

**Brief Report**

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**A low-dose combination of valsartan and low molecular weight heparin better improved glomerular permeability than did high-dose monotherapy in rats with diabetic nephropathy**Bo Jiao<sup>1,\*</sup>, Yahui Zhang<sup>1,2</sup>, Yanna Cheng<sup>1</sup>, Jianjun Gao<sup>1</sup>, Qingzhu Zhang<sup>1</sup><sup>1</sup> Department of Pharmacology, School of Pharmaceutical Science, Shandong University, Ji'nan, Shandong, China;<sup>2</sup> Department of Pharmacy, Provincial Hospital Affiliated to Shandong University, Ji'nan, Shandong, China.

**ABSTRACT:** Diabetic nephropathy is the most common and severe renal complication of diabetes mellitus. The present study sought to investigate the renoprotective effects of a combination therapy of valsartan and low molecular weight heparin (LMWH) in rats with diabetic nephropathy induced by uninephrectomy and streptozotocin. The animals were divided into five groups as follows: sham-operated rats, diabetic control rats, diabetic rats treated with 20 mg/kg/day valsartan, diabetic rats treated with 600 IU/kg/day LMWH, diabetic rats treated with a combination of valsartan and LMWH (valsartan 10 mg/kg/day and LMWH 300 IU/kg/day). The treatment regimen was maintained for 8 weeks. Treatment with valsartan, LMWH, or a combination of the two had no significant effect on blood glucose levels. However, the urine protein excretion levels significantly decreased for the three drug treatment groups; the most dramatic decreases were observed in the combination treatment group. Kidney histology was examined using periodic acid-Schiff staining and immunohistochemical staining of extracellular matrix proteins. Results indicated that histopathology improved markedly in the three drug treatment groups; combination therapy had an equal or better effect than monotherapy in terms of decreasing the abnormal thickness of the glomerular basal membrane, the ratio of the area of the mesangial region with respect to the total area of renal glomeruli, and the accumulation of collagen IV and laminin in kidney tissue. In addition, serum levels of transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ) also markedly decreased in the drug treatment groups according to ELISA. However, there were no significant differences between the combination therapy group and monotherapy group. These

results suggest that a combination of valsartan and LMWH at half the dose used in monotherapy is better at improving glomerular permeability in rats with diabetic nephropathy.

**Keywords:** Valsartan, low molecular weight heparin, transforming growth factor- $\beta_1$  (TGF- $\beta_1$ ), diabetic nephropathy

**1. Introduction**

Diabetic nephropathy is the most common complication of diabetes mellitus, often leading to end-stage kidney disease and a high risk of mortality (1). The condition is characterized by progressively increasing albuminuria and histopathological features including glomerular basement membrane (GBM) thickening and mesangial expansion due to accumulation of extracellular matrix (ECM) proteins (2). Previous studies suggested that the rennin-angiotensin system (RAS) plays an important role in progressive renal injury in diabetic nephropathy (3). Angiotensin II (Ang II), a member of the RAS family, is thought to act as a crucial mediator in this disease (4). It was indicated that Ang II induced the expression of TGF- $\beta_1$ , a key cytokine responsible for GBM thickening, mesangial expansion, and glomerulosclerosis after coupling to its receptors (5,6). Ang II type 1 receptor blockers (ARBs) were found to inhibit TGF- $\beta_1$  expression in kidney tissues and thus delay the progression of diabetic nephropathy (7).

Valsartan, a typical ARB, has been widely used in the clinical treatment of diabetic nephropathy. However, a major adverse effect of this drug is to induce a compensatory rise in renin due to the disruption of the Ang II feedback inhibition of renin production, potentially leading to renal and cardiovascular damage (8). Since the compensatory rise in renin paralleled the dosage of ARBs, reduction of the dosage of this drug may decrease the risk of its adverse effect (9).

