

## PHENOBARBITAL AMELIORATES HYPERGLYCEMIA-INDUCED ANGIOGENESIS IN DIABETIC NEPHROPATHY-POSSIBLE INTERVENTION AT THE HIF-1 $\alpha$ /VEGF AXIS

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*Hyperglycemia contributes to a cascade of inflammatory responses in kidneys that result in the development of renal hypoxia and angiogenesis with subsequent chronic renal failure. As the hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ )/vascular endothelial growth factor (VEGF) axis is a key pathway for neovascularization, the inhibition of this axis is a target for renal angiogenesis therapy. We speculate that Phenobarbital (PB) which has a potential to reduce vascularization in clinical settings might have an influence on the development of angiogenesis in diabetic kidney. The aim of the study was to explore the effects of PB on the HIF-1 $\alpha$  and VEGF expression and angiogenesis in renal tissue of rats with hyperglycemia and diabetic nephropathy. Sixty-four male Wistar rats were divided into 4 groups: control group received a single intraperitoneal saline injection; PB group received 0.05% PB orally in drinking water; diabetic group received a single intra-peritoneal STZ (65 mg/kg) injection; PB-STZ group received 0.05% PB orally two weeks before STZ administration. At the end of the experiment period (8 weeks), the kidneys were removed and used for biochemical analyses. Serum glucose, urea and creatinine levels, IL-6 levels in kidney homogenate and changes in HIF-1 $\alpha$  and VEGF gene expression were estimated. Hematoxylin-eosin staining was performed for histopathological examination. The results obtained showed that both HIF-1 $\alpha$  and VEGF gene expression and IL6 level in diabetic rat group were significantly elevated compared to that in control group, whereas in PB and PB-STZ groups, these indices were significantly down-regulated compared to the diabetic group. Abundant glomerular congestion and mesangial proliferation were detected in diabetic rat renal tissues. However, in PB-treated diabetic group, newly formed vessels were significantly decreased. These findings confirmed that phenobarbital, affecting the HIF-1 $\alpha$ /VEGF signaling pathway, ameliorates angiogenesis after hyperglycemic kidney injury.*

**Key words:** hyperglycemia, kidney injury, VEGF, HIF-1 $\alpha$ , IL6, angiogenesis.

**D**iabetic nephropathy DN refers to the condition of renal angiogenesis pathology caused by chronic ischemia in renal tissues. It is one of the most frequent complications of diabetes mellitus, affecting approximately 40% of diabetic patients and the leading cause of end-stage renal disease (ESRD).

Diabetic nephropathy (DN) is related to disturbed blood glucose metabolism with alternation and blockade of micro-vessel blood flow that leads to microcirculation dysfunction, hypoxia, ischemia of the renal tissue, and the onset of nephropathy. The

major driver in this process seems to be increased expression of VEGF-A, induced by hyperglycemia during the early phases of diabetes. This can stimulate endothelial cell proliferation with inhibition of apoptosis. Also, persistent hyperglycemia can enhance endothelial cell proliferation [1, 2].

Renal angiogenesis is a major pathological feature of diabetic nephropathy (Fig. 1). Hyperglycemia induces VEGF expression with a role of VEGF-A family in glomerular and cortical cellular proliferation and increased vascular permeability induced by TGF- $\beta$  expression. This in turn leads to glomerular

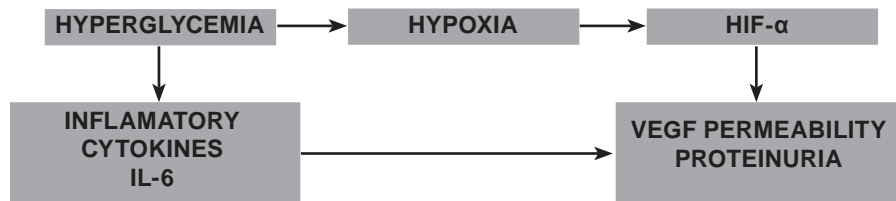


Fig. 1. Angiogenesis in DN: diagrammatic illustration of role of hyperglycemia in VEGF gene expression

injury and DN that is reduced when the TGF- $\beta$  is inhibited [3, 4]. The aim of inhibiting or controlling angiogenesis is to improve clinical outcome.

Currently, one of the major treatment approaches for DN include hypoglycemic drugs, but these may not be effective to preserve renal condition and (DN) may occur. We were focusing on preventing and delaying DN and other microvascular complications through improving glycemic control [5]. Inhibitors of VEGF, endostatin, an inhibitor of angiogenesis [6] and tumstatin [7], both prevented glomerular hypertrophy, hyperfiltration, and albuminuria in type 1 diabetic mice and attenuated the increased levels of VEGF-A and angiotensin-2.

