# Vasa Vasorum Angiogenesis through Increased Levels of $H_2O_2$, HIF-1α, NF-κB and INOS: In Vivo Study of Atherosclerosis

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***Abstract*—This study aims to determine the increased of vasa vasorum angiogenesis through $H_2O_2$, HIF-1α, NFkB and iNOS pathways in Wistar strain of *Rattus novergicus* given HFD. This research is a true experimental laboratory, with in vivo approach to post-test with control group, using 24 males wistar strain of *Rattus novergicus*, 8 weeks of age, were divided into 6 groups. Those animal model was given HFD serially for 1,3 and 5 months. Each series contained a control group with normal diet (ND). The parameter in this study are $H_2O_2$ measured by colorymetric; NFkB, iNOS and HIF-1α were measured using double staining imunofluoresence which was observed with CLSM (Confocal Lasser Scaning Microscope) in aortic smooth muscle cells; vasavasorum angiogenesis were measured from VEGFR1 levels in aortic tissue, and confirmed by HE staining for anatomical and histopathological verification. The results of ANOVA analysis, path analysis and Pearson correlations showed that HFD affect the vasa vasorum angiogenesis through $H_2O_2$, NFkB and iNOS in wistar strain of *Rattus novergicus* ($p < 0.05$). The data show that NFkB plays greater role in regulating inflammation and affect vasa vasorum angiogenesis, starting at 3 months of HFD administration. It is known from the role of iNOS on levels of VEGFR–1 that is greater than the role of HIF-1α. The conclusion of this study is the vasa vasorum angiogenesis is more dominantly influenced by inflammatory conditions.**

***Index Terms*—vasa vasorum angiogenesis, $H_2O_2$, HIF-1α, NF-kB, iNOS, atherosclerosis, hypercholesterol diet.**

## I. Introduction

Atherosclerosis is a disorder of the blood vessel wall which marked with the blockage caused by atheroma, so that blood flow becomes impaired [1]. For decades, deaths from coronary heart disease (CHD) caused by atherosclerosis in both industrialized and developing countries increased sharply [2]. In Indonesia, the morbidity and mortality rate from CHD is likely to increase and the prevalence reached 50% [3].

The risk factors of atherosclerosis are dyslipidemia, free radicals, endothelial dysfunction and inflammation [4]. Increased levels of cholesterol in the blood, especially low-density lipoprotein (LDL) produce Reactive Oxygen Species (ROS) such as OH-, RO and $H_2O_2$ that may be harmful. ROS activate NF-κB, a transcription factor that plays an important role in the chronic inflammatory process of atherosclerosis [5], [6].

Activation of NF-κB is a process when translocation of NF-Kb subunit from the cytoplasm into the nucleus happen and stimulates the expression of proinflammatory genes such as cytokines (TNF-α and interleukins), adhesion molecules (ICAM-1 and VCAM-1) and chemokines. NF-κB activation products initiate the process of atherosclerosis and endothelial dysfunction characterized by the increased of iNOS, platelet adhesion, migration and proliferation of smooth muscle cells [7]. Signs of inflammation occurred from the beginning until the development of atherosclerosis [8]. INOS expression is increased in macrophages and smooth muscle cells in the early and advanced stages of atherosclerotic lesions. Inflammation showed an important role in the development of atherosclerotic lesions affecting the coronary arteries. iNOS plays a role in inflammation through fast and massive prostanoid and nitric oxide (NO) production, both of that have proatherosclerotic effect [9].

Recent studies attempt to link the involvement of (HIF-1α) in the process of atherosclerosis. HIF-1α is a transcription factor that regulates glycolysis, angiogenesis and cell survival [10]. HIF-1α in normal oxygen conditions (unstable state) can't be detected due to hydroxylation. In hypoxic conditions, HIF-1α will be stable and undergo translocation from cytoplasm to nucleus and binds to specific HRE (hypoxia response element) promoter sites that regulate classics genes of hypoxia-responsive [11].

Manuscript received June 25, 2014; revised October 25, 2014.


doi: 10.12720/jomb.4.5.342-349

The possible role of HIF-1α in atherosclerosis is supported by the presence of angiogenesis inside the plaque as an implication of HIF-responsive genes in atherosclerosis such as VEGF [12], endothelin-1 [13] and matrix-metalloproteinase-2 [14]. VEGF binds to tyrosine kinase receptors. VEGFR-1 is also known as Flt (fms-like tyrosine kinase-1). VEGFR-1 is expressed in early vascular development and post-natal angiogenesis. VEGFR-1 is also expressed on inflammatory cells such as monocytes and macrophage, which coordinates inflammation and is an early marker of pathological angiogenesis associated with atherosclerosis [10]. Research conducted by Sluimer in 2008 suggested that hypoxia is strongly associated with angiogenesis and thrombus formation. Atherosclerosis shows the extensive, fragile, and immature process of angiogenesis vasa vasorum, which are associated with atherosclerotic plaque growth and instability [15], [16].

