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THE EFFECT OF INJECTING SIRNA ANGIOTENSINOGEN ON WISTAR STRAIN MALE RATS (RATTUS NORVEGICUS) BLOOD PRESSURE

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ABSTRACT
High blood pressure, or hypertension, is a major risk factor for various cardiovascular diseases and chronic heart failure. Although medication can help lower blood pressure, it often has side effects. Gene therapy using small interfering RNA (siRNA) has been proposed as a potential solution to regulate blood pressure by targeting proteins involved in blood pressure regulation, such as angiotensinogen. A study on rats aimed to determine the effect of intravenously administered siRNA-angiotensinogen on blood pressure. The siRNA was designed using sequence-angiotensinogen mRNA Rattus norvegicus obtained from NCBI and was given to normal and spontaneously hypertensive rats (SHR) in different doses. Blood pressure measurements were taken at various intervals after treatment, and angiotensinogen protein was isolated from blood plasma for analysis. The results showed that siRNA treatment decreased blood pressure in both normal rats and SHR, while the placebo group did not experience a decrease in blood pressure. The reduction in blood pressure in the SHR group ranged from 21-53% (SBP) and 17-58% (DBP) compared to the control group. Gene therapy using siRNA has the potential to provide long-lasting and highly specific control of blood pressure, and further research is needed to explore its effectiveness and safety in humans.
Keywords: Infrastructure Financing, Financing Schemes, Regional Waste

INTRODUCTION

The drug company today has now made a lot of progress in controlling hypertension. Potential drug that was developed to treat hypertension today has been designed to inhibit receptor ß, namely receptor ACE (angiotensin-converting enzyme), which converts angiotensin I to angiotensin II and angiotensin receptor II type 1 (AT1-R) (14) (Szalai, n.d.);(Urmila et al., 2021).

In practice, the principle of antisense gene therapy is not as easy as theory. Starting from the process of entering the antisense RNA molecule into biological system of an organism's cells (Nóbrega et al., 2020). This molecule has to deal with the presence of the enzyme nuclease everywhere, both in cells and in the blood circulation(Sayed et al., 2022). With the passage of time, and RNA antisense therapy developed into therapeutic siRNA (small interfering RNA) that implements the mechanism of RNA interference (RNAi) to try to treat several diseases like AIDS, malaria, dengue fever and cancer (Nardo et al., 2020). Not only is therapeutic, RNAi is also applied to the defense of plants from various kinds of viruses as well as an introduction to the study of genes and their functions (Genomics)(Kasi Viswanath et al., 2023).

The presence of hypertension were much struck population in developing countries, the lack of knowledge about the role of angiotensinogen in the pathogenesis of hypertension and lack of pharmacological drugs that can selectively inhibit the angiotensinogen encouraging this study. In addition, to know how big the effect of this gene therapy, in this case siRNA angiotensinogen injected intravenously with different doses on blood pressure of normal rats and spontaneously hypertensive rats (SHR) (Addison et al., 2023);(Lazartigues et al., 2023).

This research requires a draft of siRNA angiotensinogen sequence that will complement with the target mRNA sequence (in this case, angiotensinogen mRNA Rattus norvegicus), which has low homology with any mRNA sequence. The presence of low homology, allowing siRNA sequence will only be to complement with the target mRNA sequence (Hariharan et al., 2023).

RESEARCH METHODS

Preparation and Maintenance of Experimental Animals

Experimental animals used in this study were Wistar strain spontaneously hypertensive male rats (Rattus norvegicus) which is the first derivative of a cross between the spontaneously hypertensive female rats and normal male rats. Crosses are carried out in the animal enclosures which are located in the 4th Floor of the building SITH (School of Science and Technology) Bandung Institute of Technology (ITB)(Christianna et al., 2023). Hypertensive female rats which are used for the cross came from the National Bioresource Project for the Rat, Institute of Laboratory Animals, Graduate School of Medicine, Kyoto University. While normal male rats derived from SITH ITB(Somsura et al., 2023).

