/kg/day) increased urine output even in diabetic rats. However, such rise in the diuresis subsided after continuous treatment of four weeks. The diabetes associated polyuria occurs because of the hyperglycemia. OA is well known to reduce blood sugar levels and improve insulin sensitization including antioxidant activity. These effects might reduce overt urine output in the diabetic rats treated with OA. This reflects the presence of excess peroxides and hydroxyl radicals in diabetic kidney which contribute to the perturbed renal functions.^[31] Our results are in good agreement with previous report.^[7] STZ-administration brought about reduction in the levels of renal antioxidants viz. GSH, SOD and catalase. Such oxidative stress is responsible for decrease in urea clearance and creatinine clearance leading to decreased GFR.^[31] Oxidative stress can promote the formation of a variety of vasoactive mediators that can affect renal functions directly by causing renal vasoconstriction or through decreasing the glomerular capillary ultra-filtration coefficient and, thus, reduce GFR.^[32] Being a potent antioxidant, OA may contribute to reduction in the renal vasoconstriction and thereby improves GFR. Present study also established that OA protects kidney cell membranes from oxidative damage through the reduction in lipid peroxidation in kidney tissue.

At the fourth week after STZ injection rats showed significant albuminuria which indicated initiation of diabetic nephropathy. The increased urine albumin and increased urine creatinine levels in diabetic rats are considered as markers of diabetic nephropathy.^[26] Alterations in the kidney ultra-microstructures are important indicators of occurrence of diabetic nephropathy.^[14-16] Characteristic changes like podocyte abnormality, thickening of glomerular basement membrane establish the development of diabetic nephropathy. Hence in this study, kidneys were subjected to TEM examination. The diabetic control rats revealed podocyte abnormality, thickening glomerular basement membrane and increased gap between the basement cells. OA treatment protected the rats from such ultramicroscopic alterations. The increased thickness of GBM, destruction of podocytes and increase in the intracellular spaces leads to albuminuria. The observed reduction in the urine albumin level in the OA treated rats is also in congruence with this observation. Thus OA was found to protect the architecture of kidneys. A recent report states that treatment of diabetic mice with OA exerts anti-glycative effects in the mice kidneys.^[13] The direct cytoprotective effects of OA^[11] also might have contributed to preservation of the kidney ultra-structures.

Diabetic nephropathy is a complex disorder involving multiple etiological factors. Hence, use of multifunctional agents to intervene its pathogenesis is more rational. As compared to the conventional drugs, the use of multifunctional drugs is applicable in the treatment of disorders including cancer and Alzheimer's disease.^[3] In present study, OA treatment was also found to reduce the serum glucose levels in the treated rats. This hypoglycemic effect of OA might contribute to its efficacy in inhibiting progression of diabetic nephropathy. At the 55 mg/kg i. p. dose of STZ, there is a possibility of residual beta cells in the pancreas of rats. Hence, the insulinogaugeffect^[9] of OA might have partially contributed to the observed protective effects in diabetic nephropathy.

The scientific reports on OA including present study conclusively demonstrate its efficacy to intervene the pathogenesis of diabetic nephropathy through multiple mechanisms. The lower systemic toxicity of OA and its ability to act on multiple biological targets is also well known.^[2-4] OA has a favorable safety profile and has already been tested clinically.^[5] It is proposed that OA deserves further investigations through clinical trials for its role in the treatment of diabetic nephropathy.

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