

## COMBINATION OF TEMPEH AND CARROT PREVENT ATHEROSCLEROSIS WISTAR RAT: Indicated by Increase of HDL and Total Antioxidant, Decrease LDL, F<sub>2</sub>-Isoprostan, and IL-6

<sup>1</sup>Ari-Agung, I G. A., <sup>2</sup>Suryadhi, N. T., <sup>3</sup>Mantik, N. A., <sup>4</sup>Suter, I. K., <sup>5</sup>Partama, I. B. G.

<sup>1</sup>Public Health Departement, Post Graduate School, Udayana University, Bali-Indonesia

<sup>2</sup>Public Health Departement, Faculty of Medicine, Udayana University, Bali-Indonesia

<sup>3</sup>Veterinary Medicine Departement, Faculty of Veterinary Medicine, Udayana University, Bali-Indonesia

<sup>4</sup>Food Tecnology Departement, Faculty of Agricultural Technology, Udayana University, Bali-Indonesia

<sup>5</sup>Animal Nutrition Departement, Faculty of Animal Husbandry, Udayana University, Bali-Indonesia

### ABSTRACT

Coronary heart disease (CHD) is the leading cause of death, either in developed and developing countries. The disease is stimulated by the present of atherosclerosis. This study aims to investigate supplementation of combined *tempeh M-2* and carrot to prevent atherosclerosis wistar rat by increases HDL and TAC, decreases LDL, F<sub>2</sub>-Isoprostan, and IL-6. This was a true experimental study with the factorial completely randomized post-test only control group design. variables such as KN (standard feeding / pellets (50 g / kg bw / day), KP: pig lubrication: pellets (1: 9) (50 g / kg bw / day), T : lubrication pig: pellets (1: 9) with *tempeh M-2* (20 g / kg bw / day), W: pig lubrication: pellets (1: 9) with carrots (20 g / kg bw / day), and TW: pig lubrication: pellets (1: 9) with *tempeh M-2* (20 g / kg bw / day), and carrots (20 g / kg bw / day). Dependent variables in this study are serum HDL, serum TAC, LDL serum, urine F<sub>2</sub>-Isoprostan, and plasma IL-6 (with Elisa Method). Data were analyzed using the F test (two-way ANOVA), followed by LSD test. Descriptive research was also conducted in this study in order to find out the change of aortic histopathologic. The highest average levels of HDL, TAC contained on TW, which respectively amounted 68.640 ± 0.50 mg / dl, 1.454 ± 0.01 nM / mL. It showed highly significant differences (p<0.01) in the various treatments. TW treatment showed highly significant interaction effect (p<0.01) were observed for all parameters except for HDL. Average levels of LDL, F<sub>2</sub>- Isoprostan, and IL-6 lowest in the treatment TW, which respectively amounted 20.718 ± 1.33 mg / dl, 0.720 ± 0.065 ng / dl, 35.328 ± 1.000 pg/dl, showed highly significant differences (p<0.01) in the various treatments. It can be concluded that supplementation with a combination of *tempeh M-2* with carrots give the best effect, can increases HDL and TAC, and can decreases LDL, F<sub>2</sub>-Isoprostan, IL-6 significantly, and may change the hystopathology structure of aorta from endotel dysfunction to become normal.

**Keywords :** Atherosclerosis, TAC, F<sub>2</sub>-Isoprostan, IL-6 and *Tempeh M-2*.

### INTRODUCTION

Coronary heart disease (CHD) occurs as a consequence of atherosclerosis process in coronaria arteri. Among other risk factors, dyslipidemia is the major cause of atherosclerosis.<sup>1</sup> Lipid metabolism disorder stimulates atherosclerosis demonstrated by decrease of HDL and increase of LDL. Chemical change of lipid induced by free radicals generated in macrophages or endothelial cells in arterial wall oxidized LDL. LDL may create inflammation, which will lead to atherosclerosis. Research during two last