Low molecular weight heparin (LMWH) has been

\*Address correspondence to:

Dr. Bo Jiao, Department of Pharmacology, School of Pharmaceutical Science, Shandong University, Wenhua-xi Road No. 44, Ji'nan 250012, Shandong, China.  
e-mail: jiaob@sdu.edu.cn

used clinically and has renoprotective action through inhibition of the alteration of the GBM, decreasing the accumulation of mesangial matrix and reducing the abnormal excretion of albumin (10-12). Further studies suggested that LMWH has a renoprotective effect by suppressing high glucose-induced TGF- $\beta_1$  expression (13). Because valsartan and LMWH have similar mechanisms of renoprotection, *i.e.* inhibiting the expression of TGF- $\beta_1$  in kidney tissue, the present study sought to investigate the renoprotective effects of a combination of valsartan and LMWH at low doses in rats with diabetic nephropathy induced by unilateral nephrectomy and streptozotocin (STZ) injection. In addition, the serum TGF- $\beta_1$  concentration was determined in order to elucidate the underlying mechanism of inhibited TGF- $\beta_1$  expression.

## 2. Materials and Methods

### 2.1. Chemicals

Valsartan was purchased from Changzhou Kony Pharm Co., Ltd. LMWH was obtained from Qilu Pharmaceutical Co., Ltd. Valsartan was suspended in 0.5% carboxy methyl cellulose solution and LMWH was dissolved in normal sodium before use.

### 2.2. Animal model

Male Wistar rats weighing 180-220 g were purchased from the Animal Experimental Center, Shandong University, Shandong, China. The research protocol was in accordance with the institutional guidelines of the Animal Care and Use Committee of Shandong University. Rats were kept in a 12 h light/dark cycle at 25°C and fed a standard rat chow and water *ad libitum*. Before unilateral nephrectomy, rats were anaesthetized by intraperitoneal injection of sodium pentobarbital at a dose of 45 mg/kg body weight. Under sterile conditions, a right dorsolateral laparotomy was performed and the right kidney was retracted to expose the renal vessels and ureter. The vessels and ureter were ligated with cotton thread and cut between the kidney hilus and ligated portion to remove the kidney. The adrenal gland was left in place. The skin was sutured and the rats were kept in regular cages. In sham-operated rats, the thread was passed around the vessels and ureter and then removed. The kidney was returned to the abdominal cavity and the incision was sutured. After surgery, the rats were administered 50 U/d penicillin for 3 days. The wound completely healed after 2 weeks.

The uninephrectomized rats were intraperitoneally injected with STZ (Sigma-Aldrich, USA) at a dose of 45 mg/kg body weight (14). The sham-operated rats were given an equal volume of citric acid buffer solution. After 72 h, blood glucose was determined and diabetes was confirmed by BG  $\geq$  16.7 mmol/L. Rats were randomly

divided into 5 groups ( $n = 8$ /group). Group 1 consisted of sham-operated rats, group 2 consisted of diabetic rats, group 3 consisted of diabetic rats intragastrically administered valsartan at a dose of 20 mg/kg/day, group 4 consisted of diabetic rats hypodermically injected with LMWH at a dose of 600 IU/kg/day, and group 5 consisted of diabetic rats simultaneously given half the dose of valsartan and LMWH, *i.e.* 10 mg/kg/day and 300 IU/kg/day, respectively. The treatment regimen was maintained for 8 weeks.

### 2.3. Blood glucose and urine protein assay

A blood glucose assay was performed by collecting blood samples from the tails of rats in each group at the beginning and the end of the study period, respectively. A urine protein assay was performed by collecting 24 hours of urine using metabolic cages on the day before the end of the experiment. Blood glucose and urine protein were measured with an Automatic Biochemistry Analyzer (AU2700; Olympus Optical Co., Mishima, Japan).

### 2.4. Enzyme-linked immunosorbent assay (ELISA)

Blood samples were collected from the abdominal aorta of rats at the end of the study period to measure the serum levels of TGF- $\beta_1$ . TGF- $\beta_1$  was determined with a commercially available ELISA kit (Bionewtrans Pharmaceutical Biotechnology Co. Ltd., USA). Analysis was performed according to the manufacturer's recommended protocol and the methods described previously (15,16). Concentrations are presented as the mean of two measurements.

### 2.5. Histologic examination

Kidneys were completely perfused with normal saline and then removed. Kidney samples were fixed in 4% paraformaldehyde solution for 24 hours and subsequently embedded in paraffin (17). Tissues were cut into 3- $\mu$ m-thick slices for the following studies. For an examination of histomorphological features, tissues were cut into 3- $\mu$ m-thick slices and stained with periodic acid-Schiff (PAS). To characterize the expression of ECM proteins, Col-IV and LN immunohistochemical staining was performed (18,19). After incubation with anti-Col-IV (Chemicon, California, USA) or anti-LN (abcam, Cambridge, UK) antibody at 4°C, the sections were washed and treated with biotinylated anti-immunoglobulin and then washed, reacted with avidin-conjugated horseradish peroxidase H complex, and incubated in diaminobenzidine and hydrogen peroxide. The slides were then rinsed in distilled water, counterstained with hematoxylin, and mounted. Images were captured and quantified by means of a computer-assisted image analyzer, Image Pro Plus 5.1 (Media Cybernetics, Inc., Bethesda, MD, USA). Ten glomeruli were analyzed in each section (20).