Division of Experimental Group

This study used 30 male rats, 3 tails for each treatment. The rats were divided into three main groups: control group (Group 1), the placebo group (Group 2) and treatment group (Group 3) (Table 1). The treatment group was subdivided into: the first treatment group (dose of 15 mg / kg bw), treatment II group (dose of 25 mg / kg bw) and treatment III group (dose of 35 mg / kg bw). The control group did not do any injection. As for the placebo group (normal and SHR) injection was performed by giving RNAse- free water as much as 10% vol. solution per kg bw, while the treatment group (normal and THS) injection was performed by giving a solution of siRNA dose of 15 mg, 25 mg and 35 mg siRNA per kg bw. The injection is done intravenously in rats as much as 1 time after the first blood sampling (before treatment).

SiRNA Design

In determining the siRNA sequence to be tested, it is first important to know the sequence of the mRNA target (in this case, angiotensinogen mRNA of Rattus norvegicus) so that the translation process of the mRNA can be inhibited(Tan et al., 2020). The sequence of the mRNA target as much as possible should be specific and unique and does not resemble any other mRNA sequences (low homology with any other mRNA sequence). Some sequences of cDNA (complementary DNA) of angiotensinogen of Rattus norvegicus that can be targets were obtained through the method of trial targets BLAST (Basic Local Alignment Search Tool) from NCBI (National Center for Biotechnology Information) and with the help of siRNA design program from Invitrogen (Block-iT RNAi Designer TM, www.rnaidesigner.invitrogen.com / rnaiexpress), it can produce multiple siRNA sequences that are considered capable of performing RNAi mechanism against Rattus norvegicus angiotensinogen. SiRNA sequences which were used have been selected since the

percentage of GC content was large enough (52.64%) so that it will have a higher specificity by assumption.

siRNA which was used in this study was 20 nmol dried pellets that were dissolved in 10 mL of RNase-free water (no.katalog: 10977-015) to produce a working solution with a concentration of 2 lm(Zimmermann et al., 2022). This working solution was used for injection of three doses of each rat. The dried pellets were obtained from Invitrogen in a condition that has been purified by means of desalted.

Measurement of Blood Pressure

Blood pressure measurement was performed at the same time as blood sampling (immediately before blood sampling) in each group. In this study used a non-invasive blood pressure telemetry (Bard Biomedical, Sentron, USA) which is a loan assistance of co-authors in the USA for measuring blood pressure (Lee et al., 2023).

Blood Sampling

The first blood sampling was performed before treatment in all groups of rats (h-0). After that blood sampling was performed at 168 h after injection of placebo or siRNA. Blood was drawn from the tail vein of rats. Before blood sampling, rats tail were rinsed with 70% alcohol and then sliced to obtain blood by using a scalpel. The blood was then collected in eppendorf tubes. Isolated blood is 250 mL using 1 mL syringe that is divided into 10 scales.

Isolation of Blood Plasma

Plasma that was separated from blood samples was placed in a small tube containing EDTA (1 mg / mL blood sample). Plasma was separated by means of centrifugation at 10,000 rpm for 10 min and incubated at -80º C until needed for further analysis.

Preparation of Polyacrylamide Gel

Polyacrylamide gel that was used consisted of a 10% separating gel and 4% stacking gel. Separating gel solution was poured onto a glass plate between the two printers that have been prepared. After separating gel was polymerized, stacking gel solution was poured on top of it. After the gel polymerized, the comb was inserted to make the wells in the gel. The method is performed by SDS PAGE Laemmli s ystem.

Qualitative analysis of SDS-PAGE

A total of 15 mL sample of plasma proteins and 15 mL Tris-Glycine sample buffer that have included in the eppendorf tube, heated in a water bath with a temperature of 100ºC for 5 minutes and then cooled. A total of 5 mL of the sample solution was put into the wells of 10% polyacrylamide gel while wells M were included with molecular mass markers (10-220 kDa, Bench MarkTM Ladder, Invitrogen) and electrophoresed (200 volts, the current is 40mA). After electrophoresis, was used SimplyBlue TM Safestain (Invitrogen) to detect the protein bands. Excess color on the gel was removed by soaking the gel in ultrapure water. The blue color will remain attached to the protein so that it will appear as a ribbon / blue lines. Furthermore, the gel was dried on Whatman 3 mm paper with gel dryers. Data analysis was performed by comparing the electrophoretic pattern of angiotensinogen-looking with regard sighting bands from each lane.