This research aims to clarify the relationship of NF-κB and HIF-1α in inducing vasa vasorum angiogenesis in wistar strain of *Rattus novergicus* treated with hypercholesterol diet which hasn't been done before. The clarity of pathophysiological mechanisms of atherosclerosis are needed to determine the effective targeted therapy.

## II. Methods

### A. Study Group

24 male Wistar strain of *Rattus norvegicus* rats, 8 weeks old, with 150–200 g body weight, were obtained from the Pharmacology Laboratory of Faculty of Medicine, Brawijaya University, Malang, Indonesia. These rats were divided into 3 different hypercholesterol diet groups based on the duration of the treatment given (1, 3 and 5 months of treatment), and 3 different normal diet groups based on the duration of the treatment given (1,3 and 5 months of treatment).. Hypercholesterol diet in this study was a common food for the rat models with addition of 2% cholesterol, 0.2% cholic acid, and 5% lard, which was given at 30 g daily ad libitum for 3 months, obtained from the Pharmacology Laboratory of Faculty of Medicine, Brawijaya University. The measurement of parameters of this study was conducted at the Biomedical Laboratory and Central Laboratory of Biological Sciences, Brawijaya University after obtaining ethical clearance assessment by the Health Research Ethics Committee with this given number: 054 A/EC/KEPK/02/2013.

### B. Biochemical Tests

#### 1) $H_2O_2$ measurement

$H_2O_2$ levels were measured in rat plasma using a Colorimetric Hydrogen Peroxide Kit (Assay Design) (Abcam, 1 Kendall Square, Suite B2304, Cambridge, MA, 02139-1517, USA) and observed at 570 nm with an enzyme-linked immunosorbent assay (ELISA) (Life Sciences Advanced Technologies, Inc., 2900 72nd Street North, St. Petersburg, FL 33710 USA) reader.

#### 2) HIF-1α, NF-κB, and iNOS measurement

HIF-1α, NF-κB, and iNOS were measured by immunofluorescence of aortic tissues that were previously fixed with PHEMO (68 mM PIPES, 25 mM, HEPES, pH 6.9, 15 mM EGTA, 3 mM $MgCl_2$, 10% [v/v] dimethyl sulfoxide (DMSO) containing 3.7% formaldehyde and 0.05% glutaraldehyde) buffer and were processed by imumunofluoresence double labeling with anti-rat antibody HIF-1α using rhodamine secondary antibody and anti-rat antibody NF-κB using fluorescein isothiocyanate (FITC) secondary antibody (BIOS Inc. Boston, Massachusetts, USA). iNOS in smooth muscle cell derived from anti-rat iNOS antibody was colored by fluorescein isothiocyanate (FITC) and α-actin was colored by rhodamine secondary antibody (BIOS Inc. Boston, Massachusetts, USA). These three parameters were observed with confocal laser scanning microscopy and were quantitatively analyzed using Olympus FluoView software (version 1.7A) (Olympus Corporation, Tokyo, Japan).

#### 3) Angiogenesis vasa vasorum measurement

Vasa vasorum angiogenesis measurement was done by measuring levels of aortic VEGFR-1 by ELISA (Abcam, Cambridge, MA, US). Histopathological description of vasa vasorum was observed by hematoxylin and eosin staining and microscope BX 53 (Olympus Corporation, Tokyo, Japan) at 600× magnification. The amount of vasa vasorum was identified from the characteristic of aortic lumen which contains erythrocyte.

### C. Statistical Analysis

This study used analysis of variance (ANOVA) test to determine the effect of MPEE on the reduction of VEGFR-1, H2O2, HIF-1α, NF-κB, and iNOS in Wistar strain *R. norvegicus* rats with hypercholesterol administration. Analysis was continued with post hoc test with Duncan method to detect the differences of parameters among treatment groups. SPSS software (version 20; IBM Corporation, Armonk, NY, USA) was used for data analysis.

## III. Result

The number of vasa vasorum in the varying durations as you can see in Table I, ranging from 0-36. Among the ND administration groups, the number of vasa vasorum (0-7) are lower than HCD groups (0-36). The longer duration of treatment will increase the number of vasa vasorum, especially in the HCD groups. Hematoxylin Eosin (HE) staining results of vasa vasorum, were observed using a BX 53 microscope with 600x magnification, can be seen in the Fig. 1 below.

