

Evaluation of Caspase-3 And Vegf Expression in Retinopathy and Nephropathy Diabetic Rats: An Experimental Study

Habibah Setyawati Muhiddin^{1,2,3}, Fadhlullah Latama^{1,3}, Budu^{1,2,3}, Andi Muhammad Ichsan^{1,2,3}, Upik Anderiani Miskad^{2,4}, Itzar Chaidir Islam¹, Haerani Rasyid⁵

¹Ophthalmology Department, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

²Hasanuddin University Hospital, Makassar, Indonesia

³JEC Orbita Hospital and Clinic, Makassar, Indonesia

⁴Pathology Department, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

⁵Internal Medicine Department, Faculty of Medicine, Hasanuddin University, Makassar, Indonesia

ABSTRACT

Purpose: This study aimed to evaluate the timeline of the occurrence of microvascular complications due to diabetes in a diabetic rat model based on caspase-3 and VEGF expression in retinal and renal cells.

Method: This study investigated 28 adult male Wistar rats who were given 60 mg/BW streptozotocin (STZ) intravenously. The animal models were terminated after 0-6 weeks regularly. All experimental animals' retinal and renal tissues were immunohistochemically examined for VEGF and Caspase-3 markers. The results were qualitatively examined using the Kruskal-Wallis test and observed using the post-hoc Mann-WhitneyU test (sig. $p < 0.05$). Furthermore, a one-way ANOVA test was used for retinal cell apoptosis (sig. $p < 0.05$).

Results: Significant variations in caspase 3 and VEGF expression were identified between groups in retinal and renal tissue. On the retina, the apoptosis began in the third week and peaked in the fifth ($p = ??$), then the VEGF expression was highest on the x-weeks. On the renal tissue, the apoptosis started in the fourth week and peaked in the fifth week, particularly in tubular tissue ($p < 0.001$), then the greatest VEGF value was achieved with moderate-severe degrees at week 6. Moreover, VEGF expression in renal tissue was not significant in any group ($p > 0.05$).

Conclusion: The results showed that apoptosis and possible neovascularization occurred faster in retinal tissue with a higher level of VEGF and Cas-3 expression than in the renal tissue.

KEYWORDS: Diabetes mellitus, retinopathy, nephropathy. caspase-3, Vascular Endothelial Growth Factor.

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INTRODUCTION

Diabetes mellitus is a chronic metabolic disease characterized by high blood glucose levels caused by impaired insulin secretion, insulin action, or both.^{1,2} It is a complex disease that affects millions of people worldwide and has serious consequences for both individual health and the public health system. The most common types of diabetes are type 1 diabetes, type 2 diabetes, and gestational diabetes.^{3,4}

Retinopathy is a common and potentially sight-threatening complication of diabetes. High blood sugar and other diabetes-related factors can cause abnormal blood vessels in the retina, which can lead to retinopathy. There are two main types of diabetic retinopathy: non-proliferative diabetic retinopathy (NPDR) and proliferative diabetic retinopathy (PDR). NPDR is an early stage characterized by microaneurysms, hemorrhages, and retinal swelling. PDR, on the other hand, is an advanced stage in which abnormal blood vessels grow on the surface of the retina, which can lead to retinal detachment and vision loss.^{5,6} Risk factors for diabetic retinopathy include duration of diabetes, poor blood sugar control, high blood pressure, and high cholesterol. Regular eye exams, including dilated pupils, are essential for early detection and intervention. Treatment for diabetic retinopathy involves optimizing blood sugar control, blood pressure, and lipid levels to slow the progression of the disease.⁷ In some cases, laser therapy or intravitreal injections of drugs against vascular endothelial growth factor (VEGF) may be recommended to treat macular edema or abnormal blood vessel growth. Prevention is essential to reduce the effects of diabetic retinopathy. Tight glycemic control, regular eye examinations, and early management of risk factors are essential to reduce the incidence and severity of retinopathy.⁸⁻¹⁰