Data Analysis

The data was obtained and processed with Microsoft Excel 2007 and statistical analysis using SPSS 14.00 for Windows. The statistical test used was the General Linear Model-Repeated Measures (GLM-RM) with 95% confidence interval ($p < 0.05$)(Uswatun Sholikhah et al., 2020).

RESULTS AND DISCUSSION

Determination of siRNA sequence

SiRNA sequence used in this study was obtained under the provisions of that order can be paired with a specific sequence of Rattus norvegicus and angiotensinogen mRNA has low homology to any species of angiotensinogen mRNA sequences. In this study, the siRNA molecule used was 21 nucleotide base pairs with 2 hanging at the 3 'end (2-dTdT-3'hanging) with the sense sequence 5'-UCGUCUUCCAGCACGACUU dTdT-3' and anti-sense sequence 3'-dTdTAGCAGAAGGUCGUGCUGAA -5 .

The most efficient gene silencing siRNA molecule is composed of each of the 21 nucleotide sense and antisense pairs with frame 2 deoxynucleotide 3 'dangling(Boutary et al., 2023). The framework has resulted in a high specificity towards the target. 2-deoxynucleotides that has the same efficiency as ribonucleotide but is less expensive to synthesize and is more resistant to nuclease. TT hanging on siRNA was used as the framework so that it can help the formation siRNP (small interfering ribonucleoprotein particle) that will break the chains of sense and antisense targets in the same portion (5, 6). It also can increase the stability of siRNA molecules, increases resistance to RNase and increases the effectiveness of gene silencing by 10-fold. Modifications are not expected to hang on the sense to recognize a target for antisense mRNA that will serve to identify the target (Quemener & Galibert, 2022).

Side Effects And siRNA delivery Therapy

As with any other medication, there is always the possibility of side effects of this siRNA therapy. It can be derived from the lack of accuracy in predicting the proper target for RNAi. In addition, the use of therapeutic agents are less specific(Alshaer et al., 2021). Most drugs can hit more than one target. For both drugs, the secondary effects can be tolerated. Designing specific RNAi triggers is a challenge because it takes a short siRNA specific to the target. However, not all nucleotides designed to perfectly silence a target gene so that it can rise the effects off the target. However, with the proper design of nucleotides and through the experiment, siRNA therapy can exceed conventional medicines.

According to Barik (3), the majority of therapeutic molecules have the ability to achieve its goals by means of passively diffuse into or out of the cell through the cell membrane(Pei, 2022). Increasingly large and complex molecule (peptide, protein and oligonucleotide), the greater the obstacle in passing the tissue through paracellular route and cross the cell membrane (Figure 1). Until now, there has not been consensus by scientists on the system or the effective delivery of therapeutic siRNA. However, in theory there are several possible routes of delivery of siRNA include: passive diffusion, through the relationship between cells (paracellular), endocytosis and using the carrier protein.

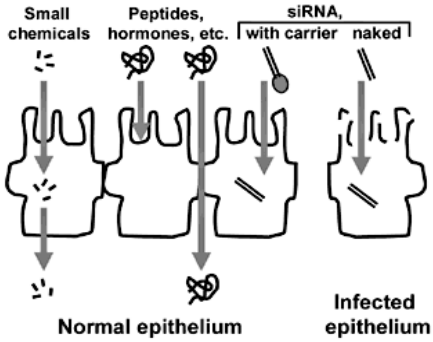


Figure 1. siRNA delivery Source :Barik, 2005

Rat Blood Pressure Before Treatment (hour-0)

Calculation of rat blood pressure before treatment was carried out on each group of experiments. It was intended to determine the blood pressure of each animal. Calculations were performed using the general linear model statistical analysis-Repeated Measures (GLM-RM). The results of the calculation of sistolic blood pressure (SBP) and diastolic blood pressure (DBP) male rats before treatment in a group of normal animals and hypertensive animals can be seen in Table 1.