One of the outcomes of DN is glomerular hypertrophy. This occurs in Streptozotocin-injected rats after 10-50 days (about 6-8 weeks) of induction with varying degrees compared to controls: glomerular hypertrophy and mesangial expansion with thickening of glomerular basement membrane. Mesangial expansion leads to the reduction in GFR that is commonly observed in diabetic patients and finally results in renal function impairment and proteinuria [8]. The abnormal angiogenesis is mediated by the VEGF-A family and is observed in diabetic patients during the first two years of onset of the disease supporting the early onset of development in early phases of DN. This abnormal angiogenesis is driven by several factors, including tissue hypoxia sensed by the hypoxia-sensing pathway (HIF-1 $\alpha$ ) [9].

Mesangial expansion and proteinuria were shown to correlate together and was found to have a strong inverse correlation with capillary surface area and GFR of patients at different stages of DN [8]. Glomerulosclerosis and albuminuria are promoted by elevated HIF-1 $\alpha$  activity, however, suppressed tubular HIF-1 $\alpha$  activity protects and prevents development of diabetes-induced tissue hypoxia, tubulointerstitial fibrosis and proteinuria [1].

HIF-1 $\alpha$  is a regulatory factor in the induction of hypoxia. Persistent elevation of HIF-1 $\alpha$  level up-regulates transcription activation of VEGF gene. As

a protective mechanism in renal hypoxia against injury with diabetes mellitus, the HIF-1 $\alpha$ /VEGF axis is considered a key for the renal neo-vessel formation. In diabetic nephropathy, HIF-1 $\alpha$  and VEGF were significantly upregulated with a positive correlation with micro-vessel formation [10]. The VEGF was increased correlating with the increased HIF-1 $\alpha$  and this occurs gradually as the hypoxia increases.

Targeting the HIF-1 $\alpha$ /VEGF axis is a key step in delaying angiogenesis and subsequent complications in the kidney.

An inflammatory response cascade induced by renal hypoxia results in renal interstitial fibrosis, hence chronic renal failure and associated with increasing levels of the inflammatory mediators, Interleukin-6 (IL-6), IL-10, and TNF- $\alpha$ .

Cytokines and chemokines including IL-6, 1- $\beta$  and IL-4 are produced in excess in diabetic kidney disease (DKD) and have a role in interstitial inflammation in DKD with decreased GFR in these cases [11]. Positive expression signals of HIF-1 $\alpha$ , HIF-2 $\alpha$  were detected, as well as VEGF and its receptor VEGFR-2 in epithelial cells of renal tubules and collecting tubules in the sheep breeds kidneys [12].

Anti-epileptic drugs (AEDs) used in the treatment of some seizures may have effects on vascular markers. Diabetes mellitus is associated with vascular changes and vascular risk markers that prompt re-evaluating the relationship between vascular risk markers and the commonly used anti diabetic drugs and AEDs that may help [13].

PB treatment was found to inhibit chondrogenesis, reduce angiogenesis and the related genes with a delay in the ossification of long bones and inhibition of cytoskeletal development in chick embryos. PB was also found to reduce vascularization and may be used in clinical settings to reduce renal angiopathy and avoid its deleterious effects in diabetic patients who often require lifelong medical treatment [14].

PB, a sedative hypnotic drug of the barbiturate family with anti-epileptic and anticonvulsant properties (AEDs) is commonly used to control sei-

zure specially in pregnant females with epilepsy. Embryonic skeleto-genesis defects were found to be a result of long-term use of the PB in pregnant females [15]. Here, we speculate that PB might have an influence on the development of angiogenesis in diabetic kidneys with reduction of proteinuria and nephropathy. To verify this assumption, we assayed the development of hyperglycemia-associated angiogenesis. Therefore, the aim was to explore the effects of PB on the HIF-1 $\alpha$  and VEGF expression and angiogenesis in DN that may provide insight into the PB use for affecting this pathway and its use in DN treatment.

### Materials and Methods

**Animals.** Sixty-four adult male Wistar rats (250-300g) were used for this study. Animals were acclimatized for 72 h before initiation of the study. All animal care and procedures followed of the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes (Strasbourg, 1986) and the National Institutes of Health Guidelines for the care and use of laboratory animals. Animals were housed in stainless steel cages and had free access to food and water ad libitum for the duration of the study. The Research Ethics Committee, Faculty of Medicine, Minia University granted the ethical approval for this research.

