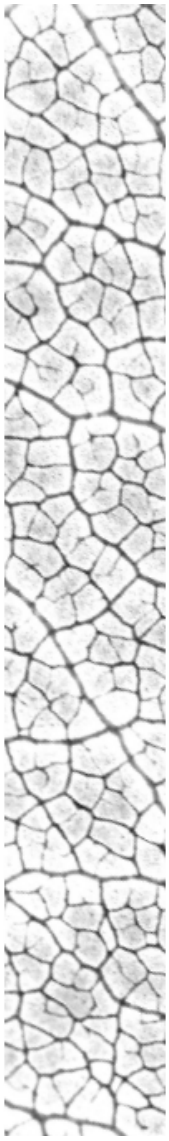

EurAsian Journal of BioSciences
Eurasia J Biosci 13, 887-891 (2019)

# The role of an angiopoietin-2 inhibitor in decreasing pericyte loss in diabetic rats

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**Abstract**

**Background:** The ratio of pericytes:endothelial cells in normal retinal vasculature is 1:1. Pericyte loss is the earliest morphological change in the diabetic retina and predominant characteristic in diabetic retinopathy. Diabetic retinopathy is associated with angiopoietin 2 (Ang-2). The aim of this study is to determine the role of an Ang-2 inhibitor in decreasing pericyte loss in diabetic rats.
**Material and Methods:** True experimental using rat model. Diabetic rats induced by intraperitoneal injection of streptozotocin (STZ). The samples were divided into 5 groups, injected intravitreally with vehicle (DMSO) or Ang-2 inhibitor with variation dose (10,20, and 30 µg). After one month the rat eyes were enucleated. Retinal digest preparation and HE staining was done to examine the retinal vasculature and count pericyte : endothel ratio.
**Results:** The number of pericytes reduced by 40% (1:2.5) after five weeks of diabetes induction in the diabetic rat model. The group receiving 10 µg Ang-2 inhibitor showed the best results in terms of reducing pericyte loss (1:1.8).The group receiving 20 µg Ang-2 inhibitor had a pericyte:endothelial ratio of 1:2.1. The pericyte loss was not decreased in the group receiving 30 µg Ang-2 inhibitor (1:2.8). Linear regression analysis revealed that there was a positive relationship between administration of the Ang-2 inhibitor and a reduction in pericyte loss in diabetic rats ($p > 0.05$).
**Conclusion:** Intravitreal administration of an Ang-2 inhibitor, at certain doses, reduced pericyte loss in diabetic rats.

**Keywords:** Angiopoietin2, pericyte loss, diabetic retinopathy

Dewi NA, Hapsari D, Refa S, Sujuti H (2019) The role of an angiopoietin-2 inhibitor in decreasing pericyte loss in diabetic rats. Eurasia J Biosci 13: 887-891.



## BACKGROUND

Diabetes mellitus is a chronic degenerative disease that has the highest rates of morbidity and mortality in the world and the incidence is increasing in developing countries. In the development of the disease, people with DM are very likely to experience complications of both acute and chronic (macrovascular or microvascular) complications (Tarr et al. 2013, Thomas et al. 2012, World Health Organization 2012).

In a study conducted by The Diab Care Asia 2008 Study reported that 42% of people with diabetes mellitus have retinopathy complications where 6.4% of them are Proliferative Diabetic Retinopathy (PDR). The risk of diabetic retinopathy increases in proportion to the duration of diabetes mellitus. Diabetic retinopathy patients at risk of blindness by 8%, where blindness is permanent and can occur in the early stages of non-proliferative diabetic retinopathy (NPDR) and advanced stage (Tarr et al. 2013, Thomas et al. 2012, World Health Organization 2012).

The state of hyperglycemia leads to changes in biochemical pathways such as aldose reductase, Advanced Glycation End Products (AGE), Protein kinase C (PKC), and Reactive Oxygen Intermediate (ROS). AGE production that increases due to changes in biochemical pathways causes excessive AGE accumulation in the retinal pericyte wherein AGE-RAGE binding leads to apoptosis. In addition, there is also an increase in inflammatory factors that can cause vascular leakage, non capillary perfusion, and endothelial damage to the retina, which may lead to clinical manifestations of diabetic retinopathy (Adamis and Berman 2008, Bouterse and Kowluru 2008, Brownlee 2001).

