Effect of Genistein on Heat Shock Protein 47 and Collagen Type IV in Diabetic Rat

Putri Dafriani¹,¹, Nur Indrawati Lipoeto², Yanwirasti², Amri Bakhtiari³ and Roza Marlinda¹

¹Sekolah Tinggi Kesehatan Syedza Santika, Padang, Indonesia
²Faculty of Medicine, Andalas University, Padang, Indonesia
³Faculty of Pharmacy, Andalas University, Padang, Indonesia

Abstract: Diabetes nephropathy (DN) is one of the most common complication in Diabetes Mellitus (DM). DN is an inflammatory process which involves immune cells and effect of genistein prevent this mechanism. However, the effects on HSP 47 and collagen type IV are not yet verified. The purpose of this study was to investigate whether the genistein can suppress HSP 47 and collagen type IV.

This study is experimental design used 25 rats. Rats were divided into five groups; normal group, hyperglycemia group, hyperglycemia by administering genistein 0.5 mg/kg, 1mg/kg, and 2 mg/kg. Streptozotocin induced 65 mg/kg administered intraperitoneal. Treatment duration is 4 weeks. After 4 weeks of blood was collected via the orbital vein and examined the levels of HSP 47 then rats’ kidneys were taken to see the levels of collagen type IV.

The average levels of HSP 47 in non diabetic control group was 1.7982 ng/ml, diabetic control 7.9424 ng/ml, STZ; G 0.5 mg/kg 5.4192 ng/ml, STZ; G 1 mg/kg 3.1152 ng/ml and STZ; G 2 mg/kg 1.849 ng/ml, with p value 0.000 (p<0.05). While Type IV Collagen in non diabetic group was 10.006 ng/ml, diabetic group 26.864 ng/ml, STZ; G 0.5 mg/kg 21.426 ng/ml, STZ; G 1 mg/kg 17.352 ng/ml and STZ; G 2 mg/kg 13.436 ng/ml with p value 0.000 (p<0.05).

Administer the genistein can reduce levels of HSP 47 and collagen type IV in diabetic rats. Genistein can reduce fibrotic mediators induced by NFkB and MAPK signaling by inhibiting the tyrosine kinase protein activation.

Keywords: Genistein, HSP 47 and Type IV Collagen.

INTRODUCTION

Diabetes mellitus (DM) is one of the most important health problems worldwide. International Diabetes Federation estimated the number of adult people living with diabetes will rise from 285 million in 2010 to 439 million in 2030. Diabetic nephropathy (DN) is seriously diabetic complications. Diabetic nephropathy is a major cause of end stage renal disease [1]. Diabetic nephropathy occurs 30-40% of all cases of diabetes [2]. Diabetic nephropathy is characterized by the occurrence of deformation of kidney structure, expansion of mesangial cells and thickening of the glomerular membrane [3].

Hyperglycemia initiated a pathological condition. Hyperglycemia activates polyol pathway which then activates protein kinase C (PKC), and formation of advanced glycation end products (AGEs). AGEs activates macrophages to produce proinflammatory cytokines [4]. Activation of NFkB plays an important role in the inflammatory process because it is a transcription factor of pro-inflammatory genes.

Oxidative stress can increase proinflammatory cytokines through several mechanisms, presence of free oxygen, the role of second messengers, activation of the transcription factor kappa beta (NFkB) and activator protein-1 (AP-1). Control of inflammatory factors will inhibit the process of sustainable fibrosis [5].

Oksidative Stress causes changes in transcription factors genes encoding cytokines, growth factors and proteins that make up the extracellular matrix [6]. In diabetic rat kidneys, Nuclear factor kappa beta activates mesangial cells [7]. NFkB also stimulates fast transcription factor genes endothelin-1 (ET-1), vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), interleukine 6, interleukin 1 and tumor necrosis α (TNF-α), which increases the development of diabetic nephropathy. TNF-α and IL-1 causes enhanced expression of chemotactic factors (MCP-1), stimulates the kidney cells produce growth factors (TGF-β, CTGF, VEGF), increasing the permeability of vascular endothelium and stimulate cell proliferation mesangial cells and improve the formation of extracellular matrix [8].

