Original article:

The effect of isoflavones on diameter change of hypo estrogenic rat abdominal aorta measured by 12-MHz ultrasound

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Abstract

Background: The decrease of estrogen level causes atherosclerosis as well as the decline of vascular relaxation ability that, in turns, causes the narrowing of blood vessel's lumen. Phytoestrogens give positive effect during the atherosclerosis process and vascular motor tone. ***Objectives:*** to find out the effect of isoflavones on the diameter change of hypo estrogenic rats abdominal aortic measured by 12-MHz ultrasound. Materials and Methods: Thirty female rats were divided into 5 groups (n = 6 each) including 1 control group (sham), one ovariectomized (OVX) group, and three test groups. The test rats were OVX group received 0.5 mg/kg BW, 2.5 mg/kg BW and 5 mg/kg BW of isoflavones. Aortic diameter was analyzed in abdominal aorta using ultrasound with 12-MHz linear probes and on histologic specimens. ***Results:*** The decreasing of abdominal aortic diameter in all groups shows no significant difference ($p>0.05$). The measurement using ultrasound does not show any difference compared with that of histologic specimen ($p=0.418$), with the sensitivity value of 64.7%, specificity of 61.5% and accuracy of 63.3%. ***Discussion:*** There is no effect on the giving of isoflavones of several doses during 4-week-period on the diameter of hypo estrogenic rat's abdominal aorta. The measurement of rat's abdominal aortic diameter using 12-MHz ultrasound is compatible with the measurement on the histologic specimens.

Keywords: Abdominal aorta; Histology; Hypo estrogenic rat; Isoflavones; Ultrasound

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Introduction

The advancement of technology has brought a lot of changes in medical world, in which it includes many research that result in many new inventions. This is also in line with the development of molecular biology, the invention of chemical agents and imaging technology. As a consequence, many things can be done from in vitro observation to evaluation of diseases by means of in vivo on experimental animal.[1,2]

Ultrasound is widely used because it is transportable, non-invasive, easy to performed, cheaper than any other modalities, and does not give radiation effect.[1,3,4] Sound wave frequency is linear to image's resolution resulted but is non-linear with the penetration power or the thickness of pictures obtained. Ultrasound examination performed to human is using 1-18 MHz frequency, depending on what organ is going to be evaluated. However, the use of ultrasound on animals with smaller size requires higher frequency. The application of ultrasound examination on rat that has 10 to 30 mm body thickness requires 33 μm wavelength (with 45 MHz frequency) to 100 μm (with 15 MHz frequency). At present, there is a new technology specially developed for the needs of experiment with experimental animal that uses linear transducer with 30-50 MHz frequencies, which is called high frequency ultrasound.[2,5] Using this device, the spatial and temporal resolution can be increased so that the results will be

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more accurate and has high reliability.[2,6,7]

Ultrasound enables the evaluation on the blood vessels anatomy, the measurement of the blood vessel's diameter and thickness, as well as the blood flow effectively. The use of ultrasound to evaluate blood vessels of experimental animal in a research to estimate the abdominal aortic aneurysm regression was initiated by Wang, *et al*.[5] Sampson, *et al.* state that the measurement of in vivo rat's abdominal aortic diameter using high frequency ultrasound shows that the result is not different from that using the in vitro measurement.[8] Another study conducted by Radu *et al*. compared the measurement of ascending aorta using US with 9 MHZ probe with that of video microscopy, and the result shows no difference as well.[9]

Blood vessels have walls consisting of three concentric layers, which are tunica intima, tunica media, and tunica adventitia. Tunica intima is the innermost layer, which consists of endothelial cells with sub endothelial tissue, which is separated from tunica media by dense elastic fiber called internal elastic lamina. Tunica media consists of smooth muscle cells with external elastic lamina on its outer part. The endothelial, intima and adventia have many receptors, one of which is estrogen receptor.[10–12]