Table 1. Comparison of SP and DP Before Treatment (Hour-0)

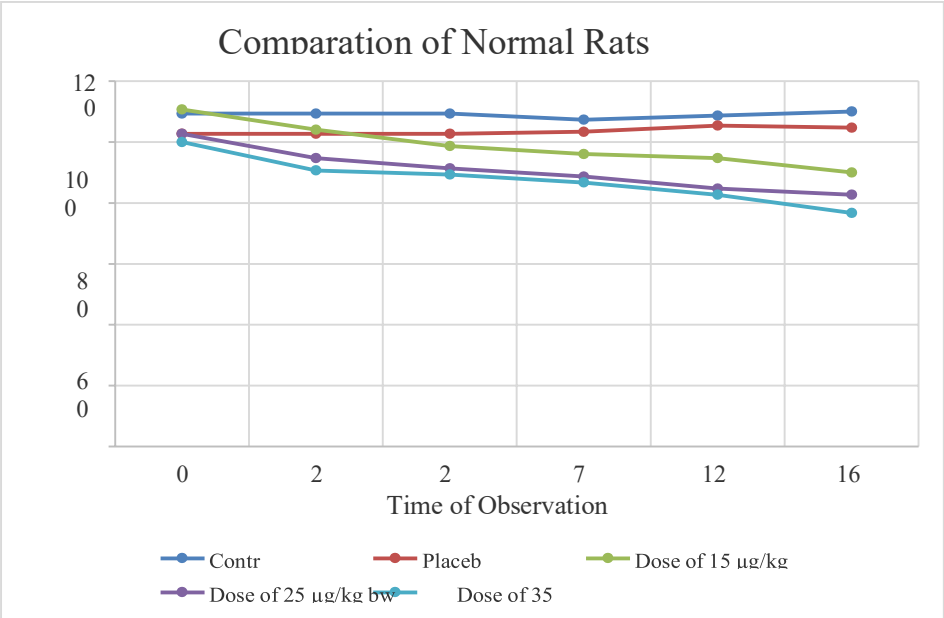
Group of rats	SBP ± Std.deviation	DBP ± Std.deviation	N (Number of Individuals)
Normal	105.07 ± 7.48	74.53 ± 7.11	15
Hypertension	205.2 ± 7.61	164.8 ± 6.27	15

Statistical Analysis of The effect of siRNA on Normal Rats Blood Pressure

Giving siRNA at a dose of 25 and 35 mg / kg bw has more effect on blood pressure reduction compared to rats by administration of siRNA dose of 15 mg / kg bw (Figures 2 and 3). While the rats that were given a placebo in the form of free water RNAse- and in the control group (untreated) showed no significant change (Figure 2 and 3).

In rats given siRNA dose of 15 mg / kg bw, there was a decrease of SBP from 110.67 mmHg to 90 mmHg until 168 hours of observation. While DBP decreased from 77.33 mm Hg to 57.33 mmHg until 168 hours of observation. While the rats given siRNA dose of 25 mg / kg bw, there was a decrease of SBP from 102.67 mmHg to 82.67 mmHg. There was a decrease of DBP from 74.67 mmHg to 54.67 mm Hg until 168 hours of observation. In rats given siRNA dose of 35 mg / kg bw, SBP decreased from 100 mmHg to 76.67 mmHg. While DBP decreased from 71.33 mm Hg to 48.00 mmHg until 168 hours of observation.

Based on the results of the GLM-RM significance test (test Pillai's Trace, Wilk's Lambda, Hotelling's Trace and Roy's Largest Root) SBP all groups of normal rats results in the Sig. <A (0,5) which means that there is an interaction between SBP and treatment (significant difference). Sig value (0.002) <α of the test Between Subject Effects (BSE) showed that there is significant treatment of the SBP group of normal rats. While the significance test at the DBP group of normal rats showed the Sig. (0.023) and (0.000) <α which means that there is an interaction between DBP and treatment (significant difference) but not significantly different at α> 1.69%. From the BSE test, the Sig. (0.009) <α, which means that there is a treatment effect on DBP of all groups of normal rats.



Fig**ure 2.** Comparison of Sistolic Blood Pressure (SBP) of Normal Control Rats With Normal rats treatedwith siRNA angiotensinogen dose of 15, 25 and 35 mg / kg bw and Observed Up for 168 Hours