## 2.6. Statistical analysis

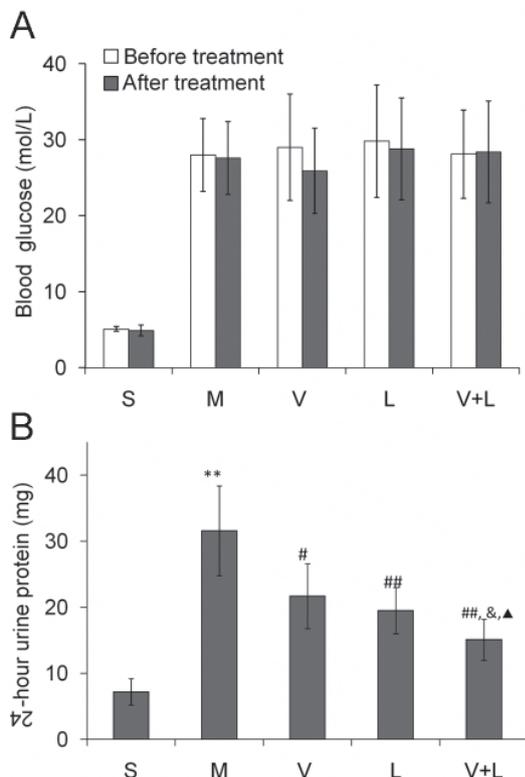
Data are expressed as mean  $\pm$  S.D. One-way ANOVA followed by LSD post hoc analysis was performed using SPSS/Win11.0 software (SPSS, Inc., Chicago, Illinois, USA);  $p < 0.05$  was indicative of a significant difference.

## 3. Results and Discussion

### 3.1. Effect of valsartan, LMWH, and a combination of the two on blood glucose and urinary protein excretion

Blood glucose levels of rats in all groups at the beginning and the end of the study period were determined. There was no significant variation in the blood glucose for all of the diabetic rats (groups 2-5) before or after the experiment (Figure 1A,  $p > 0.05$ ). Blood glucose did not change significantly before and after the experiment for any group, indicating that treatment with valsartan, LMWH, or a combination of the two did not affect blood glucose (Figure 1A,  $p > 0.05$ ).

Figure 1B shows the amount of 24-h protein excretion in urine collected on the day before the end