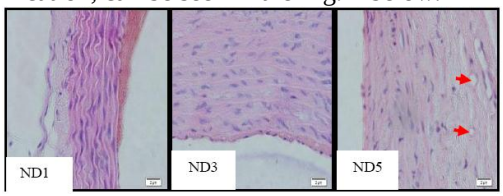


Figure 1. HE staining in various duration of ND administration showed vasa vasorum.(ND1: group which was given normal diet for 1 month, ND3: group which was given normal diet for 3 month, ND5: group which was given normal diet for 5 month, a red arrow indicates the vasa vasorum)

In the Fig. 1, the red arrows show the vasa vasorum. The vasa vasorum in ND1 and ND3 groups are almost invisible, but in the ND5 group few of vasa vasorum could be seen. HE staining of vasa vasorum in the HCD groups can be seen in Fig. 2.

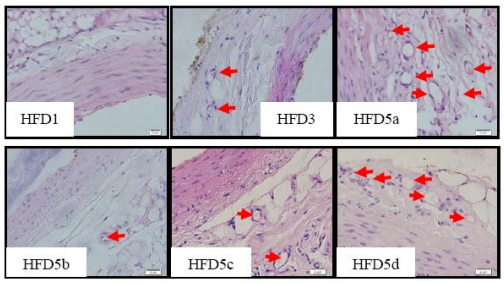


Figure 2. HE staining result showing vasa vasorum in each HCD group. (HFD 1: Group which was given hypercholesterol diet for 1 month, HFD 3: Group which was given hypercholesterol diet for 3 month, HFD 5a,b,c: Group which was given hypercholesterol diet for 5 month, the red arrow indicates the vasa vasorum).

Vasa vasorum was pointed by red arrows. Vasa vasorum still couldn't be found in HCD1 group. Vasa vasorum began to show in HCD3 group and its amount increased in HCD5 group.

Levels of VEGFR-1 on various diets duration as you can see in Table I, ranged from 9.108 to 19.989 pg/ml. Group with ND administration had lower levels of VEGFR-1 ranged from 9.108 to 16.613 pg/ml, whereas in the group with administration of HCD ranged from 10.750 to 19.989 pg / ml. The longer duration of treatment will increase the formation of VEGFR-1, both in ND and HCD group.

$H_2O_2$ levels on various diets duration as you can see in Table I, ranged from 0.131 to 2.003 pg/ml. In the groups with administration of ND had lower levels of $H_2O_2$, ranged from 0.131 to 0.197 pg / ml, whereas in HCD groups its level ranged from 0.599 to 2.003 pg/ml. The longer duration of treatment will increase the formation of $H_2O_2$, especially in the groups with administration of HCD.

TABLE I. THE AVERAGE LEVEL AND STANDARD DEVIATION OF VEGFR-1, VASA VASORUM, $H_20_2$, HIF-1A, NFKB, AND INOS IN EACH GROUP.

| PARAMETERS | GROUPS OF TREATMENT | | | | | | | | | | | | p-Value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | ND, 1 month | | ND, 3 months | | ND, 5 months | | HFD, 1 month | | HFD, 3 months | | HFD, 5 months | | |
| | $\bar{x} \pm SD$ | Min-Max | $\bar{x} \pm SD$ | Min-Max | $\bar{x} \pm SD$ | Min-Max | $\bar{x} \pm SD$ | Min-Max | $\bar{x} \pm SD$ | Min-Max | $\bar{x} \pm SD$ | Min-Max | |
| **Angiogenesis** | | | | | | | | | | | | | |
| **VEGFR-1** | 10.211 ±0.773 | 9.108-11.322 | 12.780± 1.595 | 10.087-14.276 | 13.631 ±2.179 | 10.897-16.613 | 11.349 ± 0.510 | 10.750 – 11.989 | 16.174 ±0.627 | 15.440 – 16.978 | 19.014 ±0.923 | 17.809-19.989 | 0.04 |
| **Vasa vasorum** | .00 ± .000 | 0 – 0 | 3 ± 3.098 | 1–7 | 4.67 ± 1.366 | 3 – 6 | 67 ± 516 | 0–1 | 19.33 ± 4.227 | 14 – 23 | 32.00 ± 3.225 | 29-36 | |
| **$H_2O_2$** | 0.136 ± 0.005 | 0.131 – 0.144 | 0.149 ± 0.011 | 0.137 – 0.165 | 0.178 ± 0.016 | 0.153 – 0.197 | 0.949 ± 0.363 | 0.680 – 1.675 | 1.321 ± 0.570 | 0.599– 1.987 | 1.884 ± 0.170 | 1.613-2.003 | |
| **HIF-1α** | 746.97 ±87.04 | 667.88 – 898.90 | 807.06 ±79.78 | 701.88 – 912.60 | 815.71 ± 300.69 | 432.99 – 1178.78 | 829.88 ± 77.63 | 721.98 – 962.87 | 1016.51 ± 324.89 | 771.77 – 1488.90 | 1553.66 ± 413.88 | 1100 – 2098.90 | |
| **NFKB** | 1312.33 ± 111.84 | 1199.80 – 1452.64 | 1431.19 ± 289.37 | 1109.78 – 1799.23 | 1451.80 ± 175.51 | 1315.96 – 1694.23 | 1300.28 ± 317.56 | 821.02 – 1598.80 | 1737.87 ± 335.98 | 1439.55 – 2213.89 | 1979.23 ±86.51 | 1842.86 – 2110.80 | |
| **iNOS** | 426.21 ± 112.20 | 265.12 – 567.89 | 547.54 ± 151.02 | 367.89 – 736.25 | 693.50 ±76.40 | 587.24 – 794.54 | 868.47 ± 83.84 | 741.42 – 988.68 | 1002.38 ±92.84 | 887.90 – 1100.54 | 1506.68 ± 410.16 | 1006.61 – 1987.90 | |