**Experimental design.** The animals were divided into 4 groups. Group 1 (Control group) ( $n = 16$  rats): received vehicle (1% carboxymethylcellulose) orally once daily and by a single intraperitoneal saline injection. Group 2 (PB group) ( $n = 16$  rats): received PB orally added to drinking water (0.05%) for up to 8 successive weeks. Group 3 (Diabetic group), a model group ( $n = 16$  rats), for generating proliferative DN model, received vehicle (1% carboxymethylcellulose) once daily and received STZ (65 mg/kg) by a single intraperitoneal injection. Group 4 (PB-STZ-treated group) ( $n = 16$  rats): received PB (0.05%) orally two weeks before STZ administration by a single intraperitoneal injection of STZ (65 mg/kg).

**Generation of proliferative DN rat model.** Using the previously documented method [16], proliferative DN rat model was generated according to the following steps.

To generate a T1DM state, a single dose of Streptozotocin, (Sigma-Aldrich, St. Louis, MO, USA) was injected into the peritoneal cavity of a total of 16 healthy 8-week aged SD rats, at 65 mg/kg single dosage. Rat blood glucose level was measured,

and those higher than 13.9 mmol/l were assigned to diabetic rats.

**Phenobarbital (PB) treatment.** PB was admixed to drinking water, administered to groups 2 and 4 of the rats for 8 successive weeks adjusted at a daily dose of 50mg/kg bodyweight.

The regimen for the PB dose was adjusted to two times a week and the dose in drinking water was adjusted according to body weight and the amount of water consumed by the animals [17].

**Sample collection and storage.** At the end of the experiment period (8 weeks), rats were sacrificed by decapitation under anesthesia using urethane hydrochloride (1 g/kg) intraperitoneal injection. Blood samples were collected from abdominal aorta, and then centrifuged for 10 min at 5000 rpm. Clear serum was obtained and stored at -80°C to be used for the determination of biochemical parameters. One kidney was immersed in formalin 10% for histopathological and histochemical assessment. The other kidney was snap-frozen using liquid nitrogen and stored at -80°C for RNA extraction. Kidneys were either homogenized in cold potassium phosphate buffer (0.01 M, pH 7.4) for use in different biochemical analyses, or processed for RNA extraction.

**Estimation of serum glucose, urea, creatinine and urinary protein.** Serum glucose, urea and creatinine levels were measured by chemical analyzer (TC 3300-Teco diagnostic, Anaheim, USA) using kinetic kits (Human biochemical and diagnostic kits, Wiesbaden, Germany) according to the standard manufacturer procedures. Total Protein (Urine/CSF) Assay Kit (BA0179) (American Research Products Inc.).

**Estimation of the IL-6 in renal tissue by ELISA.** IL-6 levels in kidney homogenate were measured using ELISA kits (Invitrogen Thermo-Fisher Scientific USA (LOT 192587043) according to the instructions of the manufacturer.

**Real time reverse transcriptase-polymerase chain reaction (RT-PCR).** a) **Preparation of RNA from tissue:** RT-PCR was performed according to the manufacturer's instructions. Extraction of total RNA from kidney tissue using Ribo-Zol reagent (Amresco, Solon, USA) in accordance with the manufacturer instructions was done. 5  $\mu$ g of total RNA was used for RT-PCR according to manufacture is instructions (Willowfort HERA SYBR® Green RT- q PCR Kit (WF1030300X) using primers as mentioned below in the thermal cycler (Applied Biosyst 7500 fast, Techne (Cambridge) LTD., UK).

b) *RT-PCR Reaction*: RT-PCR cycling parameters were 50°C at 15 min, 95°C at 15 min and 40 cycles of denaturation at 95°C for 15 s, annealing at 60°C and extension at 72°C were adjusted for 30 s in each stage. Relative expression of HIF-1 $\alpha$  and VEGF genes were calculated using the comparative threshold cycle method (Ct). The housekeeping gene  $\beta$ -actin was used to normalize all values.

The sets of primers used were as follows: HIF-1 $\alpha$  primer sequence (5'–3') Forward primer: 5'-AA-GTCTAGGGATGCAGCA-3', Reverse primer: 5'-CAAGATCACCAGCATCTAG-3', VEGF $\alpha$  primer sequence (5'–3') Forward primer: 5'-CGAGACGCAGCGACAAGGCA-3', Reverse primer: 5'-AC-CTCTCCAAACCGTTGGCACG-3',  $\beta$ -actin primer sequence (5'–3') Forward: 5'-GTGTGACGTTGACAT-3', Reverse: 5'-ACATCTGCTGGAAGGTG-3'

Using the formula  $2^{(-\Delta\Delta Ct)}$  reported by [18], the relative expression of each gene was calculated. They were scaled in comparison to controls. The control samples were settled at a value of 1. So, the sample results were graphed as the relative expression in comparison to the control.

