Advanced Glycation End-Product (AGEs)Level in a Diabetic Rat Model Treated with Warfarin Anticoagulant

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ABSTRACT
Background*: Diabetes mellitus is a chronic disease that is the most seriously cause of macrovascular and microvascular complications that threaten life , expensive and reduces life expectancy. The occurence of cell damage and inflammation due to AGEs and RAGE reactions generally exacerbates the risk of macrovascular complications . Pharmacological therapy given to prevent these complications is the warfarin anticoagulant . the duration of warfarin use can increase the hardening of blood vessels or vascular calcification and is compounded by AGEs accumulation and RAGE activation.* ***Objective****: To analyze plasma AGE levels as a risk factor for vascular calcification in DM and non-DM rats models treated with warfarin anticoagulants.* ***Method****: This study used a Quasi experimental method with a posttest only control group design. The total sample was 28 samples divided into 4 groups, each group consisting of 7 rats grouped into DM + Aquadest group, DM + Warfarin group, non-DM + Aquadest group , and non-DM + Warfarin group. Plasma AGE levels were measured using the ELISA method and the results were analyzed using Anova.* ***Results****: AGE Level in the DM + Warfarin group (Mean= 1.183; SD=0.16) was slightly high than AGE level in normal rat group with Warfarin induction (Mean=1,012; SD=0,02). In the normal rat group treated with Aquadest was slightly low than AGE level in the DM rat group with warfarin induction (Mean=1.147; SD=0.13). Interestingly, There was no difference in plasma AGE levels between groups (p=0,155).* ***Conclusion****: AGE levels at the onset of DM are not yet able to progressively activate vascular damage through the AGE/RAGE signaling pathway.*
Keywords : *AGE; diabetes mellitus; RAGE; warfarin*

INTRODUCTION

Diabetes Mellitus is a chronic metabolic disease characterized by hyperglycemia. The prevalence of DM, especially Type 2 DM, increases every year. Indonesia is ranked 7th in the world and *the International Diabetes Federation* (IDF) estimates that by 2025 more than 21 million people will suffer from DM (AHA, 2021; IDF, 2019). Type 2 DM, also known as "adult-onset diabetes," and accounts for approximately 90–95% of the total diabetes cases worldwide (Kaur R, 2018).

Microvascular complications can increase vascular endothelial dysfunction. It increases *reactive oxidative species* (ROS) thereby causing vascular damage through activation of the protein kinase C (PKC) pathway, increasing the hexosamine pathway, increasing glycation end products (*Advanced glycation end-product*, AGE) and polyol pathway enhancement (Prawitasari, 2019). *Advanced glycation end-product* (AGE) is a glycotoxin, a

group of highly oxidized compounds formed through non-enzymatic processes. AGEs are formed through of adding reduced sugar to free amino acids from proteins, fats and nucleic acids (Mulyati, 2016). AGEs will bind to *receptors for advanced glycation end-product* (RAGE) in body cells. When this happens RAGE will activate NADPH oxidase (NOX) in the cell membrane and protein kinase C which will then cause *chemical chains reactions*. Increased concentrations of extracellular AGEs will increase RAGE activation leading to downstream signaling through signaling proteins, such as extracellular-related kinase 1/2 (ERK 1/2), nuclear factor-κB (NF-κB), and p38 mitogen-activated protein kinase (p38 MAPK) (Kennon and Stewart, 2021).

Cell damage and inflammation due to AGEs and RAGE reactions will generally exacerbate the risk of macrovascular complications. One of the things that can arise due to high levels of AGEs and RAGE is hardening of the arteries or vascular calcification (Kay et al, 2016). This vascular calcification event will increase the risk of atherosclerosis which can then trigger macrovascular complications in type 2 diabetes mellitus patients (Albanese et al., 2018). Prevention of macrovascular complications such as thromboembolic stroke in T2DM patients is generally carried out by administering vitamin K antagonist anticoagulants such as warfarin (AHA, 2021). The dose given to patients without DM is the same as the dose given to patients with DM (Culebras et al., 2014).