Estrogen works in smooth muscle cells as well as blood vessel's endothelial cell, which has receptor for estrogen. Estrogen can cause vasodilatation if given to human and by s experimental animal stimulating prostacyclin, synthetic nitric oxide and reducing vasoconstrictor substances such as cyclooxygenase derivate, reactive oxygen species, angiotensin II and endothelin-1. In the in vivo processing, estrogen (17-β estradiol) is proven of being able to prevent the thickening of neointima after balloon catheter trauma and improving lesion on atherosclerosis condition; however, the molecular mechanism is yet to be known. Besides, estrogen can also decrease low-density lipid (LDL) accumulation, oxidation, the formation of foam cell and fatty streak on the walls, form expression of various proinflamation molecules that increases monocyte adhesion/migration and activation/migration of smooth muscle cells.[10,13]

Menopause women experience the decrease of follicles that causes the change on endocrine. The most significant effects are the decrease of estradiol level along with the increase of follicular stimulating hormone (FSH) and luteinizing hormone (LH). Those two effects result in the occurrence of atherosclerosis and the decrease of vascular relaxation ability that causes the narrowing of blood vessel's lumen. Previous studies concluded that ovariectomy on rats can cause the increase in radical oxygen species (ROS), that can actually be suppressed by giving dehydroepiandrosterone.[14]

Currently, the use of phytoestrogen, which induces biological responses such as endogen estrogen, undergoes rapid development as menopause alternative cure. [13,15] Villa, et al (2009) conclude that 6-month-phytoestrogen-therapy on menopause female can improve glucose metabolism and endothelial function, decrease the aorta's wall thickness, improve the blood pressure and increase endothelial nitric oxide synthase (eNOS) gene transcription which induces nitric oxide (NO) formation which can be seen as the result of experimentation on rats so that it will give positive effect during the process of atherosclerosis and vascular motor tone. Cowpea (*Vigna unguiculata L.)* is one of legume types that contain a lot of nutrition including flavonoids that are categorized as phytoestrogen such as isoflavones.[16]

Based on the above-mentioned facts, the researchers are interested to find out the 12-MHz ultrasound accuracy for rat abdominal aortic diameter evaluation and to see the effects of isoflavones towards rat's abdominal aorta in hypo estrogenic condition.

Materials and Methods

This study has been approved by ethical commission of Faculty of Medicine, Brawijaya University, Malang, Indonesia no. 352/EC/KEPK-PPDS/06/2014 dated 5 June 2014. The study employs true experimental design, and pre-post test control group design, which was conducted in Physiology laboratory Faculty of Medicine, Brawijaya University Malang and Radiology Department Saiful Anwar General Hospital, Malang. The study was performed from Juli to September 2014, using GE ultrasound, Logiq S6, 12 MHz linear probes.

30 female *Rattus Novergicus Wistar* strain were employed as the subjects with inclusive criteria of: 3 months of age, 150-200 grams in weight, physically healthy which was shown by their being physically active. Exclusive criteria of the subjects of experimentation were: the weight loss of more than 20% of their initial body weight or died when the experimentation was in progress.

Subjects were adapted and divided into negative control group (without ovariectomy/OVX-), positive control group (with ovariectomy/OVX+) and the treatment group (ovariectomy and being given isoflavones/OVXT). Ovariectomy was performed on the 8th day, while ultrasound was conducted on the 35th day and was repeated on the 65th day (after being given isoflavone therapy of the cowpea extract), which was

followed by termination and frozen section procedure. The sections were painted with hematoxylin eosin coloring, and were observed under Olympus microphoto microscope with 400x magnification.

The cowpea extract was made using maceration method, and was given in different doses (0.5, 2.5, and 5 mg/kg BW isoflavones level) through gastric tube.

Ultrasound was performed under anesthesia using the combination of xylazine/ketamine injected intramuscular, at the same time abdominal aortic diameter was measured vertically through the length of its hub in three different locations: proximal, medial and distal. (Figure 1)

Results

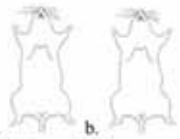

Figure 1.**Measurement of abdominal aortic diameter using ultrasound**
a.Transverse, b. Longitudinal

The measurement of diameter on histologic sample was performed using OlyVIA program by using two intersecting lines on 0o, 90o, 270o, 360o, and 45o, 135o, 225o, and 315o. (Figure 2) The diameter is the mean of those four measurements.17

Figure 2. Measurement of internal diameter on histologic specimen of blood vessel using Scion Image software with 40x (18)

Measurement of Abdominal Aortic Diameter

This study employed female rats from *Rattus Novergicus Wistar* strain*, which* already experienced sexual maturation. Ovariectomy was done to positive control group and treatment groups in order to get hypo estrogenic condition, which was similar to menopause. No rats were being excluded during the study.