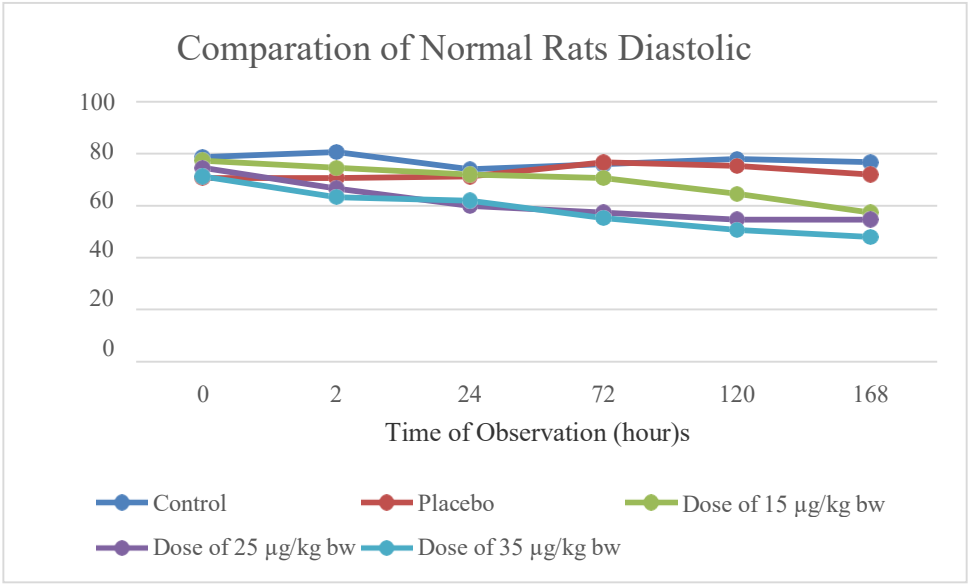


Figure 3. Comparison of DiastolicBlood Pressure (DBP) of Control Normal Rats With Normal Rats treatedwith siRNA angiotensinogen dose of 15, 25 and 35 mg / kg bw and Observed Up for 168 Hours

Statistical Analysis of The Effect of siRNA on Hypertensive Rat Blood Pressure

In Figure 4, it appears that in the hypertensive rats that were given a dose of 15 mg siRNA / kg bw, there was a decrease of SBP from 210.67 mmHg to 162.67 mm Hg until 168 hours of observation. The possibility of a decrease will occur after 168 hours, but from this study can not

be ascertained until when there is a decrease in blood pressure and also uncertain how long it takes until the blood pressure reaches a stable point. While DBP decreased from 166.67 to 134.00 mmHg. Similarly, DBP, will likely continue to decrease (Figure 5). There was a decrease in SBP of hypertensive rats that were given doses of siRNA 25 mg / kg bw which is from 194.67 mmHg to 104.67 mmHg until 168 hours of observation. In Figure 4 , SBP values approaching the normal range begins on the day of 120 hours of observation. While DBP decreased from 158.67 mmHg to 80.00 mmHg until 168 hours of observations that have been close to the values in the normal range also at 120 hours of observation (Figure 5).

In hypertensive rats that were given siRNA dose of 35 mg / kg bw, there was a decrease of SBP from 207.33 mmHg to 98.67 mm Hg until 168 hours of observation. Shown in Figure 4, the SBP approached the normal range at 120 hours of observation. Similarly, DBP decreased from 169.33 mmHg to 68.67 mmHg until 168 hours of observation. It is also an evident in Figure 4, DBP approached normal values in 120 hours of observation.

Based on the results of the GLM-RM significance test (test of Wilk's Lambda, Hotelling's Trace and Roy's Largest Root) all of SBP of hypertensive rats showed that the Sig. <A (0,5) which means that there is an interaction between SBP and treatment (significant difference). However, from Pillai's Trace test indicates that the difference of SBP between each group of hypertensive rats was not significantly different at α> 2.05%. Sig value (0.000) <α of the test Between Subject Effects (BSE) showed that there is a significant SBP difference of the hypertensive rats. While the significance test at the DBP of hypertensive rats showed that the Sig. (0,000) <α which means that there is an interaction between DBP and treatment (significant difference) but not significantly different at α> 2.66%. From the BSE test showed that the Sig. (0,000) <α, which means that there is a treatment effect on all groups of hypertensive rats based on the DBP.