Moreover, nephropathy is also known as a serious complication of diabetes. It occurs when chronically high blood sugar levels damage the small blood vessels in the renals, resulting in decreased renal function. Renal disease is a progressive disease that can eventually lead to end-stage renal disease (ESRD) and the need for dialysis or a renal transplant. The pathogenesis of diabetic nephropathy involves multiple mechanisms, including progressive accumulation of glycation end products, oxidative stress, inflammation, and activation of various growth factors.¹¹ These processes cause glomerular and tubular damage, increase vascular permeability and development of proteinuria. Risk factors for the development and progression of diabetic nephropathy include

poor glycemic control, hypertension, genetic predisposition, and the presence of other diabetic complications. Early detection is critical, and people with diabetes are advised to regularly monitor renal function through blood and urine tests.¹² Treatment of diabetic nephropathy involves a multifaceted approach. Optimal glycemic control, blood pressure control, and the use of drugs such as renin-angiotensin-aldosterone system (RAAS) inhibitors are effective in slowing the progression of renal disease. Lifestyle changes are also important, including a healthy diet and regular exercise. Prevention strategies for diabetic nephropathy include early detection and treatment of diabetes, and management of modifiable risk factors such as hypertension and dyslipidemia.^{13,14}

In the context of diabetes, Vascular endothelial growth factor (VEGF) is a key molecule involved in angiogenesis and regulation of vascular permeability. VEGF has been implicated in the development and progression of microvascular complications, including diabetic retinopathy, nephropathy, and neuropathy.^{15,16} In diabetic retinopathy, VEGF plays a critical role in the pathogenesis of abnormal blood vessel growth (neovascularization) and increased vascular permeability, leading to macular edema and retinal damage. Increased levels of VEGF have been observed in the vitreous and retina of patients with diabetic retinopathy. In diabetic nephropathy, VEGF promotes glomerular capillary dilation and abnormal vascular development, leading to increased filtration and proteinuria. Dysregulation of VEGF signaling in the renal is associated with the progression of diabetic nephropathy. VEGF is also involved in the pathogenesis of diabetic neuropathy, which causes nerve damage and impaired nerve function. It promotes neuroinflammation, disrupts the blood-nerve barrier, and induces neuronal apoptosis.¹⁷⁻¹⁹

Besides VEGF, caspase 3 expression also plays an important role in the development and progression of retinal and renal tissue damage, two major complications of diabetes.^{20,21} Caspase 3 is a key executor of apoptosis, the programmed process of cell death. Dysregulation of caspase 3 expression in retinal and renal tissues contributes to the pathogenesis of diabetic retinopathy and nephropathy. In diabetic retinopathy, Caspase 3 activation is associated with apoptosis of retinal cells, including endothelial cells, pericytes, and retinal ganglion cells.^{22,23} Chronic exposure to high glucose and retinal oxidative stress leads to mitochondrial dysfunction and the release of cytochrome c, which activates caspase 3. Increased caspase 3 activity promotes cell death, leading to retinal cell loss and progressive retinal cell degeneration. retinal tissue. In diabetic nephropathy, caspase 3 activation plays a role in renal cell apoptosis, especially in the glomeruli and tubules. Hyperglycemia and metabolic disturbances associated with diabetes induce oxidative stress and inflammation, leading to caspase 3 activation and initiation of the apoptotic pathway. Caspase 3-mediated apoptosis of glomerular endothelial cells and podocytes leads to glomerular dysfunction, proteinuria, and glomerulosclerosis. In renal tubules, caspase 3 activation promotes tubular cell apoptosis, tubular atrophy, and interstitial fibrosis.^{24,25}

This study aimed to examine caspase-3 and VEGF expression in retinal and renal cells of a diabetic rat model. The results of this study are expected to evaluate the timeline of the occurrence of microvascular complications due to diabetes between retinopathy and nephropathy.

MATERIAL AND METHODS

Design

This study is a true experimental study with a post-test-only group design. There were 35 animal subjects were included. Streptozocin (STZ) agent (Merck® No. C8H15N3O7) was used to induce the diabetic model. The animal models were divided into 7 groups: group 1 was the control group without any injection of STZ. 6 groups were injected STZ with a dosage of 1mg/kgBW through intraperitoneal. After the initial injection, blood glucose was observed 2- and 4-days post-injection which will be used as peak glucose level. 6 groups consist of subjects group that will be evaluated from one week of observation until six weeks of observation. The 1st-week group will be evaluated until one week, and the subjects will be sacrificed at the end of the week. 2nd-week group until the 6th-week group underwent the same processes through the different number of weeks of observation. After every sacrifice, enucleation of both eyes is performed to acquire the retinal tissues that will be examined using hematoxylin-eosin and immunohistochemistry evaluation.

Established animal experiment

The experimental animals were male Wistar rats (*Rattus norvegicus*), 8-12 weeks old, with body weight of 160-200 grams. All animals were kept in a room with a light-dark cycle every 12 hours, given standard feed and *ad libitum* drinking water. Induction was declared successful if blood glucose >200 mg/dl.