Pericyte is a cell that covers the capillary walls of blood vessels that interact with the endothelium. The pericyte has an important role in the stability of the blood vessels. The more the pericyte that envelops the blood vessels, the greater its function as a barrier. Ratio pericyte : endothel in normal retinal vasculature is 1:1. In diabetic retinopathy, loss of the pericyte is an early sign of damage induced by hyperglycemia. From previous studies, the loss of pericyte and vasoregression caused

Received: November 2018
Accepted: May 2019
Printed: July 2019

by apoptosis. But in recent studies mentioning in addition to apoptosis, pericyte migration may first be a sign of loss of pericyte and vasoregression. It is associated with increased Angiopoietin 2 (Ang-2) as an increased growth factor in hyperglycemic conditions and causes vascular destabilization (Abhary et al. 2010, Hammes et al. 2002, 2011, Willard and Herman 2012).

The standard of diabetic retinopathy therapy is to prevent further retinal anatomical damage so that no blindness occurs. However, the therapy is very expensive and if there has been extensive anatomical damage will produce unsatisfactory results. Therefore, management is needed in patients with diabetes in order to avoid diabetic retinopathy. The aim in this study is to determine role of Ang-2 inhibitor on decreasing pericyte loss in diabetic rats by intravitreal injection.

## MATERIALS AND METHODS

This research is true experimental research using invivo model. All experiment in this study were performed with approval from Ethics Comitee Medical Faculty Brawijaya University. Rats were divided into 5 group with 2 control groups and 3 treatment groups. Group negative control is normoglycemic rat, Group positive control is diabetic rats with DMSO intravitreal injection. Intraperitoneal STZ injection 40 mg / kgbb for 5 days were given to induces diabetes in all diabetic rat group. STZ-injected animals were considered diabetic when blood glucose levels reached stable levels (>250 mg/dl). Diabetic and nondiabetic rats were sacrificed 5 weeks after diabetes induction.

After the rats anasthesized with ketamine hidrochlorida intraperitoneally and pantocain eyedrop for eyes anesthesia, on three treatment groups we give an injection of 10 µg Ang-2 inhibitor on group 1, group 2 was given 20 µg intravitreal Ang-2 inhibitor, group 3 was given 30 µg intravitreal Ang-2 inhibitor, and each injected 5 µl in both eyes.

Diabetic and nondiabetic rats were sacrificed 5 weeks after diabetes induction, and eyes were enucleated under deep anesthesia and immediately frozen and fixed with 10% formaline. Retinal digest preparation was used for examine the ratio of pericyte: endothel like previously described (Dewi et al. 2015). Total number of pericyte were count on 3 random area (400x magnification) using image analyzing system (BX-53 ;Olympus Opticals, Hamburg, Germany). For statistical analysis, linear regression test was used to make correlation between two variable. A value of $P<0.05$ was considered statistically significant.

## RESULTS

After injection of STZ, all rats diagnosed as diabetes after 2 weeks. After four weeks diabetes were diagnosed, the mean of Ang-2 level in the normoglicemic (negative control) group was 1.89 ng / ml.

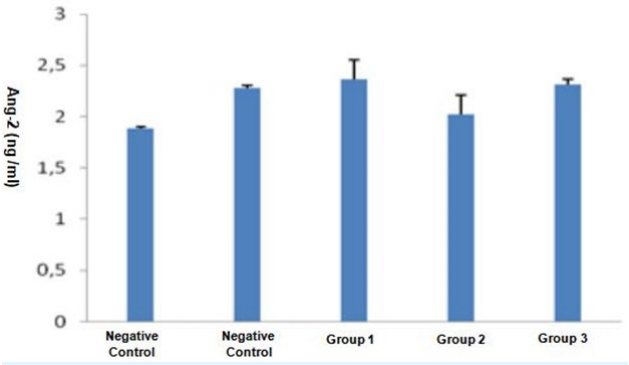


**Fig. 1.** Analysis of Ang-2 (ng/ml) levels in diabetic model mice. There were significant difference between normoglycemic rat and diabetic rats ($p<0.05$)

