Hsp70 Expression Profile in Preeclampsia Model of Pregnant Rat (Rattus norvegicus) after Giving the EVOO

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Abstract. Heat shock protein (Hsp) has long been known to protect cells from oxidative stress. In this case an increased expression is found on several cases of preeclampsia. One of the efforts to prevent preeclampsia is by giving antioxidants such as Extra Virgin Olive Oil (EVOO) or it’s better known as olive oil (Olea europaea), in the form of extra virgin known for its rich antioxidant content of tocopherols (vitamin E). The purpose of this study is to determine the expression levels of Hsp70 serum on pregnant white rat model of preeclampsia after being given EVOO. This type of research is true experiment; the subjects were female white rats and male virgin with Sprague Dawley, ± 8-11 weeks old, 180g BB / d 200g, healthy and didn’t show any physical defects. Samples were 25 animals, divided into 5 groups, which consisted of different control and treatment given to T2 (rat model of preeclampsia), T3 (rat model of preeclampsia + EVOO 0.45g/bw/day), T4 (rat model of preeclampsia + EVOO 0.9g/bw/day) and T5 (rat model of preeclampsia + EVOO 1.8g/bw/day). The determination of each group was done by simple random sampling. Result on serum levels of Hsp70 that were tested by Elisa test in rats showed the average control was 14.64 mg / ml, group T2: 22.51 mg/ml, T3: 13.62 mg/ml, T4: 15.92 mg/ml, T5: 16.09 mg/ml. ANOVA test showed the P value was 0.001 <0.005, which meant there were significant differences on serum Hsp70 levels in the control and treatment pregnant rats group. It was known that there was a significant difference level of Hsp70 serum in group of control rats with T2 (P value <0.001) after LSD test was conducted, but not so with the group T3, T4, and T5, where the difference was not significant. There was a significant difference in the levels of Hsp70 serum on group T2 and T3 (P value 0.000), T4 (0004), T5 (0000). The gift of EVOO in the treatment group which was given EVOO with even low doses was able to control the induction of Hsp70 serum levels, which was not excessive, so the process of apoptosis did not occur excessively, especially in PE models. In this case, Hsp70 served as an anti-apoptotic and it’s is suggested to further research to observe the relationship of Hsp70 and apoptotic index.

1. Background
One of the biggest causes of maternal death in Indonesia is hypertension in pregnancy or preeclampsia, [1]. Nevertheless preeclampsia can be prevented by reducing risk factors. The cause of preeclampsia until now is still unclear, but according to the theory, it is known that one reason is because of the failure of spiral arteries remodeling which results in placental ischemia. Placental ischemia and hypoxia will produce oxidants (free radicals) that will experience ultimate oxidative stress, which in this case acts as as a mediator of dysfunction and cell death [2].
Heat shock protein (Hsp) has long been known to protect cells from oxidative stress. In this case an increased expression is found on several cases of preeclampsia, especially the decrease of Hsp70 in placental. Hsp 70 expression aims to repair damaged proteins so that cell deaths due to stress agents can minimized [2]. To determine the expression of Hsp70, some studies have been conducted using the Western Blot [2, 3] method or by enzyme-linked immunosorbent assay (ELISA) [4].

One of the efforts to prevent preeclampsia is by giving antioxidants such as Extra Virgin Olive Oil (EVOO) or it’s better known as olive oil (Olea europea), in the form of extra virgin known for its rich antioxidant content of tocopherols (vitamin E). Due to ethical issue and security of materials given to the mothers and fetuses, it requires a model animal that can be used for this PE research, which are female pregnant white rats (Rattus norvegicus). In order to get this model animal NaCl 6% and acute stress (based on preliminary research) were given. Next, model animals for this experiment were given EVOO as an antioxidant.

The purpose of this study is to determine the expression levels of Hsp70 serum preeclampsia pregnant white rat models, after being given EVOO. The benefits of this research are be able to provide initial information or reference of appropriate EVOO doses which are expected to be used directly to pregnant women with a high risk of preeclampsia.

2. Research Method
This type of research is true experiment conducted in the laboratory of molecular genetics, animal laboratory of pharmacology and therapeutics department of the Faculty of Medicine, University of Padjadjaran Bandung. Subjects were female white rats and male virgin with Sprague Dawley, ± 8-11 weeks old, 180g BB s / d 200g, healthy and didn’t show any physical disabilities.