In vivo research by Brodeur et al stated that there was an increase in vascular calcification along with the duration of warfarin use, plus found accumulation of AGEs and activation of RAGE in calcified areas (Brodeur et al., 2014). This is done by carrying out a flourescent test on the affected area, showing increased expression of AGEs in the area experiencing calcification. In addition, to confirm the role of RAGE in vascular calcification, AGE agonists were used to induce RAGE, and the result was vascular calcification in areas exposed to AGE agonists (Brodeur et al., 2014). The study by Poterucha & Goldhaber also obtained the same results, namely an increase in the incidence of vascular calcification in experimental animals given warfarin (Poterucha & Goldhaber, 2016). Even in research conducted by Alppan et al with human samples also proved that warfarin increases the risk of vascular calcification (Alappan et al., 2020). This is thought to occur due to *Vitamin K Dependent Protein* (VKDP) having a role in preventing vascular calcification, but VKDP is very complex and strongly influenced by genetic factors so that many patients who take warfarin do not experience vascular calcification (Palareti et al., 2016).

On the other hand the results of research by Kay et al , stated that AGEs and RAGE reactions have an important role in the occurrence of vascular calcification (Kay et al., 2016). Based on the data above, there is still debate regarding the statement that warfarin increases the risk of vascular calcification due to decreased VKDP levels or occurs due to the presence of high levels of AGEs and RAGE activation. So researchers are interested in examining differences in plasma AGEs

levels and RAGE gene expression as a risk factor for vascular calcification in type 2 DM and non-DM rats given anticoagulants warfarin.

METHODS

Research design

This type of research is a quasi-experimental research with a posttest only control group design. Rats were randomly divided into four groups consisting of seven rats in each group: (1) DM + Aquades rats group; (2) the DM + Warfarin rat group; (3) Non DM + Aquades rats; (4) Non DM + Warfarin rats. Warfarin were given with a dose of 2 mg/kg Rats (Pradana et al., 2022). All treatments were given orally according to kgBB for 14 days, with modification (Pradana et al., 2022; Patel S, Singh R, Preuss CV, et al. 2023). Standard feed and drinking water are provided ad libitum. At the end of the experiment, rat from each group was fasted, anesthetized, and put to sleep. Blood was drawn via *the intracardia* for measurement of plasma glucose and AGE levels.

Animals

Twenty-eight male Wistar rats aged 8-12 weeks weighing between 150-200 grams were obtained from the Faculty of Medicine, University of Brawijaya, Malang. Mice were housed individually in cages, and maintained under standard conditions (12:12 h light/dark cycle at room temperature 22-25°C), rats were fed standard diet AIN-93 M by Reeves et al. with slight modification and water ad libitum (Reeves PG, Nielsen FH, Fahey GC Jr , 1993). AIN-93 M consists of 24% casein, 0.30% DL- methionine, 61% cornstarch, 1% vitamin mix, 3.5% mineral mix, 0.2% choline chloride, 5% gelatin, and 5% oil corn. Ethical approval for this study was granted from the Ethical Commission of the Faculty of Medicine, Al-Azhar Islamic University (Number: 15/EC-03/FK-06/UNIZAR/II/2022).

Diabetes Mellitus Induction

Rats were fasted for 10-12 hours, and then induced by intraperitoneal (ip) injection of 60 mg /kg body weight of streptozotocin (STZ) (Nacalai Tesque, INC), dissolved in 0.1 M citrate buffer, pH 4.5 after 15 minutes of i.p. administration of 120 mg /kg body weight nicotinamide (NA) (Sigma-Aldrich, USA) prepared in 0.9% normal saline. Mice with signs of hyperglycemia (fasting blood glucose level >200 mg / dL) after 7 days of STZ-NA administration were used for this study.

Analysis of AGE Levels

Plasma AGE levels were measured by ELISA method using the plasma AGE kit from (Finetest Rat AGE Catalog No.: ER0268).

Data analysis

All values are presented as mean ± standard deviation (SD). Plasma AGE levels were analyzed using *One- way* ANOVA. Blood glucose levels before and after treatment were analyzed using paired t test. A p value < 0.05 was considered statistically significant.

RESULTS

This research was conducted at the Research Laboratory of the Faculty of Medicine, Al-Azhar Islamic University in June 2022. The results of this study,

blood glucose level can be seen in table 1 and plasma AGE levels in the four groups can in table 2. Research results show that there was no difference in plasma AGE levels between groups, although on average plasma AGE levels in the DM group were slightly higher than the non-DM group.