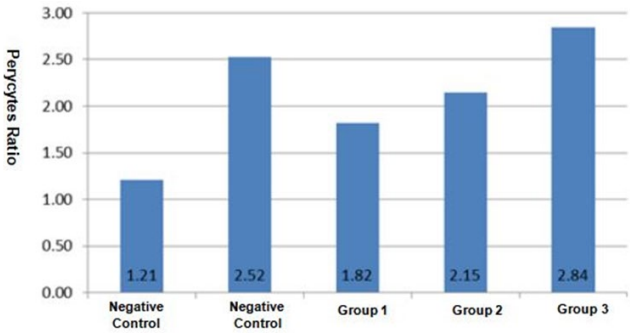


**Fig. 2.** Pericyte: edothelial cell ratio. The pericyte loss is less (closer to normoglycemic or negative control group) in group 1 and 2 which injected by Ang-2 inhibitor

In the positive control group, the mean Ang-2 level was increasing to 2.75 ng / ml. In the intravitreal injection-treated group, group 1 given of Ang-2 inhibitor 10 µg intravitreal shows Ang-2 to 2.36 ng / ml, in group 2 (20 µg intravitreal Ang-2 inhibitor) was 2.02 ng / ml, and the last group show 2.31 ng / ml Ang-2 level.

Serum Ang-2 analysis results obtained the highest angiopoietin levels in the positive control group and the lowest is in the negative control group. In the group with administration of Ang-2 inhibitor at a dose of 20 µg was the group with the lowest serum angiopoietin (**Fig. 1**).

The result of analysis of retinal digest examination obtained data of The pericyte:endothelial ratio in the negative control group was 1:1.26, and in the positive control group was 1:2.5. While in groups 1, 2, and 3 it was 1:1.82, 1:2.31, and 1:2.82, respectively (**Fig. 2**).

Pericyte loss was seen more in the positive control group where the pericyte: endothel ratio reached 1: 2.5. In group 1, where the dose of Ang-2 inhibitor 10 µg was obtained a perycite: endothel ratio 1: 1.8 where the loss of perisit was less than in the positive control group. In group 2 also, there was a lower pericyte loss rate compared to the positive control group, where the ratio of the pericyte : endothel was 1: 2.1. While in group 3, diabetic model rats were given Ang 2 inhibitor injection of 30 µg, had greater loss of pericyte (**Fig. 3**).