From the big calculation of sample, total of at least 25 samples which consisted of 5 groups (each group consisting of 5 female rats pregnant) was obtained. The determination of each group was conducted by simple random sampling. The research variables were EVOO being given to female rats which were treated orally from day 13 to 19 during the period of pregnancy. The dose consisted of three criteria: 0.45g/bw/day in group T3, and 0.9g/bw/day in group T4, and 1.8g/bw/day in group T5. 6% of NaCl to modify the model of PE in pregnant rats was obtained from the pharmacy. The treatment was given to female rats by s.c or i.m. A dose of 3 ml/day was given starting from day 6 to 12 of pregnancy period, and Hsp70 serum was measured after treatment of Elisa method was conducted.

Rats would be executed on the 20th day (before giving birth), then blood would be taken immediately by intracardia. It would be inserted into the EDTA tubes, centrifuged for 15 min at 3000 rpm and then would be taken to measure the levels of Hsp70 serum by using the Elisa method. Previously, the 25 female white rats had been acclimatized beforehand for 7 days and fed with standard pellet and water in an ad libitum (plentiful) way. A pair of while female-male rats would be mated in a cage for 3 days. The mating of the female and male would be done by putting them into a cage by evening (5 pm). If copulation occurred, it could be detected by vaginal or copulatory plug formation on the next morning, then that was counted as the first day of pregnancy.

Pregnant female white rats were divided into five groups (each group consisted of 5 rats), which consisted of the control (C) and treatment (T) group. Pregnant white rat control group was not given any treatments until day 19. The treatment group consisted of four groups of pregnant female white rats would be given 6% of NaCl with a dose of 3ml/day in a subcutan way or intramuscularly from day 6 to day 12. On day 13 group T3, T4, T5 would be given EVOO with variable doses up to day 19, while the group T2 would not be given EVOO. On the 18th day, all treatment groups would be given acute stress in the form of placement in a tube with the same size of rats for 30 minutes. It would be performed just once. One of ways to determine the occurrence of preeclampsia is by measuring blood pressure after being given NaCl 6% on the 13th day and after the administration of acute stress by using a non-invasive sphygmonanometer to the base of the female rats’ tails, as well as on the 20th day prior to execution (Results of measurement can be seen in table 1).
3. Result and Discussion

Table 1. Result of measurement of blood pressure (mmHg) on female white rats of control and treatment groups during the pre-test and post-test

| No | Group | Pre test | After the administration of NaCl 6% (day 13) | After acute stress (day 18) | After giving EVOO (Day 20) |
|----|-------|----------|---------------------------------|----------------------------|--------------------------|
| 1  | C     | 120/95   | 120/95                          | 138/73                     | 104/53                   |
|    |       | 135/71   | 135/71                          | 132/79                     | 124/87                   |
|    |       | 96/85    | 112/59                          | 124/65                     | 123/80                   |
|    |       | 102/60   | 120/59                          | 104/55                     | 85/65                    |
|    |       | 114/89   | 114/89                          | 123/63                     | 75/55                    |
| 2  | T2    | 101/83   | 170/98                          | 137/95                     | 136/107                  |
|    |       | 99/57    | 135/71                          | 137/103                    | 136/103                  |
|    |       | 101/80   | 136/100                         | 167/100                    | 131/112                  |
|    |       | 121/59   | 140/90                          | 154/112                    | 135/100                  |
|    |       | 112/59   | 131/109                         | 148/90                     | 140/95                   |
| 3  | T3    | 69/53    | 148/79                          | 81/66                      | 81/66                    |
|    |       | 110/73   | 134/82                          | 95/59                      | 110/84                   |
|    |       | 114/67   | 135/102                         | 92/67                      | 126/93                   |
|    |       | 117/96   | 154/90                          | 90/70                      | 90/70                    |
|    |       | 99/86    | 132/100                         | 100/77                     | 100/65                   |
| 4  | T4    | 130/91   | 146/100                         | 132/88                     | 124/95                   |
|    |       | 126/93   | 172/102                         | 135/91                     | 99/57                    |
|    |       | 129/75   | 139/75                          | 117/96                     | 86/63                    |
|    |       | 87/55    | 140/95                          | 128/102                    | 124/95                   |
|    |       | 101/82   | 158/113                         | 101/82                     | 111/75                   |
| 5  | T5    | 116/80   | 167/94                          | 120/86                     | 120/76                   |
|    |       | 119/100  | 167/94                          | 135/111                    | 109/85                   |
|    |       | 108/84   | 132/75                          | 120/96                     | 106/70                   |
|    |       | 103/84   | 135/75                          | 118/88                     | 96/58                    |
|    |       | 164/141  | 170/89                          | 119/76                     | 98/61                    |

