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Anti-receptor Advanced Glycation End Products Decreases Inflammatory Pathways in Retinopathy Diabetics: *In vivo* Study

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Abstract

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BACKGROUND: Diabetic retinopathy is an emerging microvascular complication of diabetes mellitus and a cause of blindness in individuals between ages 30 and 70 years, which is characterized by increased proliferation of blood vessels, vascular occlusion, angiogenesis, loss of pericytes from retinal capillaries, microaneurysms, retinal bleeding, increased retinal capillary permeability, thickening of capillary basal membranes, and infarcts that affect the retina, induced to permanent blindness.

AIM: This study aimed to find the role of receptor advanced glycation end products (RAGE) inhibition in lowering the vascularization process which causes a decrease in retinal function on diabetic retinopathy.

MATERIALS AND METHODS: This research was an *in vivo* experimental study. A total of 30 male Wistar rats (200 ± 20 g) were obtained from Eureka Research Laboratory (Palembang, Indonesia). Experimental animals were placed in cages under controlled conditions (12 h of light/dark cycles with temperatures of 22 ± 1°C and humidity of 40–60%), fed and drank *ad libitum*. White rats were induced by diabetes mellitus using alloxan at a dose of 120 mg/kgBW, intraperitoneally, accompanied by drinking 10% glucose solution for 140 days. Furthermore, experimental animals were grouped into five groups (at eight animals per group), Group 1: Normal control, Group 2: Negative control (induced diabetics retinopathy and given intravenous aquadest), Group 3: Given anti-RAGE 1 ng/mL, Group 4: Given anti-RAGE 10 ng/mL, and Group 5: Given anti-RAGE 100 ng/mL. Giving anti-RAGE was done in a single dosage and intravitreal. After the rats were sacrificed by intraperitoneal injection of 10% chloral hydrate, the evacuation of the eye's retinal tissue was then carried out, fixed in a 4% paraformaldehyde buffer for immunohistochemistry examination of the eye's retinal tissue. Evaluation of the expression of nuclear factor-κβ (NF-κB) and intercellular adhesion molecule-1 (ICAM-1) used Image J Software so that the percentage of NF-κB and ICAM-1 expression would be obtained.

RESULTS: Negative control group showed an increase in NF-κB expression in the retinal tissue of diabetic retinopathy rats. Administration of anti-RAGE showed its potential to suppress NF-κB expression in retinal tissue of diabetic retinopathy white rats as well with an increase of anti-RAGE dose from 1 ng/mL to 100 ng/mL. Activation of NF-κB causes activation of the inflammatory cascade, which is characterized by the production of pro-inflammatory cytokines, one of which is ICAM-1. Giving anti-RAGE could suppress the expression of ICAM-1 along with an increase in anti-RAGE dose.

CONCLUSION: Anti-RAGE is able to block the inflammatory process, by inhibiting the expression of NF-κB and ICAM-1 in the retinal tissue of diabetics retinopathy in white rats.

Introduction

Diabetes mellitus has been being an emerging health problem since the past two decades. The International Diabetes Foundation in 2014 estimated the number of people with type 2 diabetes mellitus worldwide at 387 million, and this number is expected to increase to 552 million by 2030. Diabetic retinopathy is a serious microvascular complication of diabetes and a major cause of blindness in individuals between ages 30 and 70 years, which is characterized by increased proliferation of blood vessels, vascular occlusion, angiogenesis, loss of pericytes from retinal capillaries, microaneurysms, bleeding, increased retinal capillary permeability, thickening of capillary basal membranes and infarcts that affect the retina of the eye, leading to blindness [1], [2], [3]

Most of all diabetes mellitus patient either type 1 or type 2 show lesions on the retina after 20 years of hyperglycemia. The upcoming lesion is a result of the hyperglycemia condition through the biochemical mechanism of the polyol pathway, the formation of advanced glycation end products (AGE), activation of protein kinase C, oxidative stress or the formation of reactive oxygen species (ROS), and hexosamine pathway. From these various pathways, the formation of AGE products becomes the mechanism that plays a significant role in initiating the incidence of diabetic retinopathy [4], [5].