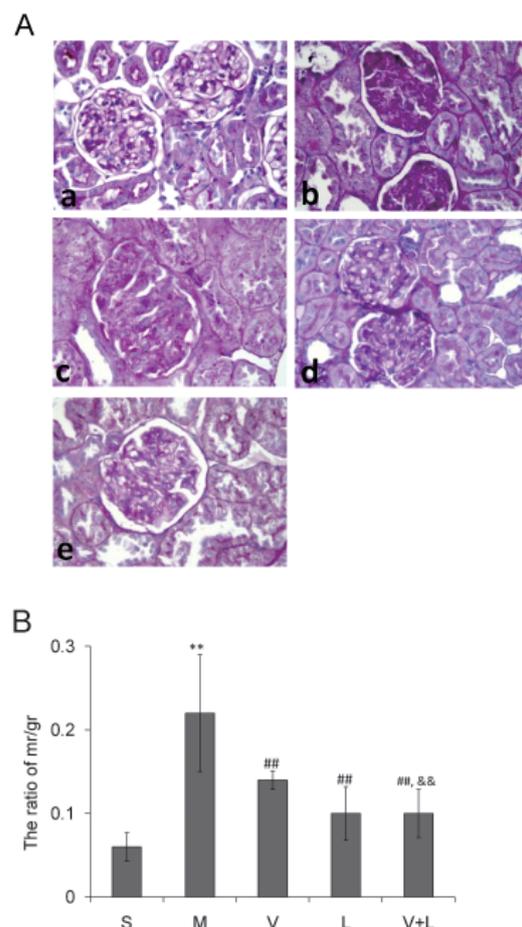


**Figure 1. (A)** Rat blood glucose levels determined at the beginning and the end of the study period; **(B)** The amount of 24 h protein excretion in urine collected during the day before the end of the study. S, sham-operated rats; M, model rats (diabetic control rats); V, valsartan-treated rats (20 mg/kg/day); L, LMWH-treated rats (600 IU/kg/day); V + L, combination (valsartan + LMWH)-treated rats (10 mg/kg/day and 300 IU/kg/day). \*\*  $p < 0.01$  vs. sham-operated group. #  $p < 0.05$ , ##  $p < 0.01$  vs. model group. &  $p < 0.05$  vs. valsartan group. &  $p < 0.05$  vs. LMWH group.

of the study. The mean amount of protein excretion in diabetic control rats was 31.6 mg, which was significantly higher than that (7.2 mg) in sham-operated control rats ( $p < 0.01$ ). The amount of protein excretion was 21.7 and 19.5 mg, respectively, in groups treated with valsartan at a dose of 20 mg/kg/d and LMWH at a dose of 600 IU/kg/day ( $p < 0.05$ , valsartan group vs. diabetic control;  $p < 0.01$ , LMWH group vs. diabetic control). Diabetic rats that received a combination of valsartan and LMWH at half the dose used in monotherapy excreted an even lower level of urinary protein, 15.5 mg, compared to those that received monotherapy ( $p < 0.01$ , combination treatment vs. diabetic control;  $p < 0.05$ , combination treatment vs. valsartan or LMWH treatment).

### 3.2. Effect of valsartan, LMWH, and a combination of the two on renal histology

Figure 2 shows the histomorphological features



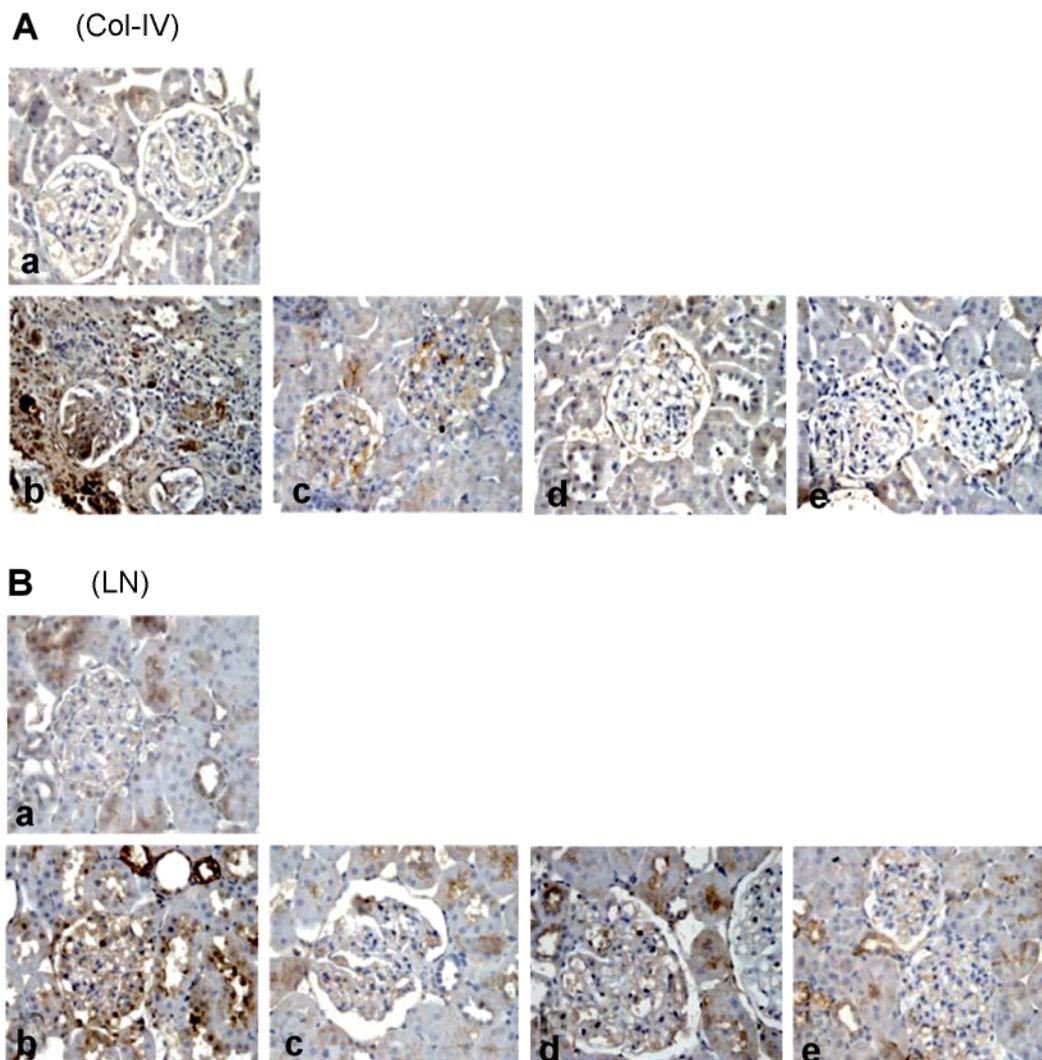
**Figure 2. Renal histology (A)** and the ratio of the area of the mesangial region (mr) with respect to the total area of renal glomeruli (rg) **(B)** for each group at the end of the study period (stained by PAS,  $\times 400$ ). S, sham-operated rats; M, model rats (diabetic control rats); V, valsartan-treated rats (20 mg/kg/day); L, LMWH-treated rats (600 IU/kg/day); V + L, combination (valsartan + LMWH)-treated rats (10 mg/kg/day and 300 IU/kg/day). \*\*  $p < 0.01$ , vs. sham-operated group. ##  $p < 0.01$  vs. model group. &  $p < 0.01$  vs. valsartan group. &  $p < 0.01$  vs. LMWH group.