From table 1 above, it can be seen that there was an increase in blood pressure after the administration of NaCl 6%, 3ml/day during the week on the group T2, T3, T4, T5 of pregnant white rats, and an increase in blood pressure on the group P2, especially systolic pressure (> 120mmHg) was settled because they were not given EVOO, but not so in group T3, T4, and T5. Meanwhile other researchers found that the exposure to 6% NaCl for 3ml/day for four weeks was reported well to increase blood pressure [5]. Provision of 6% NaCl actually was able to increase the levels of free radicals in the body that were characterized by elevated levels of MDA. It would cause hypertension and damaged tissue that could produce ROS and lead to an increase in the thickening of endothelial and soft muscle [6]. According Callera (2006), which was quoted by Roseta (2014), who explained that the sources of ROS derived from mitochondrial respiration that was contained in the blood vessels, was caused by the presence of ET-1 (endothelin-1). Besides, the free radicals could cause damage across biological membranes by attacking proteins, lipids, nucleic acids, and glikonjugat, so that cells would undergo oxidative stress6. However, on group T3, T4, T5 that were given EVOO, it was found out that TD was mostly irreversible after the administration of EVOO. In this case, EVOO as an antioxidant was proven effective in reducing oxidative stress. It was due to its content of extra hydrophilic which was proven to be very potential in providing a direct antioxidant effect on damaged cells [7].
Table 2. Result of Anova test on levels of Hsp70 serum on control and treatment rats with preeclampsia model

| No | Group | Variants |
|----|-------|----------|
|    |       | K (mg/ml)| P2 (mg/ml)| P3 (mg/ml)| P4 (mg/ml)| P5 (mg/ml)| Mean (mg/ml) | Anova test |
| 1  |       | 17.55    | 27.94     | 14.09     | 16.63     | 15.36     | 14.64        | 0.153      |
| 2  |       | 7.21     | 21.48     | 15.65     | 14.04     | 11.58     | 22.51        | 0.001      |
| 3  |       | 18.30    | 25.60     | 15.65     | 17.90     | 15.02     | 13.62        | 0.153      |
| 4  |       | 15.80    | 18.77     | 7.96      | 15.60     | 13.27     | 15.92        | 0.001      |
| 5  |       | 14.33    | 18.75     | 14.76     | 15.44     | 13.64     | 16.09        | 0.001      |

From table 2 above, can be seen that the serum levels of Hsp70 were tested by Elisa test in control rats on average of 14.64 mg/ml, group T2 (pregnant rats model of preeclampsia without giving EVOO) 22.51 mg/ml, group T3 (pregnant rats model of preeclampsia and given EVOO 0.45g/BW/day) 13.62 mg/ml, group T4 (pregnant rats model of preeclampsia and given EVOO 0.9g/BW/day) 15.92 mg/ml, the T5 (pregnant rats model of preeclampsia and given EVOO 1.8g/BW/day) 16.09 mg/ml. Before proceeding to the ANOVA test, homogeneity of variance test was given and obtained P value 0.153 > 0.05, which meant that the data distribution of Hsp70 levels in each group of pregnant rats in control and treatment groups were homogeneous, so that it can proceed with one way Anova test. From the results of ANOVA test, obtained P value 0.001 < 0.005, which meant that there was a significant difference levels of Hsp70 serum in pregnant rats in control and treatment groups after being given EVOO. In this case, the levels of Hsp70 serum in rats of T2 was higher than the control and other treatment groups (T3, T4, T5, as these groups were given EVOO after the exposure to NaCl 6%) or in other words the provision of EVOO was able to control levels of Hsp70 serum on T3, T4, T5 groups which almost close to the control group. LSD test was then performed in order to get a dose variation of EVOO that would be most influential in changing the blood levels of Hsp70 in groups of rat’s model of preeclampsia. It can be observed in table 3 below.