decade showed that there is chronic inflammation in aorta wall due to fat accumulation, which resulted in plaque destruction.<sup>2,3</sup> However the controversy lately is associated with dyslipidemia as a major risk factor for atherosclerosis. The data show that as many as 50% of patients do not have complications of atherosclerosis and dyslipidemia risk factors as much as 35-40% of all cases of CHD patients turned out to have normal cholesterol levels. Thus the need to study a lot of nonconventional biomarkers found closely related to the atherosclerosis.<sup>4,5</sup> F<sub>2</sub>-Isoprostan is the end product of lipid peroxidation.<sup>6</sup> In a state of oxidative stress increases levels of F<sub>2</sub>-Isoprostan.<sup>7</sup> F<sub>2</sub>-Isoprostan is an early predictor of atherosclerosis, and the determination of the best ox-LDL.<sup>8</sup>

**Correspondence:** I G. A. Ari-Agung  
Public Health Departement, Post Graduate School,  
Udayana University, Bali-Indonesia  
Email: [ari\\_agung@yahoo.com](mailto:ari_agung@yahoo.com)

Oxidized LDL induces the production of Interleukin-6 (IL-6), which may lead to the occurrence of atherosclerosis. Many researchers reported that there is a correlation between high lipid serum levels to incidence of atherosclerosis. Atherosclerosis is a slow progressive disease present in large to small muscular.<sup>2</sup> Increase of ion superoxide in dyslipidemia induces the production of IL-6. It has been reported in several studies that IL-6 can be used as a marker to detect early inflammation process.<sup>9</sup>

Measurement of Total Antioxidant Capacity (TAC) is an accurate measurement of oxidative stress. Oxidative stress is a problem of the role of free radicals in the biology systems of the disease, becomes very important because almost 77% related to reactive oxygen species and reactive nitrogen species. Estimated activity plays an important role in the protection cycles in diseases such as atherosclerosis, coronary heart disease, and cancer.<sup>10</sup>

Tempeh *M-2* is a local soybean tempeh with lactic acid addition in the boiling process, which significantly increase capability and contain bioactive (amino acid, isoflavon, niacin, estrogen, omega-3, lecithin, superoxide dismutase and vitamin E) compounds in the form of antioxidant that has a function as an agent of scavenger to free radicals caused oxidative stress.<sup>11,12</sup> Some researchers reported that isoflavones increased the activity of superoxide dismutase (SOD).<sup>12,13</sup> Isoflavones of Tempeh *M-2* have ability to catch free radical. Many researchers reported that the catalase enzyme activity can be induced by the intake of antioxidants (beta carotene and isoflavones).<sup>12</sup>

According to Harlinawati (2006) niacin can increase HDL cholesterol content increase significantly in patients with coronary heart disease, and individuals with low HDL cholesterol.<sup>14</sup> IL-6 can be inhibited indirectly through regulation of endogenous cholesterol synthesis.<sup>15</sup> Unsaturated fatty acids Omega-3 (linolenic acid) in tempeh can reduce the secretion of proinflammatory cytokines (Interleukin-6) and decrease the inflammation.<sup>16</sup> In addition, tempeh also contains other active substances that act as anti-inflammatory, such as phytoestrogens and niacin.<sup>12,17</sup>

Carrots contains very high level of beta carotene antioxidant, at 66 µg/g carrots, has the best antioxidant activity, beta carotene and vitamin E which can decrease atherosclerosis.<sup>18</sup> Diets low in carotenoids, but sufficient in all other nutrients produced signs of reduced blood TAC. Beta-carotene has the best antioxidant activity, contribute to the prevention and cure of cardiovascular disease.<sup>16,19</sup> Lots of research suggests beta carotene may prevent atherosclerosis.<sup>20</sup> Beta carotene as an antioxidant is very good, because the ability to reduce beta-carotene peroxy radical singlet oxygen.<sup>21</sup>

The combination of tempeh *M-2* with carrots is a harmonious combination of food (*Food Combining*

diet) which one another synergize in increasing the activity of bioactive substances they contain, especially as much a source of powerful antioxidants, which play an important role as antiatherogenic.<sup>22</sup> In general, this study aims to determine the combination of tempeh *M-2* with carrots supplementation can increase HDL and Total Antioxidant Capacity (TAC), and decrease LDL, F<sub>2</sub>-Isoprostan, IL-6, and aortic tissue damages in atherosclerosis process of Wistar .