Expression of HIF-1α on the various durations of diet as you can see in Table I, ranged from 432.99 to 2098.90 au. In the groups with the ND administration, the expression HIF-1α is lower than HCD groups, ranged from 432.99 to 1178.78 au, whereas in the group with HCD administration ranged from 721.98 to 2098.90 au. The longer duration of treatment will increase HIF-1α expression, particularly in the HCD groups. The expression of HIF-1α on the various durations of diet with immunofluorescence staining can be seen in Fig. 3.

The expression of NF-κB on various diet durations as you can see in Table I, ranged from 821.02 to 2213.89 au. In the ND groups, the expression of NF-κB is lower than HCD groups, ranged from 1109.78 to 1799.23 au, whereas in the HCD groups NF-Kb expression ranged from 821.02 to 2213.89 au. The longer duration of treatment will increase the expression NF-κB, especially in the HCD groups. The expression of NF-κB on the various durations of diet with immunofluorescence staining can be seen in Fig. 4.

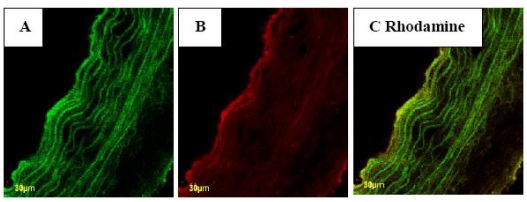


Figure 3. Imunofluoresence HIF-1α at SMC α-actin labeled. (A: α-actin FITC; B: HIF-1α rhodamine; C: double staining α-actin FTC – HIF-1α rhodamine)

INOS expression in various duration as you can see in Table I, ranged from 265.12 to 1987.90 au. ND groups had lower iNOS expression, ranged from 265.12 to 794.54 au, whereas in the HCD groups ranged from 741.42 to 1987.90 au. The longer duration of treatment will increase iNOS expression, particularly in the HCD

groups. The expression of INOS on the various durations of diet with immunofluorescence staining can be seen in Fig. 5.

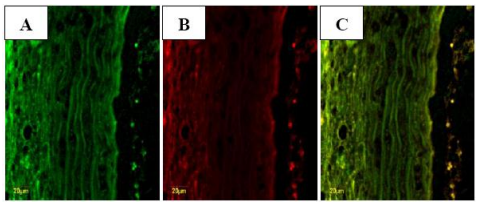


Figure 4. Imunofluorescence of NF-κB double staining with HIF-1α. (A: NF-κB FITC; B: HIF-1α rhodamine; C: double staining of NF-κB FITC and HIF-1α rhodamine)

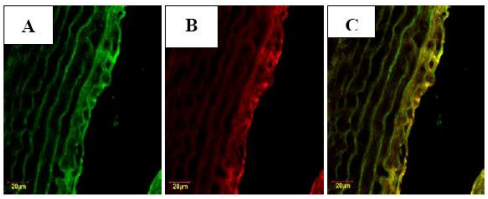


Figure 5. Imunofluresence staining results of INOS at smooth muscle cell (SMC) labeled α-actin (A : α-actin FITC; B : INOS rhodamine; C : double staining α-actin FITC – INOS rhodamine)