of kidneys from rats receiving various treatments. Diabetic control rats (Figure 2Ab) had an enlarged area of the mesangial region and thicker GBM compared to sham-operated rats (Figure 2Aa). Valsartan or LMWH treatment alone improved glomerular pathology. A combination of both drugs at half the dose provided an equal or even greater benefit (Figures 2Ac-2Ae). The ratio of the mesangial region area with respect to the total area of renal glomeruli in each group is shown in Figure 2B. Diabetic control rats had a significantly larger ratio (0.22,  $p < 0.01$ ) compared to sham-operated rats (0.06). Meanwhile, groups treated with valsartan or LMWH alone had a smaller ratio of the area of the mesangial region (0.14 and 0.10). The ratio for the combination treatment group was 0.10, which was significantly smaller than that for the valsartan treatment group and nearly equal to that for the LMWH treatment group ( $p < 0.05$ , combination treatment vs. valsartan treatment;  $p > 0.05$ , combination treatment vs. LMWH treatment).

The expression of ECM proteins Col-IV and LN in kidney tissues was examined immunohistochemically (Figure 3). The amount of Col-IV and LN in diabetic control rats had increased compared to the amount in sham-operated rats. Valsartan or LMWH treatment alone decreased the accumulation of these two proteins. The combination treatment was better at diminishing the expression of Col-IV and equivalent at reducing the expression of LN compared to sole drug treatment.

### 3.3. Effect of valsartan, LMWH, and a combination of the two on the serum TGF- $\beta_1$ level

The effect of drug treatment on the serum level of TGF- $\beta_1$  was also examined using an ELISA assay (Table 1). The mean serum concentration of TGF- $\beta_1$  in diabetic control rats was 266.4 pg/L and was higher than that (119.5 pg/L) in sham-operated rats. After 8 weeks of valsartan treatment at a dose of 20 mg/kg/day, rats exhibited significantly lower levels of TGF- $\beta_1$ ,



**Figure 3. Immunohistochemical staining of Col-IV (A) and LN (B) in rat kidney tissues at the end of the study period. a,** sham-operated rats; **b,** diabetic control rats; **c,** valsartan-treated rats (20 mg/kg/day); **d,** LMWH-treated rats (600 IU/kg/day); **e,** combination (valsartan + LMWH)-treated rats (10 mg/kg/day and 300 IU/kg/day).