## **METHODS**

### **Research Design**

This study is a true experimental research with the randomized post-test only control group design and factorial completely randomized design. In general, this study aims to determine whether supplementation of combined tempeh *M-2* with carrots increases HDL and Total Antioxidant, decreases LDL, F<sub>2</sub>-Isoprostan, IL-6 and aortic tissue damages in atherosclerosis process of Wistar rats.

### **Place and time of the study**

This research was conducted at the Experimental Animal Care Unit (UPHP) Pharmaceutical Laboratory, Faculty of Medicine, Udayana University, for the treatment of the animals, and then proceeded with the analysis of HDL, LDL, TAC, F<sub>2</sub>-Isoprostan, IL-6 and test Histopathologic of aorta in the Laboratory of Pathology of the Faculty of Veterinary Medicine, Udayana University. The study was conducted from December 2011 to October 2012.

### **Population and sample**

Target population in experimental research is all white male Wistar rats in the process of atherosclerosis. Affordable population is white male Wistar rats covering the atherosclerotic process, weighing 250 g to 300 g, aged 7 months to 7.5 months. Inclusion criteria for the sample was white male Wistar rats aged 7 months to 7.5 months, weighing 250-300 g, healthy, eating and drinking normally. Exclusion criteria were sampled white male Wistar rats with ill conditions. Criteria dropped out during the study sample was white male Wistar rats die. The number of samples used in this study were 25 Wistar rats.<sup>23</sup>

### **Research variables**

Independent variables such as KN (standard feeding/pellets (50 g / kg bw / day), KP: pig lubrication: pellets (1 : 9), T: lubrication pig: pellets (1: 9) with tempeh *M-2* (20 g / kg bw / day), W: pig lubrication: pellets (1: 9) with carrots (20 g / kg bw / day), and TW: pig lubrication: pellets (1: 9) with tempeh *M-2* (20 g / kg bw / day), and carrots (20 g / kg bw / day), and dependent variables in this study are HDL, TAC, LDL, F<sub>2</sub>-Isoprostan, IL-6 (with Elisa Method). Comparability

of test used is the F test (two-way ANOVA), Followed by multiple test Comparison Independent t-test (LSD test).

Research diagram was drawn to easily conduct the research as can be seen on Figure 1.