## IV. Discussion

Vasa vasorum angiogenesis in atherosclerosis occur as a compensation of hypoxia. It is still widely debated that vasa vasorum angiogenesis involves molecular mechanism. This study tries to explain the involvement of $H_2O_2$, HIF-1α, NF-κB and iNOS in animal models Wistar strain of Micetus novergicus of angiogenesis vasa vasorum induced by hypercholesterol diet. Angiogenesis vasavasorum in this research was observed from the increased levels of aortic tissue VEGFR-1, which were measured by ELISA. VEGFR-1 is a tyrosine kinase receptor that binds to VEGF. VEGFR-1 is expressed in early vascular development and angiogenesis post-natal. VEGFR-1 is expressed by inflammatory cells in pathological angiogenesis associated with atherosclerosis [17]. VEGFR-1 also plays a role in vascular invasion into vascular tissue which is an important step in angiogenesis vasavasorum [18]. Admission of hypercholesterol diet in this study is intended to trigger dyslipidemia as a risk factor for atherosclerosis, that will increase the production of free radicals and lead to endothelial dysfunction and inflammation.

The Increased levels of VEGFR-1 in this study indicate the occurrence of angiogenesis vasavasorum. This is confirmed by histopathological anatomy observation using HE staining to identify vasa vasorum which are formed. The longer dumiceion of treatment will increase the number of vasa vasorum, especially in the HCD group. VEGFR-1 levels in this study was influenced by the dumiceion of HCD admission, dietary factors and the type of interaction between the two. The difference in VEGFR-1 levels influenced by dumiceion factor, dietary factors and the interaction of these two factors can be explained significantly using ANOVA. Levels of VEGFR-1 in ND groups was lower than the HCD groups. The dumiceion of treatment effect positively to the levels of VEGFR-1, if the diet treatment takes longer time, it will increase VEGFR-1 levels. The average results between treatment groups explained that the average dumiceion of VEGFR-1 in each groups is significantly different from one another. The interaction effect of dietary factors and dumiceion factor significantly affect the VEGFR-1 levels. It is explained that the change pattern of VEGFR-1 on each diet group due to the length of treatment dumiceion will have a different pattern. At the first month, VEGFR-1 levels in ND and HCD group were not significantly different, whereas in the third and fifth month, VEGFR-1 levels are significantly different. The increased levels of VEGFR-1 in the HCD group are very high compared to the ND group.

Vasa vasorum angiogenesis in atherosclerosis condition involves $H_2O_2$, HIF-1α, NF-κB and iNOS in Wistar strain of Micetus novergicus which are given with hypercholesterol diet. Exposure to oxidative stress in atherosclerosis risk factors in this study triggered by administmiceion of hypercholesterol diet. Oxidative stress is a condition in which there is an imbalance between oxidant and antioxidant status, causing oxidative damage [19]. The implications of oxidative stress is the formation of Reactive Oxygen Species (ROS) such as $OH^-$, RO and $H_2O_2$. $H_2O_2$ is continuously produced in the lipid peroxidation process that occurs in the dyslipidemia conditions [20].

The increased levels of $H_2O_2$ in this study are due to the provision of hypercholesterol diet that increase LDL cholesterol levels in mices. The hypercholesterol diet which were given to the mice, contained the addition of cholesterol, cholic acid and lard so that could raise LDL cholesterol levels [21]. The use of cholesterol, cholic acid and lard aims to induce an increase in blood LDL. Lard has a higher cholesterol content compared with other animal's oils and vegetable oils. Cholic acid is given because without the addition of cholic acid, the admission of hypercholesterol diet for 8 weeks can't increase cholesterol levels and foam cell formation significantly. Sarivastava et al revealed that cholic acid supplementation is required to induce atherosclerosis in mice. In addition, the condition of hypercholesterolemia affects the reduction of surface cells' receptor level in the cholesterol binding process. Cholesterol enters the surface area and liver cells by binding to specific cell membrane receptors. The absence of receptors due to genetic factors or the presence of receptors that have been modified on the surface of cells or hepatocytes would prevent the normal cholesterol absorption after the entry of food (in the liver) or after secretion into the plasma from the liver [22].

The increased levels of LDL and total cholesterol (TC) in the preliminary studies began in the first month of hypercholesterol diet admission. It is known from the results of difference test using Duncan, showed significant differences ($p < 0.005$) in LDL and TC levels between HCD and ND groups at the first month. In this study, LDL levels increases with the addition of the diet treatment duration. LDL cholesterol levels can also be

caused by aging. From each duration of hypercholesterol diet admission, it could be observed that the elevated levels of LDL followed by increased levels of H2O2, is linear with the addition of the treatment duration. This fact is supported by the linear regression where p = 0.000 (p <0.05) showed a positive relationship between the timing of hypercholesterol diet admission with high levels of LDL cholesterol. The increased LDL levels after administration of hypercholesterol diet showed that high consumption of fat is one of the major causes of hyperlipidemia. The study examined the relationship of fat and cholesterol consumption with coronary heart disease (CHD) was begun in the 19th century. In 1847, Vogel has outlined the presence of cholesterol in atheromatous tissue. Anitschkow and Chalatow reported that cholesterol feeding in rabbits promote atherosclerosis. The differences in $H_2O_2$ levels in the ND group with HCD group due to differences in feed composition between both groups. In the HCD group, the addition of cholesterol, cholic acid and lard cause hypercholesterolemia condition. Atherogenic feeding for 8 weeks in mice can increase blood cholesterol levels and induces the formation of foam cells (foam cells) significantly [23].