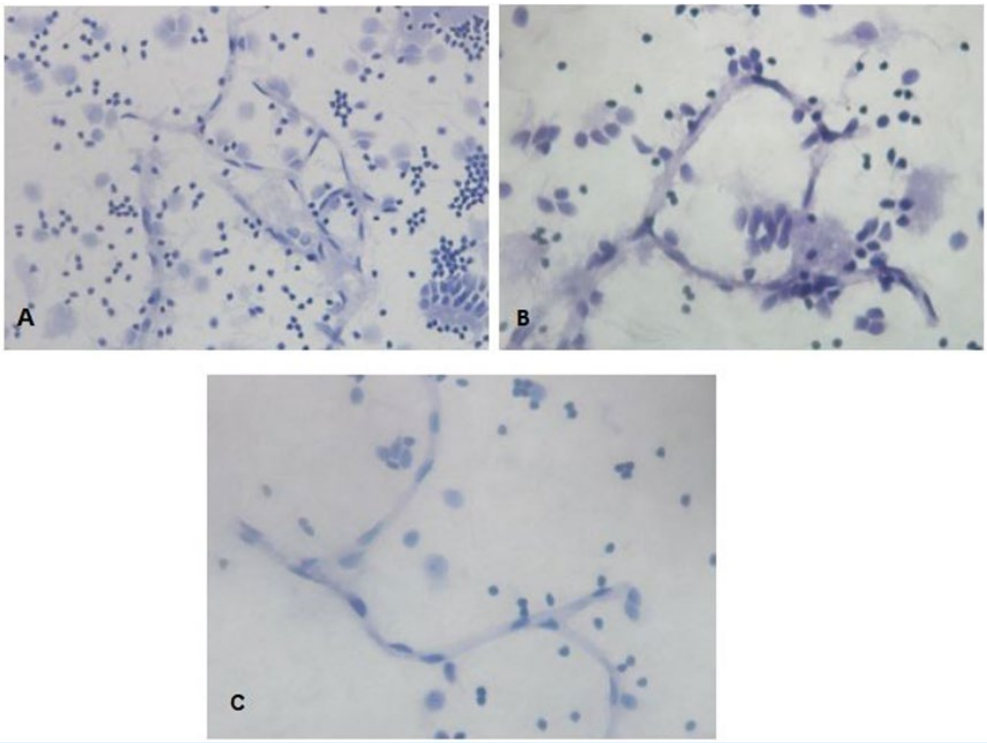


**Fig. 3.** Pericyte and endothel identification using retinal digest preparation with HE staining on (A) normoglicemic rat (negative control), (B) Diabetic rat (Positive control). The presence of pericyte less than in normoglycemic retina. Pericyte was darker and round shape (black arrow), endothel was lighter and spindle shape (white arrow). Original magnification 600x

There were significant differences between the negative control group and all treatment groups; however, there were no significant differences between the positive control group and the treatment groups. Linear regression showed no significant effect. Data analysis showed a change in the number of pericytes loss, as evident from the pericyte:endothelial ratio. Pericyte loss was seen more in the positive control group, where the pericyte:endothelial ratio reached 1:2.52. In group 1, with the 10 µg dose of Ang-2 inhibitor, the pericyte:endothelial ratio was 1:1.82, with less pericyte loss than in the positive control group. Similarly, in group 2 there was a lower loss rate compared to the positive control group, with a pericyte:endothelial ratio of 1:2.31. While in group 3, where diabetic model rats were given Ang 2 inhibitor injection of 30 µg, there was greater pericyte los, with a pericyte:endothelial ratio of 2.82: 1.

## DISCUSSION

This study aimed to determine the role of an Ang-2 inhibitor in decreasing the loss of pericytes in a diabetic rat model. In cases of hyperglycemia, there is increased Ang-2 levels, which may cause a persistent interruption of the crosstalk between the pericytes and endothelial cells, resulting in loss of pericytes and destabilization of blood vessels. Hyperglycemia induces intracellular AGEs to produce methylglyoxal (MGO) and to alter mSim3A repressor co. This change leads to a decrease in the glucose-responsive GC-box bond in the Ang-2 promotor, leading to increased Ang-2 expression. Over-expression of Ang-2 causes persistent disruption of cross-talk between pericytes and endothelial cells at the onset of diabetic retinopathy, which eventually leads to loss of pericytes and destabilization of blood vessels (Adamis and Berman 2008, Bouterse and Kowluru 2008, Brownlee 2001).

This study used five groups of normoglycemic rats. The mean blood sugar in each group after analysis with one way ANOVA showed homogeneity of the data. All rats at the start of the study met the normoglycemic criteria. In the four groups of rats induced with STZ, multiple low doses of 40 mg/dl were given for five days. In accordance with previous studies, this procedure causes a significant state of hyperglycemia after one week, and rats are classified as diabetic after two to three weeks. In this study, all rats became diabetic after two and three weeks, and remained diabetic until the fourth week.

Based on the theory that in a state of hyperglycemia there is an increase in Ang-2, in this diabetic rat model we measured serum Ang-2 at week four of diabetes induction, before enucleation. In the negative control group, the mean Ang-2 level was 1.89 ng/ml. This corresponds to average levels of Ang-2 in healthy human plasma, which are 1-3 ng/ml; whereas, levels of Ang-2 in healthy rat serum have not yet been reported

(Felcth et al. 2012, Hu and Cheng 2009, Morgan et al. 2013, Thurston and Daly 2012).