In the ultrasound examination-using probe of 12 MHz frequencies, aortic lumen and its blood flow spectrum could be well visualized. However, the tunica media and tunica intima could not be distinguished. (Figures 3 and 4) Aortic lumen diameter was vertically measured towards its long hub in three different locations: proximal, medial and distal. Cursor was located in the surface of aortic walls. (Figure 5a)

Figure 3. Ultrasound procedure
(a) Rat under anesthesia was fixed in supine position. Aorta was then identified in two sections: transverse (b) below xyphoid process and longitudinal (c).

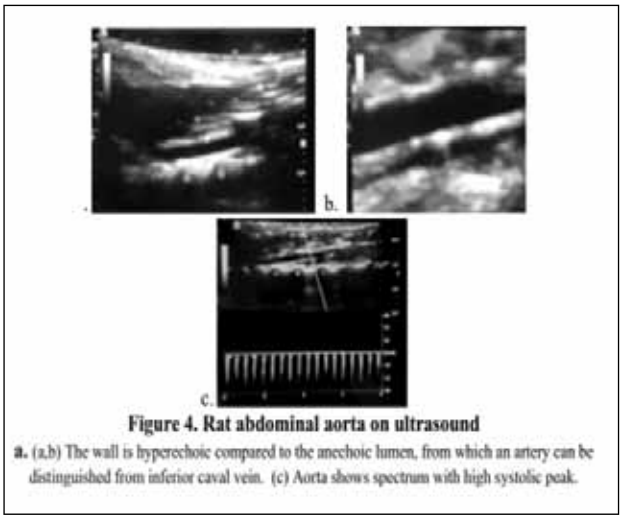

Figure 4. Rat abdominal aorta on ultrasound
a. (a,b) The wall is hyperechoic compared to the anechoic lumen, from which an artery can be distinguished from inferior caval vein. (c) Aorta shows spectrum with high systolic peak.

Histologic specimen was made from proximal aortic segment, and the diameter measurement was only conducted in the sample with whole wall and which formed full circle. (Figure 5b)

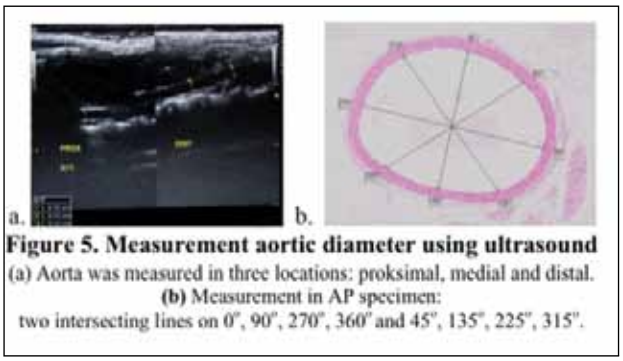

Figure 5. Measurement aortic diameter using ultrasound
(a) Aorta was measured in three locations: proksimal, medial and distal.
(b) Measurement in AP specimen:
two intersecting lines on 0°, 90°, 270°, 360° and 45°, 135°, 225°, 315°.

The result of measurement using ultrasound (before and after given the cowpea extract) and histologic specimen showed similarity, where the lowest average was found in positive control group and the highest on negative control group (Table 1).