Oxidative stress caused by hydrogen peroxide ($H_2O_2$) increases the phosporilation of tyrosine kinase, which causes neutrophils' bond to endothelial cells become stronger and changes the vascular permeability [24]. Phosporilation also activate NF-κB [6]. NF-κB activation occurs through inhibitors of kappa beta (IκB) phosporilation and ubiquination [25], causing translocation to the nucleus [26]. IκB, is a protein that binds to NF-κB subunits p50 and p65, are kept in an inactive state [27]. Oxidation stimulus associated with the degradation of IκB [28] and $H_2O_2$ activates NF-κB through those mechanism [29]. IκB contain methion residues that are sensitive to redox reactions. $H_2O_2$ increase phosporilation that caused the degradation of IκB [26]. In the walls of blood vessels, NF-κB plays an important role in the regulation of inflammatory and immune response genes which associated with atherosclerosis. NF-κB is a transcription factor that plays an important role in the process of atherosclerosis, mainly in inducing the regulation of various biological effects including inflammasi, immune system, proliferation, differentiation, tumorigenesis and cell apoptosis [30]. Many research has been widely proven that atherosclerosis is a chronic inflammatory process. Inflammation occurs due to the activation of NF-κB, where translocation occur into the nucleus and stimulates the expression of proinflammatory genes [6].

While inflammation process occur, cells undergo active metabolism that increase the oxygen demand and the declining of oxygen supply, causing hypoxia [31]. The increased hypoxia in the blood vessel wall can also be caused by the thickening of arterial walls so that the diffusion process of oxygen disrupted [32]. Hypoxia is strongly associated with angiogenesis and thrombus formation. Effects of hypoxia is mediated by macrophages as an infiltrate in the thrombus. Macrophage are cells that have the ability to change the anaerobic into aerobic metabolism fast (glycolitic turnover). This causes an increase in oxygen demand. When hypoxia occurs, cells will adapt by activating HIF-1α [11].

Oxidative stress has important implications in the pathophysiology of cardiovascular diseases including hypertension and atherosclerosis as well as in tumor progression and vascularization [33], [34]. Sufficient levels of ROS, particularly superoxide anion and hydrogen peroxide has been shown to activate a signaling cascade that mediates the response to vasoactive peptides, growth factors, cytokines, hormones and coagulation factors as well as on the physical and chemical stress [33].

HIF-1α is a heterodimer transcription factor consisting of subunits α and β [35]. HIF-1α in normoksia condition is undetected due to the hydroxylation by prolyl-hydroxylase, so that HIF-1α experienced ubiquination which is mediated by the von Hippel-Lindau protein (VHL) [36]. In hypoxic conditions, HIF-1α is stable because of the blockade of hydroxylation. HIF subunit heterodimerization then occurs and produces a variety of target gene transcription. Heterodimerization and translocation of HIF-1α/HIF1-β to the nucleus, specifically bind to the HRE (hypoxia response element) promoter sites that regulate classics hypoxia-responsive genes [37] including glucose metabolism and angiogenesis (VEGF, glucose transporter (GLUT) 1 and 3, Hexokinase (HK) 1 and 2) [13]. HIF-1α is a transcription factor that regulates oxygen homestasis and plays a key role in development, physiology and disease. The activity of HIF-1α induced by hypoxia, stimulation of growth factors and Ca2+ signal. HIF-1α mediates the adaptive response to hypoxia as in erythropoiesis, angiogenesis and metabolic reprogramming [38].

Under normal conditions, the HIF-1α subunit is synthesized in the hydroxylation of proline residues by prolyl hydroxylase domain (PHD) proteins which use $O_2$ and α-ketoglutarate as a substrat. To catalyze the deoxygenation reaction, one oxygen atom is inserted into the proline residue and the other oxygen atom is inserted into the α-ketoglutarate to form succinate and $CO_2$ [39], [40]. Prolyl hydroxylation is required for VHL protein bond that interacts with Elongin C and recruit ubiquinasi ligase complex [40], [41]. Ubiquinasi occurs because of HIF-1α degradation by the proteosome [42]. Factor Inhibitors of Hypoxia (FIH)-1 bind to HIF-1α and disrupt the function of trans-activation [43] asparagine residue 803 hydroxylation, which blocked the interaction of HIF-1α trans-activation domain with the p300/CBP co-activator [44]. Stability and transcriptional activity of HIF-1α is negatively regulated by $O_2$-dependent hydroxylation.