Sample collection and processing

Before enucleation, the experimental animals were terminated by placing the rat in a closed container filled with cotton and ether. The animals were put for 10 minutes until no motoric response, neurological reflexes, or heartbeat were found. The eye tissue was removed by the enucleation method, used tweezers to press the eyeball up to the base of the optic nerve, then cut the optic nerve until the eyeball was lifted. All eyes were fixed with 10% formalin and mobilized to the pathology laboratory. Retinal tissue was cut using a microtome with a thickness of 5 µm, then stained with hematoxylin and eosin (HE) to calculate the density of retinal ganglion and photoreceptor cells. Immunohistochemistry (IHC) examination to evaluate the expression of caspase-3 (Cat No. C9598, Sigma USA) and VEGF (Cat No. 07-1420 Merck®) in the retinal layer. Interpretation of cell density was carried out using quantitative methods using an Olympus CX23 binocular microscope with an objective magnification of 40 times. The results obtained were presented in terms of the mean with standard deviation. Expression of caspase 3 and VEGF was evaluated under 400 times magnification of microscope using the semi-quantitative technique, the grading included: Negative (<10% cell expression or tissue formation per field view); Low: (10-20% cell expression or tissue formation per field view); High: (>20% cell expression or tissue formation per field view).

Data analysis

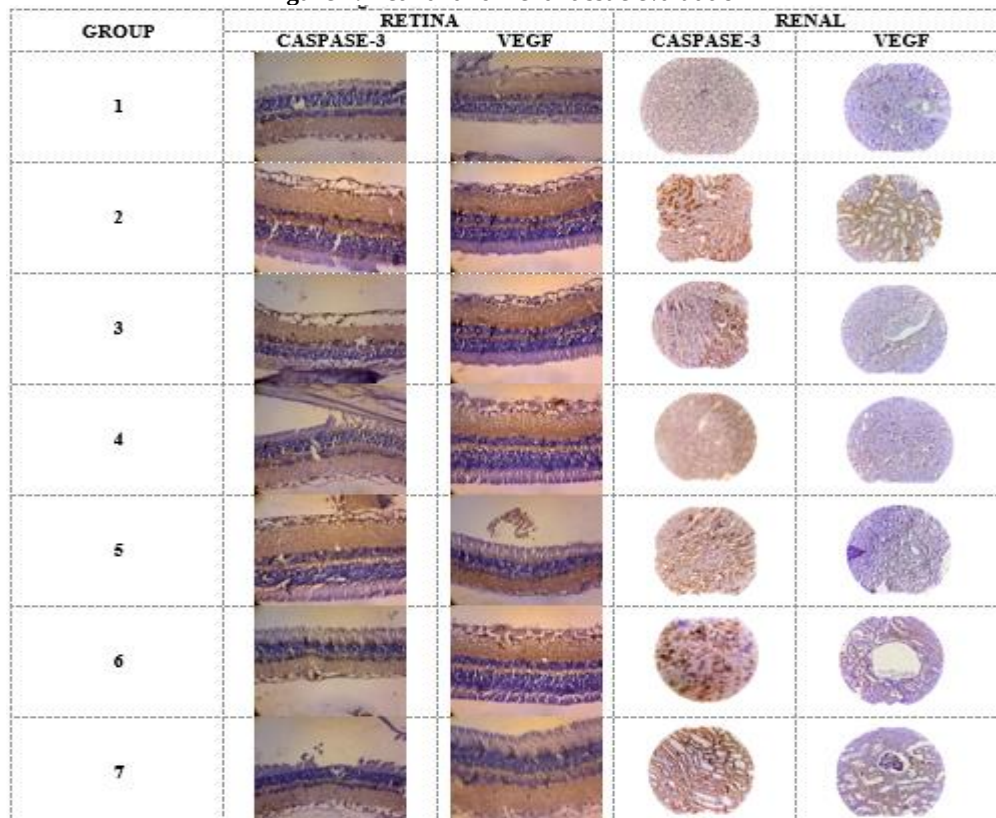
Data processing was using the Statistical Package for Social Science (SPSS® ver. 24.0) computer program. The statistical analysis used the Mann Whitney U tests. The correlation was significant if the p-value remained <0.05.