The interaction between AGE and its receptor, receptor AGE products (RAGE), increases the production of intracellular ROS and upregulation of nuclear factor-κβ (NF-κβ) transcription factors and their products, endothelin-1, vascular cell adhesion molecule-1, intercellular adhesion molecule-1 (ICAM-1),

vascular endothelial growth factor, pro-inflammatory cytokines interleukin-1 α (IL-1 α), IL-6, and tumor necrosis factor α . This will cause the activation of the inflammatory process which will cause tissue damage accompanied by a wound healing process, in the form of a collagenization and fibrosis process, which in turn, will end in damage to the function of the tissue, in this case retinal tissue [6], [7], [8].

AGE inhibition through RAGE receptor inhibition is a therapeutic promising to inhibit the inflammatory cascade, which forms the basis of the pathophysiology of diabetic retinopathy. This study is a continuation of the previous research, which seeks to see the role of RAGE inhibition in decreasing the vascularization process which causes a decrease in retinal function in the case of diabetic retinopathy. Efforts to explore the mechanism of action pathway continue in this study, where the inflammatory pathway is the target of further exploration [8].

Materials and Methods

This research is an *in vivo* experimental study. A total of 30 male Wistar rats (200 \pm 20 g) were obtained from Eureka Research Laboratory (Palembang, Indonesia). Experimental animals were placed in cages under controlled conditions (12 h of light/dark cycles with temperatures of 22 \pm 1°C and humidity of 40–60%), fed, and drank *ad libitum*. All animal treatments and experimental procedures were approved by the Ethics and Humanities Commission of the Faculty of Medicine, Universitas Sriwijaya (No.243/kptfkunsri-rsmh/2019).

White rats were induced to be diabetes mellitus using alloxan at a dose of 120 mg/kgBW, intraperitoneally, accompanied by drinking 10% glucose solution for 140 days. Furthermore, experimental animals were grouped into five groups (at eight animals per group), Group 1: Normal control, Group 2: Negative control (induced diabetics retinopathy and given intravenous aquadest), Group 3: Given anti-RAGE 1 ng/mL, Group 4: Given anti-RAGE 10 ng/mL, and Group 5: Given anti-RAGE 100 ng/mL. Giving anti-RAGE was done in a single dosage and intravitreal.

After the rats were sacrificed by intraperitoneal injection of 10% chloral hydrate, the evacuation of the eye's retinal tissue was then carried out, fixed in a 4% paraformaldehyde buffer for immunohistochemistry examination of the eye's retinal tissue. After the tissue that had been put into the fixation fluid, the next process was dehydrated using alcohol and xylene, then paraffinized and then cut as thick as 5 μ m using a rotary microtome (Leica). Next, it was placed on coated-object glass. Then, rehydration is carried out on the tissue using xylene and alcohol

with a concentration of 96%, 90%, 80%, and 70% and rinsed with tap water. The next stage, retrieval antigen was carried out with the HIER (Heat Induced Epitop Retrieval) method, where the slides were put into a citrate buffer solution, then heated at a temperature of 95°C for 60 min. Then, NF- κ B 1: 700 (Cloud Clone) antibody was painted; ICAM-1 antibody 1: 700 (Cloud Clone), followed by overnight incubation at 4°C. The next stage was to paint with a secondary antibody, Biotinylated-Horseradish Peroxidase, incubated for 1 h, at room temperature. Next, chromogen was given to the tissue. Furthermore, the dehydration process was again carried out using concentrated alcohol and xylene. The next step was mounting and evaluating the expression of NF- κ B and ICAM-1 using Image J Software so that the percentage of NF- κ B and ICAM-1 expression would be obtained.

All data were presented as mean \pm standard deviation and all statistical analyzes were performed with the SPSS 25 (IBM) program. One-way ANOVA followed by *post hoc* analysis was carried out to assess differences in the mean expression levels of each protein. $p < 0.05$ was determined as an indication that there were significant differences in the mean levels.