*Histopathological Studies (hematoxylin and eosin stain)*. Sixteen rats in each group were sacrificed at all time points. The kidneys were removed for preparation of tissue slides. Hematoxylin-eosin (H&E) staining was performed to examine for glomerular congestion and fat globules within the glomerular tuft of capillaries, Thickening of interstitial blood vessels (Endarteritis), and Mesangial expansion. Three fields were randomly selected from each slide (X200) to examine staining pattern.

Kidneys were rapidly fixed in Bouin's solution for 24 h, cleaned with xylene, and then prepared by paraffin processing and tissue slides 7  $\mu$ m were cut by Leitz 1512 Microtome. Sections were stained by H&E stain (H&E; Sigma-Aldrich, Egypt) for routine histological examination [19]. Histological findings of the kidney tissue were assessed by a pathologist.

*Immuno-histochemical studies (IHC)*. IHC kits were obtained from Novus Biologicals USA 10730 E Briarwood Avenue Centennial, Co 80112 USA.

*Statistical analysis*. Data were collected, revised, verified, coded, then entered PC for statistical analysis and graph blotting using the software Statistical Package for Social Sciences, SPSS version 20 (SPSS, Chicago, IL, USA). Results are expressed as means  $\pm$  SEM. Differences in groups were compared using analysis of variance (ANOVA), then multiple comparisons were made using post hoc test. For all tests, *P*-value was considered statistically significant if less than 0.05.

## Results

*PB alleviates hyperglycemia induced changes in diabetic rats*. To investigate the ameliorative effect of PB, the diabetic rats were treated with PB or not for 10 weeks. As shown in Table 1 and Fig. 2, the 18-weeks-old diabetic rats exhibited typical diabetic features, including increased Fasting blood glucose (FBG) and other parameters for kidney functions including total urea, creatinine, urinary protein.

Administration of PB prevented the increase of (FBG), urea, creatinine and urinary protein in diabetic rats significantly suggesting that PB might exert a glucose-lowering effect but does not aggravate kidney injury.

*PB prevents the DN development via regulating HIF-1 $\alpha$ /VEGF signaling*. HIF-1 $\alpha$  expression in the diabetic group was significantly elevated compared to that in the control group ( $P < 0.05$ ) (Table 2). However, in the PB and PB-STZ, HIF-1 $\alpha$  was significantly downregulated compared to the diabetic group ( $P < 0.05$ ). As these changes of the HIF-1 $\alpha$  went by, VEGF expression level was also upregulated in the diabetic group and further downregulated in the PB and PB-STZ compared to the diabetic group ( $P < 0.05$ ).

Table 1. Effects of STZ-induced diabetes, PB and PB-pretreated diabetes on renal functions, serum glucose and total urinary protein levels

Groups	Control	Diabetic	PB	PB + Stz
Glucose, mg/dl)	88.000 $\pm$ 2.265	434.875 $\pm$ 6.113 <sup>ac</sup>	94.875 $\pm$ 2.903 <sup>b</sup>	140.125 $\pm$ 2.178 <sup>abc</sup>
Urea, mg/dl	39.625 $\pm$ 1.007	106.000 $\pm$ 2.526 <sup>a</sup>	43.125 $\pm$ 0.464 <sup>bc</sup>	44.875 $\pm$ 0.569 <sup>b</sup>
Creatinine, mg/dl	0.800 $\pm$ 0.018	3.062 $\pm$ 0.049 <sup>ab</sup>	0.956 $\pm$ 0.011 <sup>b</sup>	1.046 $\pm$ 0.022 <sup>b</sup>
Total protein, mg/day	4.075 $\pm$ 0.052	5.662 $\pm$ 0.130 <sup>a</sup>	4.262 $\pm$ 0.113 <sup>bc</sup>	4.350 $\pm$ 0.126 <sup>b</sup>

Note. Data are presented as mean  $\pm$  SEM. Significance at  $P < 0.05$  probability level. <sup>a</sup> $P < 0.05$  Diabetic group versus Control, <sup>b</sup> $P < 0.05$  PB versus Diabetic group, <sup>c</sup> $P < 0.05$  PB-STZ versus PB group