### Procedure

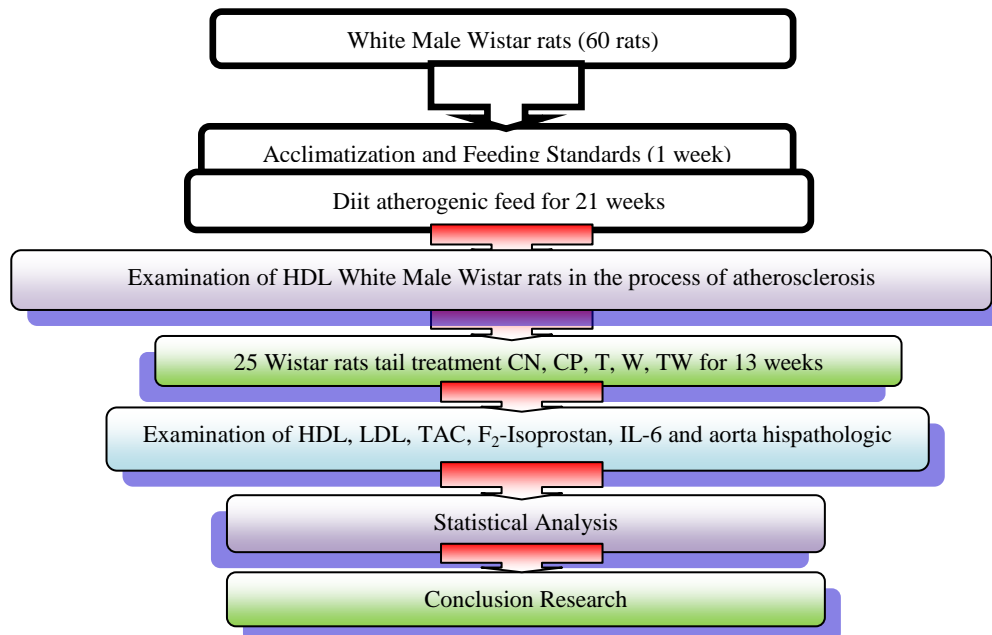


Figure 1. Research Flow Chart

CN (Control Negative/standard feeding), CP (Control Positive/atherogenic feeding), T (Atherogenic feeding and tempeh *M-2*), W (Atherogenic feeding and carrot), TW (Atherogenic feeding, tempeh *M-2* and carrot)

### RESULTS

#### Increase of HDL and TAC levels

The highest level of HDL and TAC occurred in the treatment of atherogenic feeding and combination treatment of tempeh *M-2* with carrots (TW) by amount of  $68.46 \pm 0.50$  mg/dL, and  $1.454 \pm 0.01$  nM / mL, respectively. There were highly significant ( $p < 0.01$ ) increase HDL and TAC in the combination of tempeh *M-2* with carrots, treatment showed highly significant interaction effect ( $p < 0.01$ ) except for HDL. Tempeh *M-2* give effect increase higher HDL and TAC, respectively for 27.48% and 59.56% compared to the effect of the carrots treatment.

#### Decrease of LDL, F<sub>2</sub>-Isoprostan and IL-6

The lowest levels of LDL, F<sub>2</sub>-Isoprostan and IL-6 occurred in the treatment of atherogenic feeding and combination of tempeh *M-2* with carrot (TW) by

amount of  $20.718 \pm 1.33$  mg / dL ,  $0.720 \pm 0.065$  ng / dL, and  $35.328 \pm 1.000$  pg / dL, respectively and showed highly significant differences ( $p < 0.01$ ) in the various treatments, the combination of tempeh *M-2* with carrots can decrease highly significant ( $p < 0.05$ ) levels of LDL, F<sub>2</sub>-Isoprostan and IL-6. TW treatment showed highly significant interaction effect ( $p < 0.01$ ). The influence of tempeh *M-2* is lower than carrot of F<sub>2</sub>-Isoprostan and IL-6 occurred, respectively for 30.28%, and 28.05%. The influence of carrots is lower than tempeh *M-2* of LDL occurred for 18.84%. The combination of tempeh *M-2* with carrots showed highly significant interaction effect ( $p < 0.05$ ) in all biomarkers were observed, except in HDL level, which can be seen on Table 1.

Tests showed aorta histopathologic, TW treatment can be as antiatherogenic best, can be seen in Figure 2.

Table 1  
 Data of HDL, TAC, LDL, F<sub>2</sub>-Isoprostan and IL-6  
 At Atherosclerosis Process in Wistar Rats

Treat ment	HDL (mg/dL)	TAC (nM/mL)	LDL (mg/dL)	F <sub>2</sub> -Isoprostan (ng/mL)	IL-6 (pg/mL)
KN	37,08 ± 0,48d	0,56 ± 0,02d	33,92 ± 0,92b	3,03 ± 0,07b	168,85 ± 11,29b
KP	28,68 ± 3,32e	0,44 ± 0,03e	39,01 ± 2,43a	5,26 ± 0,07a	222,67 ± 10,56a
T	49,65 ± 2,87b	0,87 ± 0,04b	24,15 ± 0,90d	0,96 ± 0,53d	39,76 ± 1,64d
W	44,31 ± 2,67c	0,65 ± 0,02c	28,05 ± 1,52c	2,05 ± 1,56c	49,67 ± 1,44c
TW	68,46 ± 0,50a	1,45 ± 0,01a	20,72 ± 1,33e	0,72 ± 0,07e	35,33 ± 1,00e
TXW	ns	s	s	s	s

CN=standar feeding/pellets (50 gr/kgbw/day); CP=pig lubrication:pellets(1:9) (50 gr/kgbw/day); T=pig lubrication:pellets (1:9) with tempeh *M-2* (20 g/kgbw/day); W=pig lubrication:pellets (1:9) with carrots (20 g/kgbw/day); TW=pig lubrication:pellets(1:9) with tempeh *M-2* (20 g/kgbw/day) and carrots (20 g/kgbw/day). ns : non significant, s : significant

Notes: Mean value followed by different letter indicates highly significant different (p<0,01), LSD for posttest.