Results

It is believed that NF- κ B is the main regulator in the activation of pro-inflammatory cytokines. From Table 1, it was clearly stated that negative control group has an increase in NF- κ B expression in the retinal tissue of diabetic retinopathy rats. Furthermore, there was higher expression of NF- κ B in negative control group which was shown by brownish image in Figure 1. The administration of anti-RAGE showed its potential to suppress NF- κ B expression in retinal tissue of diabetic retinopathy white rats as well with an increase of anti-RAGE dose from 1 ng/mL to 100 ng/mL.

Activation of NF- κ B causes activation of the inflammatory cascade, which is characterized by the production of pro-inflammatory cytokines, one of which is ICAM-1. Figures 2 and 3 show that in diabetic retinopathy was an increase in the expression of ICAM-1 in the retinal tissue of white rats. Giving anti-RAGE can suppress the expression of ICAM-1 along with an increase in anti-RAGE dose.

Table 1: Level of NF- κ B expression in retinal tissue retinopathy diabetics

S. No.	Group	NF- κ B (%) \pm SD	p value*
1.	Negative control	70.62 \pm 2.95	0.00
2.	RAGE 1 ng/mL	66.98 \pm 5.08	0.00
3.	RAGE 10 ng/mL	48.22 \pm 4.27	0.00
4.	RAGE 100 ng/mL	26.66 \pm 3.28	0.00
5.	Normal	5.45 \pm 1.31	-

*VS normal; ANOVA, *post hoc* Bonferroni; $p < 0.05$. RAGE: Receptor advanced glycation end products, NF- κ B: Nuclear factor- κ B.

Discussion

This study found a significant difference between the treatment groups in diabetic retinopathy white rats that had been given anti-RAGE antibodies against NF- κ B expression where in higher dose of

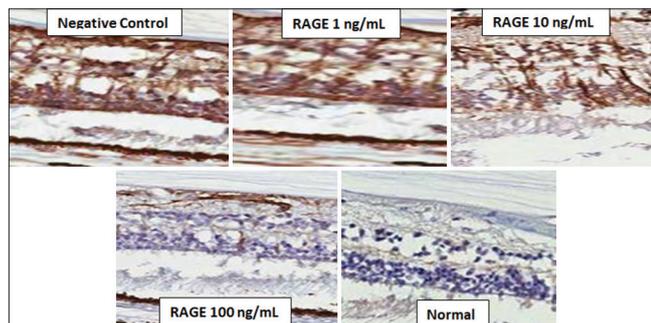


Figure 1: Effect of anti-receptor advanced glycation end products (RAGE) on nuclear factor- κ B expression in retinal tissue retinopathy diabetics rats. Immunohistochemistry assessment. Normal: Normal group, Negative control: Retinopathy diabetics rats, RAGE 1 ng/mL: RAGE 1, 10, and 100 ng/mL: Anti-RAGE doses 1, 10, and 100 ng/mL + Retinopathy diabetics. Magnification $\times 400$

anti-RAGE antibody (100 ng/mL) was more effective than the administration of a dose of 10 ng/mL because higher dose obtained percentage of NF- κ B expression of $26.66\% \pm 3.28$ while giving 10 ng/mL of $48.22\% \pm 4.27$. AGE-RAGE bonds were able to induce endothelial dysfunction and cause hyperpermeability.