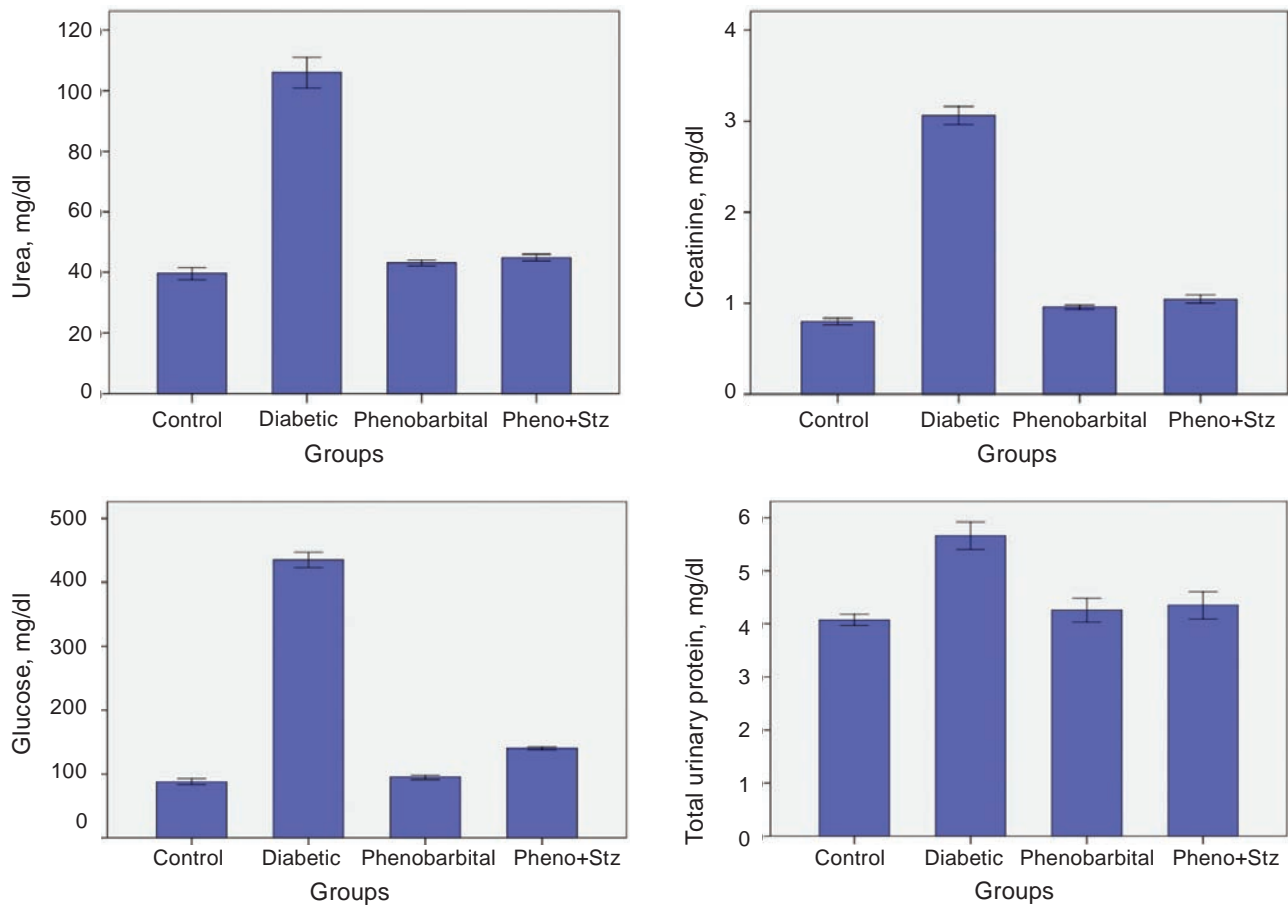


Fig. 2. Serum level of glucose, urea, creatinine and total urinary protein levels in different groups of rats

Table 2. RT-PCR values of HIF-1 $\alpha$  and VEGF expression levels in all groups of rats

Groups	Control	Diabetic	PB	PB + Stz
HIF-1 $\alpha$	1.0 $\pm$ 0.00	7.378 $\pm$ 0.191 <sup>ac</sup>	1.514 $\pm$ 0.032 <sup>ab</sup>	2.245 $\pm$ 0.117 <sup>abc</sup>
VEGF	1.0 $\pm$ 0.00	7.071 $\pm$ 0.127 <sup>ac</sup>	1.283 $\pm$ 0.050 <sup>b</sup>	1.522 $\pm$ 0.143 <sup>ab</sup>

Note. Mean  $\pm$ SEM,  $n = 16$ . Significance at  $P < 0.05$  probability level. <sup>a</sup> $P < 0.05$  Diabetes versus control group, <sup>b</sup> $P < 0.05$  PB versus Diabetic group, <sup>c</sup> $P < 0.05$  PB-STZ versus PB group

Table 3. ELISA levels of IL-6 in homogenized kidney tissue in all groups

Groups	Control	Diabetic	PB	PB + Stz
IL-6	10.581 $\pm$ 0.134	22.480 $\pm$ 0.213 <sup>ac</sup>	11.097 $\pm$ 0.185 <sup>b</sup>	14.712 $\pm$ 0.17 <sup>9abc</sup>