When cells acutely undergo hypoxia, hydroxylation reaction is inhibited due to loss of substrat ($O_2$) and or increase mitochondrial ROS production, which can inhibit the hydroxylation of the ferrous ion oxidation in catalysis process [40], [45]. The loss of hydroxylation activity improves the stability and function of HIF-1α trans-activation, causing dimerization with HIF-1β. HIF-

1 bond is identified by 5'-(A / G) CGTG-3 'sequence [46] on targeted gene and increases the transcription of target genes sequence into mRNA [38]. The principle mechanisms of extracellular signal transduction into the nucleus is the binding of growth factors, cytokines and other ligands to tyrosine kinases receptor and G-protein coupled receptor on the cell surface, leading to the activation of phospatidylinositol 3-kinase (PI3K) and MAP kinase pathways. Signal transduction through this pathway stimulates the survival, growth and proliferation of cells. The cells growth and proliferation consequence is unavoidably increase $O_2$ consumption, so it is considered as a signal transduction pathway that precedes the activity of HIF-1 as occurs in conditions that do not depend on $O_2$ [47]-[50]. Table 1 of correlation matrix between variables showed that the expression of HIF-1α has a significant relationship ($p < 0.05$) with the expression of NF-κB. The data showed that the increased expression of HIF-1α associated with inflammatory processes that are regulated by NF-κB activation that occurs in the conditions of hypercholesterol diet admission.

NF-κB plays an important role in atherosclerosis in which the activation of this transcription factor in vascular cells such as endothelial cells, smooth muscle cells and macrophage causes increased expression of various genes that encode cytokines, leukocyte adhesion molecules, matrix metalloproteinase, cyclooxigenase-2 and iNOS [51]. Furthermore, NF-κB activation may increase the recruitment and activation of inflammatory cells in the vessel wall. The activated NF-κB can be identified in endothelial cells, smooth muscle cells and macrophage on human atherosclerotic lesions [52] where it contributes to the dysregulation of vascular smooth muscle cell function. On the other side, a wide variety of stimuli, like local factors (vascular injury), LDL modification, metabolic factors, microbial agents and products, cytokines and lymphocytes also leads to the activation of NF-κB [6]. The exposure of macrophage, smooth muscle cell (SMC) and endothelial cells to cytokines, such as IL-1 and TNF-α, can induce iNOS which produces large amounts of NO during the inflammatory process [53].

NO is a relaxation factor produced by endothelial cells. NO increased while the conversion of L-arginine to L-citrulin in the process of NADPH formation which produce electrons, a reaction catalyzed by NO synthase (NOS), which consists of 3 isoforms [53]. iNOS is the type II isoform which has a high inducible capacity. The exposure of macrophage, SMC and endothelial cells to cytokines such as IL-1 and TNF-α induce iNOS, which produce large amounts of NO during the inflammatory process [53]. The iNOS expression increase in macrophages and vascular smooth muscle cells in the early and advanced stages of atherosclerotic lesions. Inflammation showed a large role in the development of atherosclerotic lesions affecting the coronary arteries. iNOS plays a role in inflammation through the fast and massive production of prostanoid and NO, which both of them have the pro-aterosclerotic effect [9]. In accordance with the results of this study, there is an increase in iNOS levels in each group with the administration of various hypercholesterol diet duration. Increased levels of iNOS in this study indicate the inflammatory process as a consequence of the activation of NF-κB. NF-κB is a transcription factor that plays an important role in the process of atherosclerosis, particularly in inducing the regulation of inflammation, immune system, proliferation, differentiation, and apoptosis of cells [5].

Atherosclerosis shows the extensive process of angiogenesis and vasa vasorum, associated with atherosclerotic plaques growth and instability [15], [16], [54]. Angiogenesis is a major impact of tissue hypoxia. The occurrence of angiogenesis supports the existence of hypoxia in atherosclerosis. The possible role of HIF-1α in atherosclerosis is supported by the presence of angiogenesis intra-plaque as a consequence of responsive genes to HIF-1α activation, such as VEGF [12], [13], endothelin-1 [13], [55] and matrix-metalloproteinase-2 [13], [14]. Hypoxia is strongly associated with angiogenesis and thrombus formation. The effect of hypoxia is mediated by macrophages as an infiltrat in the trombus. Hipoksia occurs when oxygen supply decreases and or the increasing of the demand. Hypoxia also evolved from an increased need for oxygen, the result of the high demand for oxygen in metabolically active inflammatory cells [31].