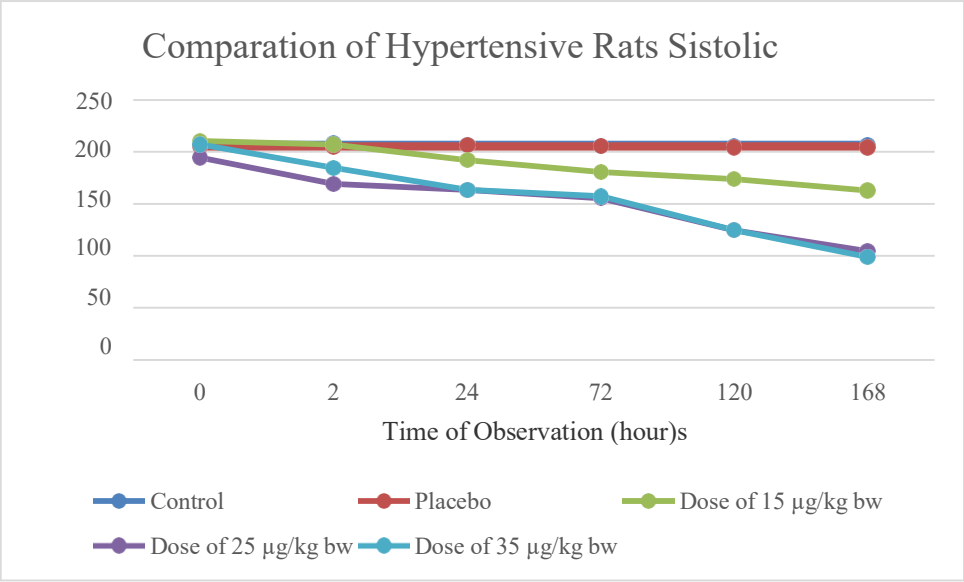


Figure 3. Comparison of DiastolicBlood Pressure (DBP) of Control Normal Rats With Normal Rats treatedwith siRNA angiotensinogen dose of 15, 25 and 35 mg / kg bw and Observed Up for 168 Hours

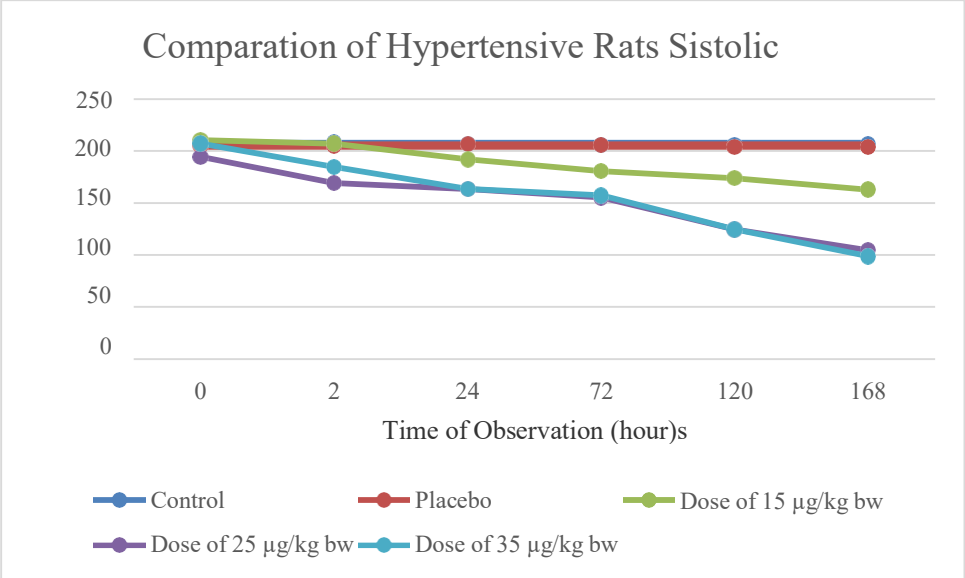


Figure 4. Comparison of Sistolic Blood Pressure (SBP) of Hypertensive

Effect of siRNA on Normal and Hypertensive Rat Blood Pressure

Based on the mechanism of action of siRNA treatment, the possibility of a reduction in blood pressure in normal and hypertensive rats (three doses) due to the success of the antisense siRNA induces angiotensinogen mRNA cuts the two types of rats. The more angiotensinogen mRNA clipped the less protein angiotensinogen which is produced to split into AGT I by renin. As you well know,AGT I is the precursor of AGT II which in turn will affect other factors of blood pressure regulation that will ultimately lead to dilation of the blood vessels, especially in the kidneys.