RESULTS**Table 1. Retinal and Renal tissue evaluation**

GROUP	RETINA				RENAL			
	CASPASE-3 EXPRESSION	p- value*	VEGF EXPRESSION	p- value*	CASPASE-3 EXPRESSION	p- value*	VEGF EXPRESSION	p- value*
1 (n=5)	Neg: 5 Low: 0 High: 0	0.006	Neg: 5 Low: 0 High: 0	<0.001	Neg: 5 Low: 0 High: 0	0.003	Neg: 5 Low: 0 High: 0	<0.001
2 (n=5)	Neg: 0 Low: 5 High: 0		Neg: 5 Low: 0 High: 0		Neg: 3 Low: 1 High: 1		Neg: 3 Low: 2 High: 0	
3 (n=5)	Neg: 2 Low: 2 High: 1		Neg: 4 Low: 1 High: 0		Neg: 1 Low: 3 High: 1		Neg: 1 Low: 3 High: 1	
4 (n=5)	Neg: 1 Low: 3 High: 1		Neg: 2 Low: 2 High: 1		Neg: 0 Low: 3 High: 2		Neg: 0 Low: 4 High: 1	
5 (n=5)	Neg: 1 Low: 1 High: 3		Neg: 1 Low: 2 High: 2		Neg: 0 Low: 4 High: 1		Neg: 0 Low: 3 High: 2	
6 (n=5)	Neg: 1 Low: 1 High: 3		Neg: 1 Low: 2 High: 2		Neg: 0 Low: 2 High: 3		Neg: 0 Low: 3 High: 2	
7 (n=5)	Neg: 1 Low: 3 High: 1		Neg: 1 Low: 1 High: 3		Neg: 0 Low: 2 High: 3		Neg: 0 Low: 3 High: 2	

*Mann-WhitneyU test

In table 1, it can be seen that there are significant differences in the calculated expression of caspase-3 and VEGF both in the retina and in the kidneys of experimental animals. In the retina, cell death begins to form at week 4 while neovascularization at week 5. Meanwhile, in the kidneys, cell death and neovascularization begin to occur in the 3rd week. This can be seen with a p-value <0.05 for all variables tested.

Figure 1. Retinal and Renal tissue evaluation

DISCUSSION

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels, resulting from defects in insulin secretion, insulin action, or both.²⁶ It is a complex condition that affects millions of people worldwide and has significant implications for individual health and public healthcare systems.^{27–29} In cases of diabetes mellitus, retinal tissue damage is a common and significant complication known as diabetic retinopathy. It is a leading cause of vision loss and blindness in adults worldwide. The condition is characterized by progressive damage to the blood vessels of the retina, the light-sensitive tissue at the back of the eye.^{30,31}

The pathogenesis of retinal tissue damage in diabetic retinopathy involves several interconnected mechanisms. Prolonged exposure to high blood glucose levels leads to the formation of advanced glycation end products (AGEs) and oxidative stress. These processes contribute to the dysfunction and damage of retinal cells, including endothelial cells, pericytes, and retinal ganglion cells.³²

The damage to retinal tissue in diabetic retinopathy also involves inflammatory processes. Inflammatory mediators, such as cytokines and chemokines, are released, promoting the recruitment and activation of immune cells in the retina. Chronic inflammation contributes to the breakdown of the blood-retinal barrier and the development of edema, further impairing retinal function.^{33–35}

In cases of diabetes mellitus, kidney tissue damage is a significant consequence and a major cause of morbidity and mortality. Diabetic kidney disease, also known as diabetic nephropathy, is a progressive condition characterized by structural and functional abnormalities in the kidneys. It is a leading cause of end-stage renal disease (ESRD) globally.^{11,36} The underlying mechanisms of kidney tissue damage in diabetes are multifactorial and involve a complex interplay of metabolic, hemodynamic, and inflammatory processes. Prolonged exposure to high blood glucose levels leads to the formation of advanced glycation end products (AGEs) and activation of various intracellular signaling pathways. These processes contribute to oxidative stress, inflammation, and cellular dysfunction in the kidneys.^{37,38}

The hallmark of kidney tissue damage in diabetic nephropathy is the presence of glomerular lesions, including glomerular basement membrane thickening, mesangial expansion, and podocyte injury. These changes disrupt the filtration barrier of the glomerulus, leading to increased permeability and loss of proteins, particularly albumin, in the urine (albuminuria). Persistent albuminuria is an important clinical marker of kidney damage and predicts the progression of diabetic nephropathy.^{12,14}

Alongside glomerular lesions, tubulointerstitial damage occurs in diabetic kidney disease. Tubular cells undergo structural and functional alterations, leading to tubular atrophy, interstitial fibrosis, and inflammation. These changes further contribute to the decline in kidney function.^{39,40} The renin-angiotensin-aldosterone system (RAAS) plays a significant role in the development and progression of kidney tissue damage in diabetes. Activation of the RAAS system contributes to increased renal vascular resistance, hypertension, and glomerular hyperfiltration, exacerbating kidney injury.^{41,42} Inhibition of the RAAS pathway with angiotensin-converting enzyme inhibitors (ACE inhibitors) or angiotensin receptor blockers (ARBs) is a cornerstone of therapeutic management in diabetic nephropathy, as it helps to reduce proteinuria, slow the progression of kidney disease, and lower cardiovascular risk.^{38,43}