In the positive control group, the mean Ang-2 level was 2.75 ng/ml. In the intravitreal injection-treated groups of mice, after four weeks of diabetes induction, there were increases in Ang-2 compared to the control group, i.e., 2.36 ng/ml in the 10 µg Ang-2 inhibitor group, 2.02 ng/ml in the 20 µg Ang-2 inhibitor group, and 2.31 ng/ml in the 30 µg Ang-2 inhibitor group. The Ang-2 levels in the treatment groups were higher, but not up to two times higher, than the levels of Ang-2 in the control group. In another study conducted on mice that were induced with diabetes for six months, there was an increase in Ang-2 by 1.87 times compared to healthy mice. Over-expression of Ang-2 not only occurs in hyperglycemia, but can also occur in melanoma, colorectal, neuroendocrine, lung, chronic diseases, metabolic diseases, inflammatory conditions, and sepsis (Felcth et al. 2012, Holopainen et al. 2012, Hu and Cheng 2009, Morgan et al. 2013, Thurston and Daly 2012).

Administration of an Ang-2 inhibitor by intravitreal injection has never been done before. The selection of doses of the Ang-2 inhibitor in this study was based on previous studies in which the Ang-2 inhibitor was administered intraperitoneally for colon cancer management, and based on a study conducted by Holopainen et al. which used an Ang-2 inhibitor to reduce lung cancer metastases. Holopainen et al. reported that subcutaneous administration of an Ang-2 inhibitor, 30 mg/kg bb, in mice can inhibit angiogenesis and reduce the formation of new blood vessels in the retina (Thurston and Daly 2012).

The Ang-1 and Ang-2 affinity of Tie-2, as a specific receptor, is almost the same; thus, an increase in Ang-2 is able to shift Ang-1 to bind to the Tie-2 receptor, and then induce the intracellular pathways below it. The excessive expression of Ang-2 causes remodeling of blood vessels with decreased endothelial integrity caused by increased endothelial hypoxia. This results in increased leakage, which is marked by increased peripheral endothelial ratios. Administration of an Ang-2 inhibitor will inhibit the work of Ang-2, thus increasing endothelial cell junctions and improving the basal membrane. In the case of hyperglycemia, the loss of pericytes triggers disruption of the endothelium, leading to endothelial death and the formation of acellular capillaries (Felcth et al. 2012, Holopainen et al. 2012, Hu and Cheng 2009, Morgan et al. 2013, Thurston and Daly 2012).

Dysfunction or loss of pericytes affects endothelial stability and ultimately affects retinal blood vessels. In the case of diabetic retinopathy, the loss of pericytes is the first thing to happen histologically. Because of the high incidence of diabetic retinopathy, pericytes are very important cells (Allt and Lawrenson 2001, Pfister et al. 2008, Rangasamy et al. 2011).

This study used three different doses of the Ang-2 inhibitor for each treatment group, respectively. Group 1 (10 µg) and group 2 (20 µg) appeared to exhibit inhibited migration of the spiral, even though the indices were relatively similar. However, in group 3 (30 µg) there was more migration of the pericytes. This is probably caused by a toxic dose. A study conducted by Holopainen et al., using a dose of 30 mg/kg bb administered subcutaneously for lung cancer metastasis therapy, also reported that the retina obtained fewer peripherals than the endothelium.

Ang-2 serum levels also decreased after intravitreal injection of the Ang-2 inhibitor. This is probably due to the systemic effect caused by the Ang-2 inhibitor. As has been shown before, Ang-2 inhibitors can be used for cancer therapy.

The weakness in this study is that it did not examine the effective dose range and toxic dose, so we cannot know the most effective dose for inhibitory effects on migration or the minimum dose required to induce toxic effects.

## CONCLUSION

There was an increase in Ang-2 levels in the diabetic model rats, and there was a significant difference between the levels of Ang-2 in normal rats compared to the DM rats. There were no significant differences between the groups of DM rats. The number of narrows decreased in diabetic model rats by 40%, and the group administered 10 µg of the Ang-2 inhibitor showed the best results in terms of a reduced pericyte loss of 44.44%. The group administered the Ang-2 inhibitor at a dose of 30 µg did not show any effects on reducing the loss of pericytes. This is probably because this dose may be a toxic dose to the retina.

## ACKNOWLEDGEMENTS

All Author thank to the 42nd Indonesian Ophthalmologist Association (IOA) Annual Meeting.

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