Table 3. Levels of Hsp70 in pregnant female rats in control and treatment groups using LSD test

| No | GROUPS | P value |
|----|--------|---------|
|    | GROUP  |         |
| 1  | C      | 0.001   |
|    | T2     | 0.000   |
|    | T3     | 0.000   |
|    | T4     | 0.000   |
|    | T5     | 0.000   |
| 2  | T2     | 0.000   |
|    | C      | 0.004   |
|    | T3     | 0.000   |
|    | T4     | 0.000   |
|    | T5     | 0.000   |
| 3  | T3     | 0.004   |
|    | C      | 0.000   |
|    | T2     | 0.000   |
|    | T3     | 0.000   |
|    | T4     | 0.000   |
|    | T5     | 0.303   |
| 4  | T4     | 0.000   |
|    | C      | 0.303   |
|    | T2     | 0.000   |
|    | T3     | 0.941   |
|    | T5     | 0.675   |
From table 3 above, it can be seen that there were significant differences in levels of Hsp70 serum in the control group of rats which T2 P value < 0.001, but it was not the case with group T3, T4, T5, where there were not significant. This meant that the levels of Hsp70 serum in that group was approximately equal to the control group. Then the group of rats that were given doses of EVOO with different variations was able to control the balance of Hsp70 induction in the blood with PE models, so that the levels were not much different from the control group. Observed on the result of table 3 above, especially in the treatment group, it was known that there were significant differences in serum Hsp70 levels of group T2 and T3 (P value 0.000), T4 (0.004), T5 (0.000). This meant that the administration of EVOO with any doses was able to suppress the induction of Hsp70 in the blood. It did not increase as happened in T2. Based on the results of LSD test, it can be seen that the variation of different doses (starting at a low to high) didn’t show any significant differences, which meant that the administration of EVOO regularly in very low dose was able to prevent apoptosis in cells undergoing oxidative stress in preeclampsia.

In normal pregnancy, it is known that there is an increased production of free radicals and lipid peroxides in late pregnancy compared to women who are not pregnant. At the same time, there is an increase of antioxidant during pregnancy, aiming to maintain the balance of oxidants during pregnancy. However there is an increase production of ROS on pathologies pregnancy such as PE, in which, it’s thought to be one of the main causes of reperfusion failure in placental development. This is the cause of a placental debris accumulation in apoptosis pathway. In this case the induction of heat shock proteins (HSPs) with an increase of Hsp70 during stress response as the derivative, turns out to prevent cell damage caused induced stress by preventing denaturation of proteins and or repair the damage, in this case to protect cells from excessive apoptosis on PE as response to oxidative stress [2].

Through increased expression of Hsp70, it can block the process of cell death, or to the more extreme, it is able to support or reduce the growth of these cells. This is probably the cause of an increase in maternal complications and fetal, thus it requires the development of appropriate therapies, one of which is the provision of a wide range of antioxidants, such as vitamin E, β-carotene, ask orbit acid and glutathione along with decreased activation of free-tied iron that have been widely tested in the case of PE [8,9,10]. Vitamin E is able to affect the signal of cytokines on placental trophoblastic and also has effects on the maternal immune cells in early and late pregnancy [8], in this case are EVOO and the antioxidant content of tocopherols (vitamin E). So that, on researches that have been done, it shows that EVOO is able to maintain the levels of Hsp70 so it doesn’t increase. Other studies have also revealed that the increase in serum Hsp70 in PE seems to reflect the occurrence of systemic inflammation, oxidative stress, damage cells, and liver, and apparently this is not related to patients’ characteristics such as age, parity, BMI (body mass index), blood pressure, gestational age, birth weight, and some other laboratory parameters [4].

One of the one important components of olive oil is a tocopherol (vitamin E), consists of alpha tocopherol, beta, gamma, and delta. Alpha has the highest concentration, which is nearly 90 percent of total tocopherols. Therefore, this oil is ideal as an antioxidant. The color of pure olive oil is mostly contributed by chlorophyll, fœofitin, and carotenoids. Chlorophyll and fœofitin can protect against oxidative oil in an obscure condition, while carotenoids protects is from oxidative in bright conditions. These three pigments facilitate oil absorption in the body [11,12].

4. Conclusion
In the group of rats T2, the levels of Hsp70 serum is higher compared to other treatment groups (T3, T4, and T5) and control group. The gift of low dose of EVOO in the treatment groups could control the induction levels of Hsp70 serum, so it will not be excessive. Thus, the process of apoptosis does not occur in excess, especially in PE models. Hsp70 in this case works as anti-apoptotic. It is then suggested to further research to see the relationship of Hsp70 and the apoptotic index.
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