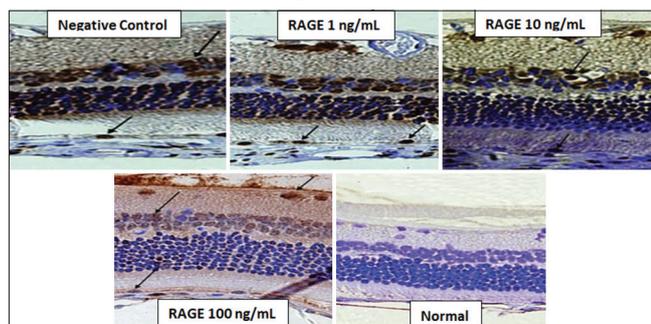


Figure 2: Effect of anti-receptor advanced glycation end products (RAGE) on intercellular adhesion molecule-1 (ICAM-1) expression in retinal tissue retinopathy diabetics rats. Immunohistochemistry assessment. Normal: Normal group, Negative control: Retinopathy diabetics rats, RAGE 1 ng/mL: RAGE 1, 10, and 100 ng/mL: Anti-RAGE doses 1, 10, and 100 ng/mL + retinopathy diabetics. Magnification $\times 400$

At the cellular level, the AGE-RAGE bond could induce nuclear activation and translocation of NF- κ B (a transcription factor capable of responding to adhesion molecules in endothelial or leukocytes) which would initiate vascular disorders. Activation of NF- κ B would also induce activation of pro-inflammatory cytokines. Activation of NF- κ B also played a role in the occurrence of apoptosis, because it made an increase in the factor of proapoptosis bax (bax-caspase protease pathway). RAGE expression depends on the amount of NF- κ B, which was a positive feedback response to increased RAGE expression through AGE-RAGE interactions. Others study concluded

that anti-RAGE treatment in diabetic retinopathy mice could lower pro-inflammatory cytokines such as NF- κ B and IL1 β [3], [9], [10], [11], [12], [13].

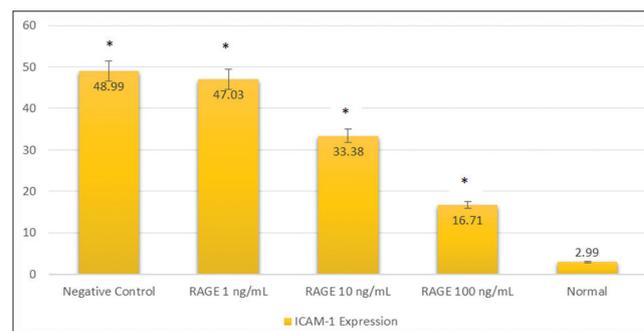


Figure 3: Effect of anti-receptor advanced glycation end products on intercellular adhesion molecule-1 expression in retinal tissue retinopathy diabetics rats. * $p < 0.05$ versus normal; ANOVA, post hoc Bonferroni

This research also found a notable difference between the treatment groups in white rats with diabetic retinopathy models who had been given anti-RAGE antibodies to ICAM-1 expression where the dose of anti-RAGE antibody at a dose of 100 ng/mL was more effective than lower dose (10 ng/mL) because administration of 100 ng/mL obtained percentage of ICAM-1 expression of 16.71 ± 3.10 while administering 10 ng/mL of 33.38 ± 5.09 . Giving anti-RAGE 100 ng/mL is considered more effective in reducing ICAM-1 expression.

NF- κ B has correlation and relationship with ICAM-1 expression. NF- κ B activation activates the inflammatory cascade, which is characterized by secretion of pro-inflammatory cytokines and mobilization of neutrophils at the inflammatory site. The mobilization of neutrophils will cause activation of ICAM-1, which is an adhesion molecule of neutrophils, so as to facilitate the movement and mobilization of neutrophils. The results of this study indicate that an increase in NF- κ B is followed by an increase in ICAM-1 in diabetic retinopathy. Anti-RAGE is able to break the cascade of NF- κ B activation through the activation of AGE receptors (RAGE) [14], [15], [16], [17] This study reinforces previous studies also showing the potential of anti-RAGE in preventing angiogenesis activation in diabetics retinopathy. The ability to inhibit angiogenesis in anti-RAGE administration through inhibition of the inflammatory pathway through inhibition of NF- κ B and ICAM-1 [8].

Conclusion

Anti-RAGE is able to block the inflammatory process, by inhibiting the expression of NF- κ B and ICAM-1 in the retinal tissue of diabetics retinopathy in white rats.

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