Note. Mean  $\pm$ SEM,  $n = 16$ . Significance at  $P < 0.05$  probability level. <sup>a</sup> $P < 0.05$  Diabetes versus control group, <sup>b</sup> $P < 0.05$  PB versus Diabetic group, <sup>c</sup> $P < 0.05$  PB-STZ versus PB group

*PB inhibits inflammation in the renal tissue of diabetic rats.* In the present study, we investigated the relationship of inflammatory marker IL-6 in the diabetic, phenobarbital, and PB-STZ groups. Measured by ELISA, IL-6 levels were significantly higher in the diabetic group compared to the control

group. The levels were mildly higher in the phenobarbital group and the control but significantly lower than the diabetic group ( $P < 0.05$ ) (Table 3).

*Histopathology changes in different groups.* In this study, histological alterations have been observed in all groups compared to the control nor-

mal one (Fig. 3, A). while there are not any pathological changes in the control group, the diabetic group (Fig. 3, B) shows mesangial proliferation (Asterix), glomerular congestion (green arrow), and fat globules within the glomerular tuft of capillaries (red arrow). Moreover, phenobarbital group (Fig. 3, C) section from phenobarbital group shows mesangial expansion (yellow arrow). PB-STZ group (Fig. 3, D), a section from PB-STZ group shows mesangial expansion (black arrow).

**Immunohistopathology Result of (VEGF) in kidney tissue.** In this study, immunohistopathological changes have been observed in all groups compared to the control normal one (Fig. 4, A). Positive VEGF expression in glomeruli of nonneoplastic kidney tissue (red arrow) and mild expression in tubules and negative expression in stromal cells (red circle), as shown in (Fig. 4, A), while in diabetic group (Fig. 4, B) immuno-histochemical staining of VEGF of renal cortex section (red circle) shows positive reaction for VEGF expression in the tubules and glomeruli (green arrow) as shown in (Fig. 4, B). Moreover, phenobarbital group (Fig. 4, C) in which Renal cortex show weak staining for VEGF predominantly involving PCT, with localization of staining to brush border of cells (groups of proximal tubules) (red circle) as shown in (Fig. 4, C), while PB-STZ group. Tubular staining for VEGF in this group decreased in the glomerulus (green arrow) but markedly increased in the proximal tubular segment (red arrow) as shown in (Fig. 4, D).

## Discussion

Hyperglycemia, considered to be a cause of hypoxia, promotes the hyperglycemic damage induced by neovascularization. (HIF-1 $\alpha$ )/VEGF axis is a key pathway for neovascularization and even involved in abnormal angiogenesis. Under hyperglycemic conditions, abnormal angiogenesis occurred in the retina with upregulation of VEGF that correlates with expression of HIF-1 $\alpha$  [20]. We hypothesized that the inhibition of this axis is a target for renal angiogenesis therapy. In the current study, we selected VEGF and HIF-1 $\alpha$  as the targets. The PB treatment of model group significantly reduced HIF-1 $\alpha$  and VEGF expression. HIF-1 $\alpha$ /VEGF is one of the key pathways contributed to diabetic angiogenesis. To further confirm the effect of PB on HIF-1 $\alpha$ /VEGF signaling in the kidney, the RT-PCR was performed. Compared with normal rats, the diabetic rats showed markedly higher protein expression of

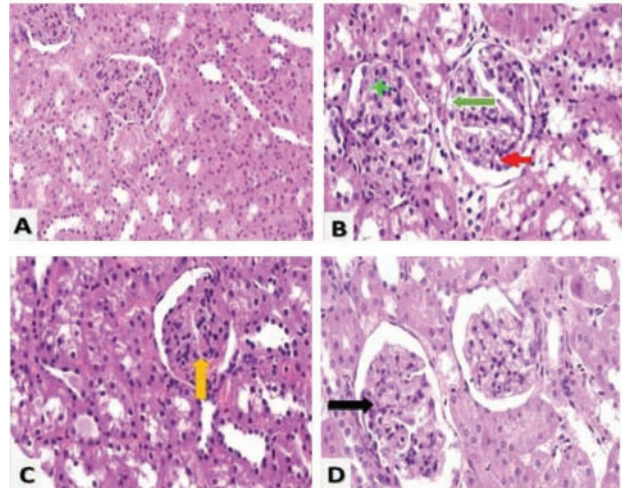


Fig. 3. Histopathological examination of the kidney tissue. Microscopic images of H&E stained sections (A) control group, (B) Diabetic group, (C) PB group and (D) PB + STZ group (200 $\times$ )