Activation profibrotic cytokines such as (TGF-β) in renal tissue causes fibrosis in the kidney. Fibrosis is
accumulation extracellular matrix. The dominant extracellular matrix in the kidney is collagen type IV [9]. The synthesis of collagen type IV is mediated by heat shock protein (HSP) 47 [10]. Heat shock protein (HSP) 47 is a collagen-binding glycoprotein that is located within the endoplasmic reticulum. HSP 47 important in the development of fibrosis by preventing the misfolding of collagen [11]. Increased expression of HSP 47 was associated with increased collagen accumulation in renal fibrosis [12]. HSP 47 expression is increased in kidney of diabetic rats induced by streptozotocin [13]. Animal studies that fibrosis obtained by lowering the levels of HSP 47 can reduce the production of collagen and progression of fibrosis [14].

The use of herbs and natural medicines has become an alternative in DM and DN. One of them is soy. Beside as an anti diabetic, soy is one of source of nutrition for humans. Most of Asian people use it as a food ingredient [15].

Isoflavones is one of flavanoid that have affect as antioxidant and inhibitor tyrosine kinase. The Soy of isoflavone consist genistein, daidzein and glyceitin.Genistein (4',5,7-trihydroxyisoflavone)is one of the major soy isoflavones [16]. Genistein has a very interesting biological activity. Genistein can decrease the activity of genes that regulate cell proliferation, cell cycle, inducing apoptosis, inhibiting the activation of NF-κB and have antioxidant effects [17].

The positive effect of genistein on diabetic renal are still insufficiently presented in the literature, so the purpose of this study is to investigated whether the genistein can reduce fibrosis by decreasing HSP 47 and collagen type IV.

**MATERIAL AND METHODS**

**Chemicals**

Genistein and streptozotocin (STZ) were purchased from Sigma Aldrich. Kits for the assay of HSP 47 and collagen type IV were purchased from elabscience. The chemicals and solvents used in the study were purchased from farmacology laboratory Andalas University, Indonesia.

**Diet**

Diet of non diabetic group is starch and diabetic groups is genistein. Genistein is given by oral use stomach tube at a dose of 0.5 mg/kgw/day, 1 mg/kgw/day, 2 mg/kgw/day. 100 mg of genistein dissolved in 100 ml (1mg/1ml). It was gave for 30 days.

**Animals**

At the beginning of the experiment, Male wistar rats weighing between 150 and 200 g were purchased from farmacology Laboratory Animal Research (Padang, Indonesia). The animals were all individually housed in stainless steel cages in an air-conditioned room with controlled temperature (20–22 °C) and automatic lighting (alternation 12-h periods of light and dark) and fed an AlN-93 [18]. The experimental procedures were done according to the guidelines of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA) and approved by the Institutional Ethical Committee of Animal Care, Andalas university, Indonesia (NO: 104/KEP/FK/2015).

**Experimental Design**

The animals were divided into two groups: a nondiabetic control and a diabetic group. Diabetes was induced by a single injection of STZ (50 mg/kgw; Sigma, USA). STZ dissolved in a 0.1 mol/L citrate buffer (pH 4.5) and then it injected into the intraperitonium. The control rats were only injected with the citrate buffer. Diabetes was confirmed in the STZ-treated rats by measuring the fasting blood glucose concentration 48-h post-injection. The rats with blood glucose level above 350 mg/dL were considered to be diabetic and were used in the experiment. The diabetic rats were randomly divided into four sub-groups, diabetic controls (STZ), diabetic rats given genistein (STZ-G; 0.5 mg/kgw), (STZ; 1 mg/kgw) and (STZ; 2 mg/kgw). In this study, 25 rats were used (five controls and 20 diabetic).

After 30 days of treatment, the rats were anesthetized with ether following a 16-h fast. Blood samples were taken from the abdominal aorta using heparin-coated syringes for plasma and regular syringes for serum. Plasma and serum were obtained by centrifuging the blood at 3000 rpm for 15 min at 4 °C. The kidneys were removed and rinsed with physiological saline. All samples were stored at -70 °C until analyzed.