In addition to metabolic and hemodynamic factors, inflammation and fibrosis are important contributors to kidney tissue damage in diabetes. Inflammatory mediators, such as cytokines and chemokines, promote the recruitment and activation of immune cells, leading to interstitial inflammation and fibrotic changes. The activation of profibrotic signaling pathways, including transforming growth factor-beta (TGF- β) and connective tissue growth factor (CTGF), further promotes the deposition of extracellular matrix proteins and the development of fibrosis.⁴⁴

In cases of diabetes mellitus, the expression of caspase 3 plays a significant role in the development and progression of retinal and kidney tissue damage, two major complications of diabetes. Caspase 3 is a key executor of apoptosis, a process of programmed cell death. Dysregulated caspase 3 expression in the retinal and kidney tissues contributes to the pathogenesis of diabetic retinopathy and nephropathy.^{20,45}

In diabetic retinopathy, the activation of caspase 3 is implicated in the apoptosis of retinal cells, including endothelial cells, pericytes, and retinal ganglion cells. Prolonged exposure to high glucose levels and oxidative stress in the retina triggers mitochondrial dysfunction and the release of cytochrome c, leading to the activation of caspase 3. Increased caspase 3 activity promotes cell death, contributing to the loss of retinal cells and the progressive degeneration of retinal tissue.^{46–48}

In diabetic nephropathy, caspase 3 activation plays a role in the apoptosis of renal cells, particularly in the glomeruli and tubules. Hyperglycemia and metabolic disturbances associated with diabetes induce oxidative stress and inflammation, leading to the activation of caspase 3 and the initiation of apoptotic pathways. Caspase 3-mediated apoptosis of glomerular endothelial cells and podocytes contributes to glomerular dysfunction, proteinuria, and the development of glomerulosclerosis. In the tubules, caspase 3 activation promotes tubular cell apoptosis, tubular atrophy, and interstitial fibrosis.^{38–40}

In diabetic mellitus cases, the expression of vascular endothelial growth factor (VEGF) plays a crucial role in the development and progression of retinal and kidney tissue damage, two major complications of diabetes. VEGF is a potent angiogenic factor that stimulates the growth of new blood vessels. However, in the context of diabetes, dysregulated and excessive VEGF expression can lead to pathological neovascularization and contribute to tissue damage. In diabetic retinopathy, VEGF

overexpression in the retina is a hallmark feature.^{16,49,50} Chronic hyperglycemia and metabolic changes associated with diabetes trigger VEGF upregulation as a compensatory response to the hypoxic microenvironment in the retina. Increased VEGF levels promote the proliferation and migration of endothelial cells, leading to the formation of abnormal blood vessels. These new vessels are fragile, leaky, and prone to hemorrhage, causing macular edema, retinal ischemia, and vision impairment. VEGF-induced vascular permeability is a critical factor in diabetic macular edema, a common vision-threatening complication in diabetic retinopathy.^{18,51,52}

In diabetic nephropathy, VEGF also plays a significant role in the development of kidney tissue damage. Initially, the kidneys respond to hyperglycemia by upregulating VEGF expression, aiming to enhance angiogenesis and restore oxygen supply. However, persistent and excessive VEGF production leads to abnormal capillary growth and glomerular hyperfiltration. The resulting glomerular endothelial cell damage and increased permeability contribute to proteinuria, a key feature of diabetic nephropathy. Furthermore, VEGF promotes the proliferation of mesangial cells, leading to glomerular hypertrophy and extracellular matrix accumulation, which ultimately progress to glomerulosclerosis and renal fibrosis.⁵³⁻⁵⁵

CONCLUSION

Dysregulated of vascular and cell apoptosis give us an information that VEGF and caspase 3 expression contributes to retinal and kidney tissue damage in diabetic mellitus cases. By this study, it is prove that the nephropathy appears before retinopathy. Activation of caspase 3-mediated apoptosis may offer potential strategies for preventing and managing tissue damage in diabetic patients. Moreover, dysregulated expression of VEGF in retinal and kidney tissue damage is a critical factor in promotes pathological angiogenesis, vascular permeability, and tissue fibrosis in diabetic retinopathy and nephropathy.

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