Table 1. The mean of abdominal aortic diameter measurement using ultrasound

Group	Mean ± SD		
	First ultrasound (mm)	Second ultrasound (mm)	histologic specimen (mm)
OVXT 0.5	1.3 ± 0.12	1.2 ± 0. 10	1.2 ± 0.51
OVXT 2.5	1.3 ± 0.19	1.2 ± 0.10	1.3 ± 0.98
OVXT 5	1.2 ± 0.19	1.2 ± 0.23	1.2 ± 0.13
OVX+	1.2 ± 0.19	1.1 ± 0.11	1.2 ± 0.89
OVX-	1.4 ± 0.19	1.3 ± 0.23	1.2 ± 0.98

Compatibility of the results of rats' abdominal aorta diameter measurement using 12-MHz ultrasound compared with histology examination

Examinations toward aortic diameter on the histologic specimen and second ultrasound after giving isoflavones showed that *p*-value > 0.05 which means there is no significant difference between both of them. In order to define the accuracy of US examination, ROC curve with cut-off point used is the mean scores of histology examination result as the gold standards, that is 1.3 mm. It can be calculated that diagnostic ultrasound value in the measurement of the diameter of abdominal aorta in comparison with histology examination is as follows: sensitivity: 11/17x100% = 64.7%, specificity: 8/13 x100% = 61.5%, accuracy: (11+8)/30x100% = 63.3%.

The effects of giving isoflavones on the diameter change of abdominal aorta

In order to find out the different result of the aortic diameter measurement between groups in ultrasound examination before and after giving isoflavones, *One Way Anova* was used. Based on table 2, value p>0.05 showed up in all measurements, which means they do not have significant difference. The changes on abdominal aortic diameter on each group can be seen in line diagram, which average final diameter is smaller than initial diameter in all groups with almost similar difference, which is 0.1 mm. Using *pair t-test* analysis, it shows that the changes are not different significantly (p>0.05) to five groups. (Table 3)

Table 2. OneWay Anova Test

Variable	p-value
D1	0.267
D2	0.124
D1-D2	0.838

D1: initial diameter, D2: final diameter

Table 3. Pair t-test

Group	Mean ± SD	*p-value*
OVXT 0.5	0.0889 ± 0.1148	0.116
OVXT 2.5	0.0667 ± 0.2044	0.461
OVXT 5	0.0111 ± 0.2157	0.904
OVX+	0.1333 ± 0.1535	0.087
OVX-	0.0444 ± 0.0834	0.249

In order to know the effect of isoflavones, the first thing to be observed is whether the data fulfill the terms of linearity. The graph shows that there is a linearity with determinant coefficient $R^2 = 0.201$. Then, Pearson correlation test results in p=0.275, which means there is no effect of isoflavones on abdominal aorta diameter.

Discussion

This research evaluated rat abdominal aorta using ultrasound with 12 MHz frequency linear probes. In the examination, aortic lumen could be visualized well to all subjects. However, tunica intima and tunica media could not be distinguished, which becomes the weakness of this examination if it is compared with high frequency ultrasound. It happens because of the different axial resolution, which is the ability to distinguish two different structures, which are located close to the parallel position towards the direction of sound propagation. The influencing factors are pulse length and frequency; if the pulse length is bigger than the distance between the two structures, the two structures could not be distinguished. High frequency ultrasound has shorter pulse length that it may increase image resolution.[2,5,6]

The image produced is good enough since the probe with ±10 MHz frequency has the ability of 3-4 cm network penetration, suitable with body size of rats. However, the thickness of tunica intima and media ±60 µm needs the frequency of 40 – 60 MHz, which has the ability of network penetration for about 1-2 cm with the resolution up to 50 µm. The

flow of abdominal aorta can be displayed quite well by using color Doppler technique, likewise the flow spectrum that is taken from desired specific area (sample volume). It may happen because the targeted area can be identified well in the description of two dimensions.[19,20]

This research resulted in the measurement result of abdominal aortic diameter using 12 MHz US is suitable with the measurement on histologic specimen with level of confidence 95%. It is in line with the research conducted by Radu, *et al*. to 111 male rats to compare the measurement result using 9 MHz US with video microscopy as gold standards examination. It can be concluded that there is compatibility between both examinations with the bias only 0.23 mm and level of confidence 95%.[9]

Ultrasound imaging is an effective examination to assess blood flow and measure the diameter and thickness of blood vessel walls. Ultrasound with 7 MHz frequencies has sensitivity 91-94% and specificity 85-99% in measurement to detect stenosis in human blood vessel. This research used 12 MHz probe to measure rat abdominal aortic diameter, resulted in sensitivity value of 64.7%, specificity of 61.5%, and accuracy of 63.3%. The values obtained could not approach 100% since the device used belongs to low frequency, whereas for obtaining optimum result, it needs high frequency with the range between 30-50 MHz as generally used in experiments in developed countries.