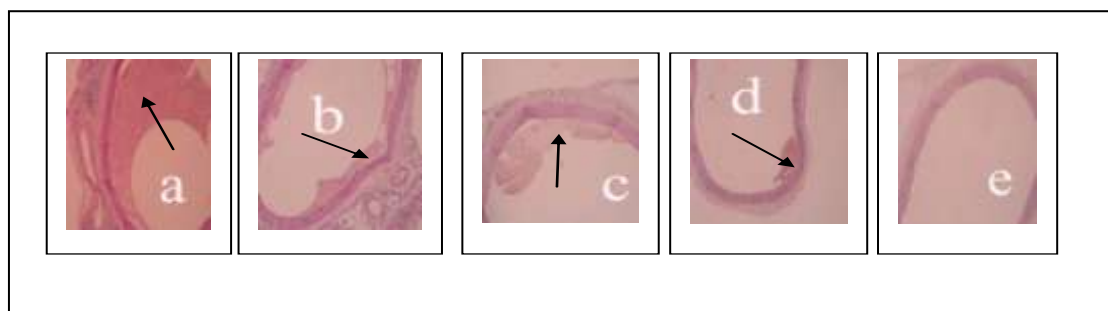


Figure 2

Profile of Aorta of the Experimental rat

a. In the treatment of atherogenic feeding /Positive Control / CP; b. In treatment of standard feeding/ Negative Control/CN; c. In the treatment of atherogenic feeding with carrot/W; d. In the treatment of atherogenic feeding with tempeh *M-2*/T; e. In the treatment of atherogenic feeding with combination of tempeh *M-2* with carrots/TW

Figure 2a shows aorta in treatment of atherogenic feeding / Positive Control (CP), which mostly contain patches of fatty (fatty streak) (indicated by the arrow), then the reduced content of fatty patches (fatty streak) the treatment standard feed / negative control (CN) (Figure 2b), the treatment of atherogenic with carrots (W) (Figure 2c), the treatment of atherogenic with tempeh *M-2* (T) (Figure 2d), respectively. The treatment of atherogenic treatment with a combination of tempeh *M-2* with carrots (TW) has not looked fatty patches (Figure 2e).

In the treatment of atherogenic feed / positive control / CP (Figure 2a) and the treatment of the standard feeding / negative control / CN (Figure 2b) almost the entire endothelial dysfunction (endothelial expressed). In the treatment of atherogenic and carrots (W) are still visible endothelial dysfunction (Figure 2c). In the treatment of atherogenic feeding with tempeh *M-2* (T) are still visible endothelial

dysfunction (Figure 2d). On treatment with a combination of tempe *M-2* with carrots is not visible presence of endothelial dysfunction (Figure 2e).

## DISCUSSIONS

### Increase of HDL and TAC levels

Treatment atherogenic feeding in this study raises three atherosclerosis risk factors that increased lipid metabolism, oxidative stress, and inflammation, as shown in Table 1, and Figure 2. In this research, it was obtained that supplementation of combined tempeh *M-2* with carrot increase HDL and TAC levels in atherosclerosis process of Wistar rats, Table 1 reveals that the highest increase of 68.640 ± 0.50 mg / dL, and 1.454 ± 0.01 nM / mL, respectively, and showed highly significant increase (p<0.01) (Table 1). Tempe *M-2* to give effect increases rat serum HDL 27,48% higher than the influence of the treatment carrots. This is in accordance with the opinion of