DISCUSSION

This study aims to determine plasma AGE levels in rats (*Rattus norvegicus*) DM and non-DM models given anticoagulants warfarin as a risk factor for vascular calcification. Based on the analysis of plasma AGE levels, it was found that there was no difference in plasma AGE levels between groups in both the DM and non-DM rat groups. This study provides direct evidence regarding the role of AGE in *early onset* DM *rats,* those results were influenced by 2 important factors: First, increased levels of protein glycation and AGE compounds in circulation are seen in diabetes patients early on, but the series of processes to produce AGEs and the formation of AGEs from glucose (hyperglycemia) takes days, weeks, even months to complete (Vetter WS, 2015). The level of glycated AGE is determined by the balance between the rate of formation and elimination processes. Proteins that have a shorter half-life with a high elimination rate are initially glycated and at the same rate as proteins with a longer circulating half-life but the glycation process does not become AGE . Therefore AGEs can be degraded or eliminated more quickly (Vetter WS, 2015).

The main group of AGEs is carboxymethyl lysine (CML), carboxyethyl lysine (CEL), pentosidine , glucosepane , methylglyoxal lysine dimer, glioxal dimer lysine, and glycolic acid lysine amide (Henning and Glomb, 2016). Senatus & Schmidt (2017) stated that even though healthy, elderly subjects may have a higher accumulation of AGEs compared to younger subjects with DM and complications, so that the production and accumulation of AGEs are in line with the normal aging process. Many factors influence such as the rate of accumulation of AGE ligands, absolute concentrations of ligands, and individual susceptibility to AGE formation. This situation could occur in our study.

The scientific explanation of this first situation like process AGE- uptake and degradation. This process started with AGE- modified proteins cleared through mediated endocytosis receptors and degradation lysosomes. Receptors *scavenger* on monocytes, in particular macrophages, and type cell others in a manner continuously binds to AGE-modified proteins and inserts them to in track degradation endolysosomal. Receptors *scavengers* play an impotant role in clearing glycated and AGE-modified proteins from circulation process. This is exactly the uptake of oxidized LDL (Vetter SW, 2015). The role of warfarin in stimulating vascular calcification due to AGE formation was not found in this study. This happened because of *early conditions onset* DM and currently, there is evidence that albumin glycation in diabetic patients can have a statistically significant effect on drug binding to albumin, but this effect appears to be relatively small and more detailed studies are needed .

Secondly, plasma AGE concentration is closely related to the severity of diabetes complications (Brodeur MR, 2014). This study was designed to evaluate AGE levels in the early phase of DM as a risk factor for vascular damage, but these results are not different from normal conditions. This condition can occur because of the glyoxalase system which is present in all cells and efficiently removes dicarbonyl stress. Glyoxalase I (GLO I) and glyoxalase II (GLO II) in the body will naturally detoxify AGEs (Sergi D, 2021). Interestingly, exercise training in aged rats resulted in activation of GLO1, with consequent reduction in the formation of MG and CML, along with lower RAGE expression in the aorta (Gu et al., 2014). Studies in cell cultures and model organisms show that overexpression of GLO1 is associated with inhibition of AGEs inducing oxidative stress, whereas downregulation of GLO1 expression is associated with increased levels of AGEs accumulation (Khalid N *et al*, 2022). This condition may also occur in this study. However, plasma AGE levels slightly difference in DM and non - DM rats given warfarin.

CONCLUSION

Based on the results of this study, there was no difference in plasma AGE levels between groups, although the the mean plasma AGE levels in the DM group were slightly higher than the non-DM group.

SUGGESTION

Further research is needed regarding to serum albumin levels and vascular calcification levels due to anticoagulant administration and comparison of AGE/RAGE levels in *early* and *late onset* DM Type 2.

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Table 1. Blood Glucose Level

Group	Pre-test Blood Glucose Level (mg/mL)	Posttest Blood Glucose Level (mg/dL)
DM + Warfarin	63.3 ± 6.34	238.8 ± 155.9
DM + Aquadest	77.0 ± 5.36	281.1 ± 129.8
Non DM + Warfarin	65.6 ± 8.47	90.0 ± 23.6
Non DM + Aquadest	67.6 ± 8.91	98.6 ± 33.2

Data are presented as mean ± standard deviation.

Table 2. Plasma AGE levels

Group	AGE levels (ng/mL) (Mean ± SD)
DM + Warfarin	1.183 ± 0.16
DM + Aquadest	1.147 ± 0.13
Non DM + Warfarin	1.012 ± 0.02
Non DM + Aquadest	1.129 ± 0.15

Data distribution was tested with Shapiro-Wilk : $p \geq 0.05$. Data are presented as mean ± standard deviation. *One-way ANOVA* test : $p=0.115$ $p \geq 0.05$. The confidence interval is 95% (95% CI).