**Assay of Inflammatory Markers**

**HSP 47 and Collagen Type IV**

Add Sample: Add 100µL of Standard, Blank, or Sample per well. The blank well is added with Reference Standard & Sample diluent. Solutions are added to the bottom of micro ELISA plate well, avoid inside wall touching and foaming as possible. Mix it gently. Cover the plate with sealer we provided.
Incubate for 90 minutes at 37°C. Biotinylated Detection Ab: Remove the liquid of each well, don’t wash. Immediately add 100µL of Biotinylated Detection Ab working solution to each well. Cover with the Plate sealer. Gently tap the plate to ensure thorough mixing. Incubate for 1 hour at 37°C.Wash: Aspirate each well and wash, repeating the process three times. Wash by filling each well with Wash Buffer (approximately 350µL) (a squirt bottle, multi-channel pipette, manifold dispenser or automated washer are needed). Complete removal of liquid at each step is essential. After the last wash, remove remained Wash Buffer by aspirating or decanting. Invert the plate and pat it against thick clean absorbent paper. HRP Conjugate: Add 100µL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 minutes at 37°C. Wash: Repeat the wash process for five times as conducted in step 3. Substrate: Add 90µL of Substrate Solution to each well. Cover with a new Plate sealer. Incubate for about 15 minutes at 37°C. Protect the plate from light. The reaction time can be shortened or extended according to the actual color change, but not more than 30minutes. When apparent gradient appeared in standard wells, user should terminate the reaction. Stop: Add 50µLof Stop Solution to each well. Then, the color turns to yellow immediately. The order to add stop solution should be the same as the substrate solution. OD Measurement: Determine the optical density (OD value) of each well at once, using a micro-plate reader set to 450 nm. User should open the micro-plate reader in advance, preheat the instrument, and set the testing parameters. After experiment, put all the unused reagents back into the refrigerator according to the specified storage temperature respectively until their expiry.

Statistical Analysis

All data are presented as the mean ± SE. The data were evaluated by a one-way ANOVA using the SPSS program, and the differences between the means assessed using posthoc bonferroni. Statistical significance was considered at p < 0.05.

RESULTS

Effect of Genistein on HSP 47

ANOVA test result found significant difference in average levels of HSP 47 between diabetic group and non diabetic control group. The average levels of HSP 47 in non diabetic control group was 1.7982 ng/ml, while diabetic control 7.9424 ng/ml, STZ; G 0.5 mg/kgw 5.4192 ng/ml, STZ; G 1 mg/kgw 3.1152 ng/ml and STZ; G 2 mg/kgw 1.849 ng/ml, with p value of 0.000 (p < 0.05). This means that there is positive effect of genistein on the levels of HSP 47. Post hoc Bonferroni were used to see the significance difference between treatment groups . There are no significant differences between STZ group with STZ group G 0.5 mg/kgw, whereas there are significant differences between the STZ group with STZ group G 1 mg/kgw and STZ G 2 mg/kgw.

![Figure 1: Mean of HSP 47 level between non diabetic control group and diabetic group.](image)

Effect of Genistein on Collagen Type IV

There is significant differences in average levels of Type IV Collagen between non diabetic control group and diabetic group. The average levels of Type IV Collagen in non diabetic group was 10.006 ng/ml, diabetic group 26.864 ng/ml, STZ: G 0.5 mg/kgw 21.426 ng/ml, STZ; G 1 mg/kgw 17.352 ng/ml and STZ; G 2 mg/kgw 13.436 ng/ml with p value 0.000 (p <0.05). This means that there is the effect of genistein on the levels of type IV collagen. To see the difference among the treatment groups, Post Hoc Bonferroni test were used. There are significant differences among STZ group with STZ G 0.5 mg/kgw, STZ G 1 mg/kgw and STZ G 2 mg/kgw.