Harlinawati (2006) that niacin (vitamin B3) in tempe *M-2* can significantly increase the content of HDL cholesterol in patients with coronary heart disease, and individuals with low HDL cholesterol levels.<sup>14</sup> It is also emphasized by Mindell (2008) that the amino acid composition of tempeh increased HDL cholesterol levels by improving blood glucagons (the hormone released by the pancreas), can alter the performance of the HMG-CoA reductase (Coenzym A), which is very important in the synthesis of HDL cholesterol.<sup>17,28</sup> Effects thyroid research on animals has shown that tempeh can increase blood plasma levels of thyroxine, a hormone produced by the thyroid gland, increasing HDL cholesterol.<sup>17</sup> Alrasyid (2007) mentions that the molecular analysis of tempeh isoflavone genistein apparently shows a similar structure with 17  $\beta$ -estradiol. Supporting the mechanism of action of this substance in the plasma lipid profile improvement., Genistein (17  $\beta$ -estradiol exogenous) indirectly affects lipolysis by lipolytic stimulate hormone-sensitive lipase enzyme or by increasing the lipolytic effect of epinephrine.<sup>29,30</sup> Substances suspected to have the properties of tempeh normalize blood lipids is protein, PUFA fatty acids, fiber, niacin, vitamin E, carotenoids, isoflavones and calcium.<sup>31</sup>

Tempe *M-2* increases the effect of rat serum TAC 59,56% higher than the influence of the carrots treatment. This is because the tempeh *M-2* is an excellent source of antioxidants, such as protein, vitamin E, omega-3, vitamin B2, zinc (Zn), copper (Cu), selenium (Se), ferrum (Fe), isoflavones, SOD.<sup>16,17</sup> Soybean isoflavones may increase the activity of enzymes SOD, catalase and glutathione peroxidase. These findings are in line with the research of Catherine (2009) and Mary (2011) which states that isoflavone supplementation would increase TAC. This could happen because of the possibility of activation of Nrf2 (Nuclear factor erythroid related factor 2) in the nucleus resulting in increased gene expressing involved in the synthesis of endogenous antioxidant enzymes.<sup>27</sup> This is in accordance with the opinion of Hastuti (2005) that HDL cholesterol also increases antioxidant activity.<sup>1</sup> Confirmed also by Widowati (2007) that elevated levels of HDL will increase TAC.<sup>25</sup> This could happen due to the increased possibility of HDL activates Nrf2 (Nuclear factor erythroid related factor 2) in the nucleus resulting in increased gene expressing genes involved in the synthesis of endogenous antioxidant enzymes, there by increasing the levels of TAC.<sup>27</sup>

The combination of tempeh *M-2* with carrots is a harmonious combination of excellent food, each other synergize in increasing the activity of bioactive substances it contains, which is acting as anti-atherogenic (Farida and Amalia, 2009). Average serum TAC levels are highest in the combination of tempeh *M-2* with carrots is  $1.454 \pm 0.01$  nM / mL. The result of

the analysis of the diversity of the levels of serum TAC Wistar rats showed significant differences ( $p < 0.05$ ) in the various treatments, the combination of tempeh *M-2* with carrots showed a significant interaction effect ( $p < 0.05$ ), and the highest level of serum TAC. This is in accordance with the opinion of Sunita (2009) availability of beta-carotene increased in the availability of vitamin E and other antioxidants in tempeh *M-2*.<sup>16</sup> Various studies have found that tocopherol ( $\alpha$  or  $\gamma$ ) is contained in the tempeh *M-2* protects beta carotene from autooxidation.<sup>35</sup>

Beta carotene and isoflavones has important role in inducing the body's antioxidant status.<sup>36</sup> Isoflavones tempeh *M-2* and beta-carotene may increase the activity of catalase. Isoflavones and selenium affect the activity of the enzyme glutathione peroxidase.<sup>12</sup> Diet low in beta carotene, but enough in all other nutrients produced signs reduced total antioxidant capacity of blood.<sup>37</sup>