**Table 1. Rat serum TGF- $\beta_1$  concentrations in each group at the end of the study period**

Group	Dosage	TGF- $\beta_1$ level (pg/L)
Sham-operated rats	–	119.5 $\pm$ 23.8
Diabetic control rats	–	266.4 $\pm$ 41.1**
Valsartan-treated rats	20 mg/kg	186.7 $\pm$ 38.3 <sup>###</sup>
LMWH-treated rats	600 IU/kg	203.5 $\pm$ 33.5 <sup>###</sup>
Combination (valsartan + LMWH)-treated rats	Valsartan, 10 mg/kg LMWH, 300 IU/kg	202.7 $\pm$ 37.0 <sup>###</sup>

\*\*  $p < 0.01$ , vs. sham-operated rats; <sup>###</sup>  $p < 0.01$ , vs. diabetic control rats.

186.7 pg/L on average, compared to diabetic control rats ( $p < 0.01$ ). Rats treated with LMWH at a dose of 600 IU/kg/day also exhibited significantly lower levels of TGF- $\beta_1$ , *i.e.* 203.5 pg/L ( $p < 0.01$ ) on average. In the combination treatment group, rats administered 10 mg/kg/day valsartan and 300 IU/kg/day LMWH had significantly lower levels of TGF- $\beta_1$ , 202.7 pg/L on average, compared to diabetic control rats ( $p < 0.01$ ). However, no significance differences between the combination treatment group and valsartan or LMWH group were observed.

The present study investigated the renoprotective effects of valsartan, LMWH, and a low-dose combination of the two in rats with diabetic nephropathy induced by uninephrectomy and STZ. Valsartan, LMWH, or a low-dose combination of the two did not affect blood glucose levels in the rats. Treatment with valsartan or LMWH alone significantly decreased albuminuria, the abnormal thickness of the GBM, the ratio of the mesangial region area with respect to the total area of renal glomeruli, and the amount of ECM proteins Col-IV and LN that accumulated in kidney tissue. A combination therapy of valsartan and LMWH at half the dose used in monotherapy was better than monotherapy in terms of diminishing urine protein excretion and ameliorating renal histopathology and was equivalent to monotherapy with respect to reducing the level of serum TGF- $\beta_1$ . These findings implicate that a low-dose combination of valsartan and LMWH is better at improving glomerular membrane protein permeability in rats with diabetic nephropathy.

Kidney cells such as mesangial cells and podocytes are able to synthesize all of the components of RAS such as renin, the (pro)renin receptor, angiotensinogen, and Ang II receptors independently of the systemic RAS, thereby making the kidney capable of maintaining a high level of local Ang II (21,22). Hyperglycemia may activate the intrarenal RAS, leading to accumulation of Ang II in the kidney (22-24). Treatment of rat mesangial cells cultured *in vitro* with Ang II increased the expression of TGF- $\beta_1$  and ECM proteins while the competitive inhibitor of Ang II, saralasin, inhibited that expression (6). These results coincide with the current findings that blocking the Ang II pathway with valsartan decreased the serum level of TGF- $\beta_1$  in rats with diabetic

nephropathy. The current results are also consistent with findings from previous studies that LMWH significantly diminished the serum level of TGF- $\beta_1$  in diabetic rats. TGF- $\beta_1$  plays a key role in increasing glomerular permeability by stimulating ECM protein synthesis, increasing matrix protein receptors, and altering the protease/protease-inhibitor balance, thereby inhibiting matrix degradation (25). Thus, these results suggest that inhibiting TGF- $\beta_1$  expression with valsartan or LMWH is associated with ameliorated glomerular permeability.

The current study showed that a low-dose combination of valsartan and LMWH offered better renoprotection in terms of decreasing urine protein excretion, indicating that combination therapy is better at ameliorating glomerular permeability. Although combination therapy had the same level of inhibition of TGF- $\beta_1$  expression as did monotherapy, a low-dose combination of valsartan and LMWH resulted in additional inhibition of TGF- $\beta_1$  expression. Since lower dosages of valsartan and LMWH were employed in the combination therapy, adverse effects of these drugs might thus be reduced. Given these facts, the current study suggests that a low-dose combination of valsartan and LMWH is better at treating diabetic nephropathy. However, further studies are needed to clarify the mechanism underlying the augmented renoprotective effects of this combination therapy.

In conclusion, the current data demonstrated that a low-dose combination of valsartan and LMWH was better at ameliorating glomerular permeability than was monotherapy at high doses in rats with diabetic nephropathy. This study hints at the benefits of combining valsartan and LMWH when treating diabetic nephropathy.

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