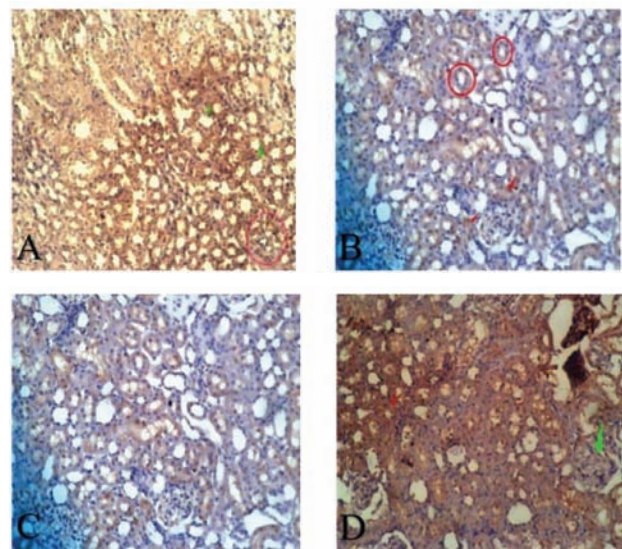


Fig. 4. Immunohistopathological examination of the kidney tissue. Microscopic images of (A) control group, (B) Diabetic group, (C) PB group and (D) PB+STZ group (250 $\times$ )

HIF-1 $\alpha$ , VEGF, in renal tissue (Table 2). PB treatment inhibited the gene expression of HIF-1 $\alpha$ , VEGF in diabetic kidney, suggesting that PB could prevent DN development by downregulating HIF-1 $\alpha$ /VEGF signaling.

In our study, HIF-1 $\alpha$  and VEGF expression were consistently trending with renal angiogenesis, and this is an indication of the impact of renal hyperglycemia on VEGF expression via the HIF-1 $\alpha$

pathway, thus modulating renal angiogenesis. In this work, we found that the downregulation of HIF-1 $\alpha$  and VEGF was deterred by the drug PB, resulting in the reduction of renal newly formed vessels. A marked difference in the extent of mesangial VEGF staining by IHC was detected between diabetic and control kidneys. A significant widespread expression of VEGF was observed in the glomerular epithelial cell and tubular segments in the early phase of DKD. These changes in VEGF played a crucial role in angiogenesis during DN development [21]. HIF-1 $\alpha$ /VEGF is one of the key pathways contributed to diabetic angiogenesis. To further confirm the effect of PB on HIF-1 $\alpha$ /VEGF signaling in the kidney, the RT-PCR was performed. Compared with normal rats, diabetic rats showed markedly higher protein expression of HIF-1 $\alpha$  and VEGF in renal tissue (Table 2). PB treatment inhibited the gene expression of HIF-1 $\alpha$ , VEGF in diabetic kidney, suggesting that PB could prevent DN development by downregulating HIF-1 $\alpha$ /VEGF signaling. To identify the effect of PB on angiogenesis in diabetic rats, we used the diabetic rat model to further investigate whether PB affected angiogenesis. Our results showed that PB treatment restricted angiogenesis and decreased the expression of angiogenesis-related factors, HIF-1 $\alpha$ , and VEGF which could support the above observations (Table 2). Those angiogenesis-related genes play crucial roles in angiogenesis during the process of diabetic nephropathy [22]. As a leading cause of end-stage renal disease, inflammatory mediators in DN have been implicated in the pathogenesis of DN [23], thus considered an inflammatory disease. To assess the renal damage caused by the action of these molecules, the expression of IL-6 as one of the cytokines implicated in this inflammatory disorder in renal tissue was analyzed. The result of renal slide H&E staining showed abundant glomerular congestion in diabetic rat renal tissues, along with prominent mesangial proliferation. However, in treated diabetic group, newly formed vessels and mesangial proliferation were significantly decreased, whilst in control group, no significant change of renal vascular morphology was found. Significantly, larger amount of VEGF expression was found in diabetic group by IHC. The high HIF-1 $\alpha$  expression of VEGF in the tubules and glomeruli in diabetic group, suggests that DN pathogenesis was correlated with renal tissue hypoxia. After renal hypoxia, positive expression of

VEGF is observed in renal tissue, and is positively correlated with local HIF-1  $\alpha$  expression.

Under hypoxia stress, HIF-1 $\alpha$  upregulated the VEGF expression to induce prominent angiogenesis in DN pathology, and this was consistent with previous studies. As HIF-1 $\alpha$  level was reduced in the treated group, VEGF was remarkably suppressed. Concomitantly, IL-6 levels were also reduced in treated group. This is consistent with a previous study that showed the involvement of IL-6 in the control of VEGF expression and new vessel formation [24].