In the result of measurement, there is a deviation in the mean score as much as 0.1 up to 0.2 in all subject groups. It happens because of variation of measurement location even though all were conducted in proximal segment. Other factors that may influence are breathing movements, aortic pulsation, and operator dependence. To avoid the last factor, Sampson *et al*. used one sonographer to measure abdominal aortic diameter. Yet, variation still exists both between subjects and the same subjects when the re-measurement was conducted. This measurement also showed that the biggest variation happened in suprarenal and infrarenal segments, which were caused by remodeling as the result of aneurysm and atherosclerosis process.[8,21]

Variation also happened in the measurement result by using ultrasound compared with measurement to histologic specimen. Some factors causing this are measurement location may not be exactly the same, since it is impossible to give marker to state measurement location. On the other hand, the elasticity of blood vessel walls is lost in postmortem condition and the separation of blood vessel from connective tissue surround it when the aorta lifting causes minor changes to the specimen when filling and embedding were conducted. Previous studies have shown the measurement of thickness of tunica intima in ultrasound was bigger than that in histology preparations. Besides, the tissue damaged during dissection and contraction in extraction also influence ex vivo measurement result. However, in this current research, choosing only intact tissue for the measurement [1,16] minimized those. Failure may happen in measurement using ultrasound to the longitudinal section since the shape of lumen is quite eccentric. It may be avoided by measuring the axial section, which all contour of lumen can be displayed. However, it is hard to make sure that the section we take is perpendicular to the long axis from the blood vessel.[21]

By the end of the research, it was obtained that there is no difference in the initial measurement result of abdominal aortic diameter before giving isoflavones to five subject groups, which means that all samples are homogeneous. In the second measurement after giving isoflavones, constriction of diameter to all groups happened that it might happen as the result of aging process so that it influenced endothelial, subendothelial layer, and lamina elastic internal. In rats, there was a change in which endothelial cells became irregular with phagocyte cell invasion and sub endothelial became thicker and the disintegration of lamina elastic internal. As a result, there was migration of smooth muscle cells from tunica media to sub endothelial layer and plague formation.[22–24]

Patel states that aorta has slightly different characteristics with other blood vessels, in which it is more vulnerable to vasodilatation effect than occlusion as a result of aging. The increase of blood pressure and atherosclerosis may accelerate this process and cause aneurysm. In atherosclerosis, the damage of tunica media happened which results in wall depletion and degradation of matrix protein extracellular, which function to keep the integrity and the mechanic strength of aortic wall. This explains why in atherosclerosis continuing process, it is possible that it does not appear to be lumen narrowing. The previous studies concluded that internal diameter of descending aorta, the thickness of tunica intima and cross-sectional area of lumen decreased after given estrogen preparations to female rats, which had been OVX.[11,12,25]

Decreasing levels of estrogen triggers a series of processes which causes atherosclerosis and the

decrease of the ability of vascular relaxation which results in narrowing blood vessel's lumen. Estrogen functions to decrease LDL accumulation, oxidation, and foam cell formation as well as fatty streak to the wall, expression of many proinflammatory molecules which increase adhesion/migration of monocytes and activation/migration of smooth muscle cells and also reduce the production of vasoconstrictor.[10–12] However, in this research, the effect of giving phytoestrogen which has similar effect with estrogen still does not show up, perhaps it needs longer time. As the previous studies that used ovariectomized hamster showed that there was no difference in aortic diameter to the group given flaxsheet diet for 4 months or not, and did not find any plague to all subjects. The changing of aortic diameter and the effect of dietary administration with some concentration to treatment group just showed in evaluation, which was conducted 4 months later.

It concluded that the result of diameter measurement of rat abdominal aorta using 12-MHz ultrasound is compatible with the measurement result in histologic specimen as gold standards. There is no effect in giving isoflavones in few certain dosages for four weeks.

Conflict of Interest:

The authors declare that there is no conflict of interests regarding the publication of this paper.

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