DISCUSSIONS

Effect of Genistein on Levels of HSP 47

Levels of HSP 47 STZ group 7.9424 ng/ml higher than the levels of HSP 47 non diabetic group 1.7982 ng/ml. This suggests that hyperglycemia can cause elevated levels of HSP 47. The chemical reaction between sugar products with a protein that occurs over several days to several weeks to produce irreversible protein derivative called AGE (Advanced Glycation End Products). Derivatives This causes thickening of
collagen and endothelium, accelerated atherosclerosis, glomerular dysfunction, reduced formation of nitric oxide and alter the composition and structure of the extracellular matrix [19].

The formation of AGE also stimulate excessive secretion of anti-growth factors such as insulin-like growth factor 1 and transforming growth factor beta which causes decreased glomerular surface area for filtration. AGEs stimulate HSP 47 through increase in TGF-β. AGE also increased the expression of HSP 47 mRNA and collagen type IV. Giving AGE inhibitors can inhibit the buildup of matrix ekstrasuler [20].

Fibrogenic factors such as transforming growth factor (TGF) -β1 generated by the activation and changes the phenotype of cells resident and inflammatory cells that cause disease in humans and animals fibrosis experiments that subsequently increases the production of collagen. TGF-β1 affect the extracellular matrix. HSP47 plays an important role in fibrosis enhanced by increasing regulation of transcription factors to collagen. Thus, TGF-β1 can stimulate the expression of HSP47 [12].

The current study found genistein administration at a dose of 0.5 mg/kg (p = 0.001), 1 mg/kg (p = 0.000) and 2 mg/kg (p = 0.000) can significantly reduce the levels of HSP 47. Rosmarinus officinalis polyphenols can reduce HSP activity 47. As the result, it can protect cells against the heat and the temperature increase [21]. Flavanoid apigenin is one that efficiently inhibit HSP 90 in pancreatic cancer that inhibit the proliferation and migration of pancreatic cancer cells [22]. Other flavonoids group which can decrease the expression of HSP 90, which stimulates apoptosis of prostate cancer cells are quercetin [23]. Genistein is one of inhibitor tyrosine kinase, which activated inflammation cytokine. Genistein can inhibit inflammation process and reduce levels of HSP 47.

**Effect of Genistein on the Levels of Collagen Type IV**

The levels of collagen type IV on STZ group 26.864 ng/ml higher than the levels of collagen type IV non diabetic group 10.0060 ng/ml. This suggests that hyperglycemia cause increasing levels of type IV collagen. Hyperglycemic conditions stimulate the formation of extracellular matrix. The pathological changes on glomerular basement membrane initiated differences of glomerular filtration [24].

Study results found the dose of genistein,0.5 mg/kg (p = 0.000), 1 mg/kg (p = 0.000) and 2 mg / kg (p = 0.000) in diabetic rat can reduce levels of type IV collagen significantly. Hyperglycemic conditions stimulate the formation of extracellular matrix. Other research [25] found that the administration of genistein concentrations of 5μmol/l can inhibit the formation of collagen type IV.

Genistein, an isoflavone in soya can inhibit proliferation and collagen formation through suppression of protein tyrosine kinase. This is a signaling protein that promotes the growth and differentiation of cells. By suppressing the accumulation of growth factors, collagen type IV can be reduced [26]. Genistein may also decrease in renal cell apoptosis and reduce the formation of extracellular matrix such as type IV collagen and laminin in diabetic rats that could protect their kidneys due to the conditions of hyperglycemia [25]. Genistein can reduce collagen type IV with decrease level of HSP 47.

**CONCLUSION**

Administration of genistein can reduce levels of HSP 47 and collagen type IV in diabetic rats. Genistein is inhibitor tyrosine kinase and as the result, prevent the inflammation process. Decreasing inflammation cytokine will be continued as lowering fibrosis process on kidney diabetic. Thus, investigating inflammatory cytokine will be a plus.

**CONFLICT OF INTEREST**

There were no conflict of interest found among the authors in this work.

**ACKNOWLEDGMENTS**

The research supported clinical investigator in Biomedic and Farmacology laboratorium Andalas University Indonesia.
REFERENCES