Provision of siRNA in rats with three different doses resulted in a decrease in SBP and DBP are quite drastic. The higher the dose given, the greater the effects of the decrease in SBP and DBP. According to Mulyana (9), the decrease of blood pressure depends on the concentration of drug in blood plasma, in this case the siRNA. The higher dose of siRNA was injected, the higher the concentration of siRNA in the blood plasma so that the more siRNA is distributed to the liver cells. Blood pressure decreased significantly due to the depression in the heart muscle that decreases cardiac output and eventually cause dilation of blood vessels. Dilation of blood vessels can lead to permanent possibility of severe hypotension (low blood pressure).

Giving siRNA in hypertensive rats with three different doses also resulted in a decrease in blood pressure of up to 168 hours of observation. However, in the hypertensive rats that were given siRNA dose of 25 mg / kg bw, SBP and DBP reached the normal range in 168 hours of observation. While at the lower dose (15 mg / kg bw), SBP and DBP are still too high. While at a higher dose (35 mg / kg bw), SBP and DBP reached a low value. As in normal rats, a decrease in blood pressure was produced very dramatically, so might cause an influence on the rat itself both physically and psychologically. The effect may include dizziness, nausea and difficulty breathing due to decreased cardiac output and stress. But it still needs to be confirmed with longer follow-up time again.

Qualitative analysis of angiotensinogen Ribbon With SDS-PAGE Method

According to the literature, the molecular weight of Rattus norvegicus angiotensinogen is KD 51.981 (1). Results of SDS-PAGE gel is shown in Figure 6. In the wells H1 (hypertension dose of 15 mg / kg bw), N2 (normal dose of 25 mg / kg bw) and the well NC (normal control), bands of angiotensinogen was too thin so that it was so hard to do observations. Protein buildup on the top of the gel may be caused by the aggregation of some proteins. In addition, there may be a protein molecule that has a size smaller than the pore size of the gel 10% or degradation resulting in pieces of protein molecules. The existence of the tape may also be caused by uneven electric field of electrophoresis. It showed that, the bands of thick angiotensinogen was detected in wells H3 (hypertension dose of 35 mg / kg bw), H2 (hypertension dose of 25 mg / kg bw) and wells CH (control hypertension). While bands that were thin angiotensinogen appeared on wells N3 (normal dose of 35 mg / kg bw) and the well N1 (normal dose of 15 mg / kg bw).

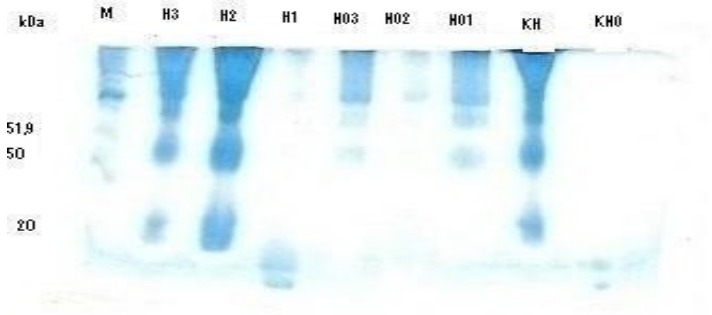


Figure 6. Result of SDS-PAGE electrophoresis of Blood Plasma Protein Samples of Each Group of Rats on 168 Hours Observation

CONCLUSION

From this study, it can be drawn some conclusions as follows: Intravenous injection with siRNA sequence 3'dTdTAGCAGAAGGUCGUGCUGAA-5 '(antisense) can lead to lower blood pressure in hypertensive rats and normal rats,intravenous injection of the antisense sequence produces translational inhibition of angiotensinogen which resulted in a decrease in SBP and DBP in hypertensive rats groups respectively: 21.29% (SBP) and 17.96% (DBP) (dose of 15 µg / kg bw), 49.35% (SBP) and 51.02% (DBP) (dose of 25 µg / kg bw) and 52.26% (SBP) and 57.96% (DBP) (dose of 35 µg / kg bw) and angiotensinogen has been proved to be one of the regulation factors of blood pressure.

From this research, the author suggests to do further research to determine: the time required to achieve stable normal blood pressure, the correlation between the concentration of angiotensinogen and blood pressure levels and side effects that may occur if the turn off hit another genes.

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