#### **Decrease of LDL, F<sub>2</sub>-Isoprostan and IL-6**

The lowest levels of LDL, F<sub>2</sub>-Isoprostan and IL-6 occurred in the treatment of atherogenic feeding and combination of tempeh *M-2* with carrot (TW), and showed highly significant differences ( $p < 0.01$ ) in the various treatments, this is because the results of this study showed the significantly highest level of HDL occurred in the treatment of atherogenic feeding and combination treatment of tempeh *M-2* with carrots (TW). HDL acts as a vacuum cleaner as possible excess LDL,<sup>38</sup> until can be decreased oxidation of LDL in the blood and tissues.<sup>31</sup> F<sub>2</sub>-Isoprostan is the end product of lipid peroxidation.<sup>6</sup> Oxidized LDL so induces the production of Interleukin-6 (IL-6). Pectin contained in carrots has play an important role to reduce levels, of LDL cholesterol, and increase HDL cholesterol levels making it useful for preventing atherosclerosis.<sup>19</sup>

LDL is a similar product prostaglandin formed in vivo from non-enzymatic peroxidation arachidonic acid by free radicals.<sup>7</sup> Much of arachidonic acid as a phospholipid layer on the outer membrane of LDL precursor compound formation of F<sub>2</sub>-Isoprostan. F<sub>2</sub>-Isoprostan is a product of lipid peroxidation.<sup>6</sup> F<sub>2</sub>-Isoprostan is a similar product prostaglandin formed in vivo from non-enzymatic peroxidation of arachidonic acid by free radicals.<sup>7</sup> F<sub>2</sub>-Isoprostan is the best biomarker for oxidative stress status and lipid peroxidation in vivo, with high sensitivity and specificity,<sup>32</sup> describes the process of calcification in the coronary arteries.<sup>33</sup>

The combination of tempeh *M-2* with carrots showed a highly significant interaction effect ( $p < 0.01$ ) in all biomarkers were observed, except for HDL. This happens because the combination of tempeh *M-2* with carrots is a combination of food (*Food Combining* diet) is very harmonious with one another synergize in increasing the activity of bioactive substances they

contain, especially as much a source of powerful antioxidants, which play an important role as antiatherogenic,<sup>21</sup> can be shown from showed histopathologic aorta tests TW treatment can be as antiatherogenic best.

Availability of beta-carotene carrots increased in the presence of vitamin E tempeh (Sunita, 2009).<sup>16</sup> Various studies have found that tocopherol ( $\alpha$  or  $\gamma$ ) protects beta-carotene from autooxidation.<sup>39</sup> Beta carotene to strengthen the potential of  $\alpha$ -tocopherol as a peroxy radical scavenger. Murray *et al.* (2009) confirmed that beta-carotene is an antioxidant that has a significant role in capturing peroxy free radicals in tissues at low oxygen partial pressure, because it is effective at low oxygen concentrations, beta carotene supplement antioxidant properties of vitamin E is effective at higher oxygen concentrations.<sup>6</sup>

### CONCLUSIONS

It can be concluded that supplementation with a combination of tempeh M-2 with carrots give the best effect, can increase HDL and Total Antioxidant Capacity, and can decrease LDL, F<sub>2</sub>-Isoprostan, IL-6 highly significant, and may improve aortic tissue. TW treatment showed highly significant interaction effect ( $p < 0.01$ ) except for HDL. In the tempeh M-2 treatment influence higher than carrots on HDL, TAC, F<sub>2</sub>-Isoprostan and IL-6 observation, except the observation LDL.

### FUTURE WORK

We found that supplementation with a combination of tempeh M-2 with carrot is necessary to functional food is antiatherosclerosis. Further research regarding of non-invasive biomarkers such as Lp-PLA<sub>2</sub>, which specifically may reflect early atherosclerotic process.

### ACKNOWLEDGMENT

The author would like to thank Prof.drh N.Mantik Astawa, Ph.D for aids and access to the Laboratory of Pathology of the Faculty of Veterinary Medicine, Udayana University, during analysis of the observed parameters. Thanks was also great to drh. Ida Bagus Oka Winaya, M.Si for aids and access during histopatologic aorta testing at Laboratory of Pathology of the Faculty of Veterinary Medicine, Udayana University.

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