Mesangial expansion is one of the key structural changes observed in the glomerulus 5-7 years after development of DN. It was found to reduce the GFR observed in diabetic patients with a correlation with proteinuria constituting a cause of reduction in renal functions in diabetic patients [8]. Mesangial expansion is a process of fibrosis with aberrant proliferation of mesangial cells and excess production of matrix proteins that accumulates in the central region of the glomerulus [25].

Hyperglycemia transiently mediates mesangial cell proliferation that is significant in STZ-induced diabetes in rats. *In vitro* studies showed that the incubation of mesangial cells in high glucose concentrations resulted in the accumulation of matrix components such as collagen I, collagen IV, fibronectin, and laminin [25, 26]. The role of hyperglycemia in mediating mesangial cell proliferation has been studied *in vitro* and *in vivo*, and this hyperglycemia-induced proliferation is transient and starts a few days after induction of diabetes and then decreases over a period of 30 days down to reach the normal levels [27, 28].

**Conclusion.** Our data demonstrated that, during the process of renal angiogenesis and hypoxia, HIF-1 $\alpha$  expression was significantly enhanced. The decrease in HIF-1 $\alpha$  in renal tissue, reduced VEGF expression and inhibited angiogenesis, this offers a chance for the basis for the use of phenobarbital in prevention and treatment of DN. However, pharmac-epidemiologic studies are needed to determine the long-term vascular effects of using the AEDs as a supplement in treatment of DN.

**Conflict of interest.** The authors have completed the Unified Conflicts of Interest form at [http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi\\_disclosure.pdf](http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf) and declare no conflict of interest.

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## ФЕНОБАРБИТАЛ ПОСЛАБЛЮЄ ІНДУКОВАНИЙ ГІПЕРГЛІКЕМІЄЮ АНГІОГЕНЕЗ ЗА ДІАБЕТИЧНОЇ НЕФРОПАТІЇ: МОЖЛИВЕ ВТРУЧАННЯ НА РІВНІ HIF-1 $\alpha$ /VEGF

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Гіперглікемія сприяє каскаду запальних реакцій у нирках, які призводять до розвитку ниркової гіпоксії та ангіогенезу з подальшою хронічною нирковою недостатністю. Оскільки система гіпоксія-індукований фактор-1 $\alpha$  (HIF-1 $\alpha$ )/судинний ендотеліальний фактор росту (VEGF) є ключовим шляхом неоваскуляризації, пригнічення цієї системи є мішенню для терапії ниркового ангіогенезу. Ми припускаємо, що фенобарбітал (ФБ), який має потенціал для зменшення васкуляризації, може впливати на розвиток ангіогенезу в нирках за діабету. Метою дослідження було вивчення впливу ФБ на експресію HIF-1 $\alpha$  і VEGF та ангіогенез у тканині нирок щурів із гіперглікемією та діабетичною нефропатією. Шістдесят чотири щури-самці лінії Вістар було розділено на 4 групи: контрольна група отримувала в/о одноразову ін'єкцію фізіологічного розчину; група ФБ отримувала 0,05% ФБ перорально з питною водою; група тварин із діабетом отримувала в/о одноразову ін'єкцію стрептозоцину (СТЗ) (65 мг/кг); група ФБ-СТЗ отримувала 0,05% ФБ перорально за два тижні до введення СТЗ. Наприкінці експеримен-

ту (8 тижнів), нирки було видалено і використано для біохімічних аналізів. Досліджено рівні глюкози, сечовини та креатиніну в сироватці крові, рівень ІЛ-6 в гомогенаті нирок та зміни експресії генів HIF-1 $\alpha$  та VEGF. Для гістопатологічного дослідження використовували забарвлення гематоксилін-еозином. Показано, що експресія генів HIF-1 $\alpha$  та VEGF, а також рівень ІЛ-6 у групі хворих на діабет щурів були значно підвищені порівняно з контрольною групою, тоді як у ФБ та ФБ-СТЗ групах, ці показники були значно знижені порівняно з діабетичною групою. У тканині нирок діабетичних щурів виявлено гломерулярні скупчення та мезангіальну проліферацію. Однак у діабетичній групі, які отримували ФБ, кількість новоутворених судин помітно зменшилася. Одержані результати підтверджують, що фенобарбітал впливає на сигнальний шлях HIF-1 $\alpha$ /VEGF, зменшуючи ангіогенез за гіперглікемічного пошкодження нирок.

**Ключові слова:** гіперглікемія, пошкодження нирок, VEGF, HIF-1 $\alpha$ , ІЛ-6, ангіогенез.

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