Hypoxia occurs in macrophage foam cells, but not all macrophages experiencing hypoxia. Most of hypoxia depends on the environment of micro-inflammation although only have a minor contribution to the reduction of oxygen supply. Hypoxia cells activates HIF-1α as in the study conducted by Sluimer in 2008, found the presence of HIF-α in atherosclerosis with hypoxia [56]. Some of the non-hypoxic stimuli can also activate HIF-1α, such as lipopolysaccharide [57] and angiotensin II [58], hypoxia is a significant stimulator of HIF-1α to induce angiogenesis to repair the oxygen flow. Those are consistent with the opinion of localized hypoxia in human atherosclerosis is associated with HIF, VEGF and angiogenesis intra-plaque. The hypothesis that hypoxia in macrophage stimulates HIF-1α and angiogenesis in the progression of atherosclerosis is supported by the effects of hypoxia pro-angiogenic on macrophage. This is illustrated by an increase in cytokine production [31], LDL oxidation [59] and lipid [60] in vitro, which all the processes associated with the development of macrophage foam cells, lipid and or necrotic core, and plaques instability [15]. There is a speculation that hypoxic macrophages contribute to atherosclerotic plaque instability [56].

Plaque angiogenesis plays an important role in the development of atherosclerosis. Vasa vasorum angiogenesis and medial infiltration provides nutrition to the development and expansion of the intima, thus preventing cell death and contributes to plaque growth and stabilization of the initial lesion. However, in more advanced plaques, inflammatory cell infiltrations occur, and the production of pro-angiogenic cytokine in large quantities, that are likely responsible for the induction of

uncontrolled mikrovessel neointimal proliferation, occur simultaneously. This resulted in the production of immature and fragile neovessel resembling the tumor development. These conditions contribute to the development of unstable haemorrhagic rupture [61].

## V. Conclusion

The administration of hypercholesterol diet affect the vasa vasorum angiogenesis through elevated levels of $H_2O_2$, HIF-1α, NF-κB and iNOS in Animal Models Wistar strain of *Rattus novergicus*. Vasa vasorum angiogenesis occurred since the third months of hypercholesterol diet treatment, in both of the VEGFR-1 parameters and anatomical histopathology with HE staining. Vasa vasorum angiogenesis that occurs in atherosclerosis conditions in this study involves a pathway that begins with the increased levels of $H_2O_2$ as an indication of oxidative stress. Oxidative stress triggers the inflammatory process by NF-κB as a primary regulator. Activation of NF-κB Influence on the expression of HIF-1α, as proven in this study that NF-κB becomes an upstream of HIF-1α.

## Acknowledgment

This work is supported by the Medical Faculty of Brawijaya University research center in 2013. This work was performed under the benevolence of the Head of the Medical University and with the kind collaboration of the Department of Cardiology and Laboratory of Pharmacology, Faculty of Medicine, Brawijaya University, Malang, Indonesia.

## References

[1] S. A. Price and L. M. Wilson, *Patofisiologi: Konsep Klinis Proses Penyakit*, EGChal, pp. 60-65, 2006

[2] L. A. Reynolds and E. M. Tansey, *Cholesterol, Atherosclerosis and Coronary Disease in The UK 1950–2000*, London: Wellcome Trust Centre27, 2006

[3] H. Sulastomo, "Sindroma koroner akut dengan gangguan metabolik pada wanita usia muda pengguna kontrasepsi hormonal," Departement of Cardiology and Vascular Faculty of Medicine University of Indonesia, 2010.

[4] D. Sargowo, M. Setiawan, and Hafisalevi, "Effect of extract from pericarp of mangosteen as antioxidant rats models of atherosclerosis," *Jurnal Kardiolog*, vol. 33, 2012.

[5] G. Bonizzi and M. Karin, "The two NFκB activation pathway and their role in innate and adaptive immunity," *Trends Immunol*, vol. 25, pp. 280-288, 2004.

[6] C. Monaco, E. Andreakos, S. Kiriakidis, M. Feldmann, E. Paleolog, "T cell-mediated signalling in immune, inflammatory and angiogenic processes: The cascade of events leading to inflammatory diseases," *Curr Drug Targets Inflamm Allergy*, vol. 84, pp. 695, 2003.

[7] E. Andreakos, C. Smith, and S. Kiriakidis, "Heterogeneous requirement of IκB kinase 2 for inflammatory cytokine and matrix metalloproteinase production in rheumatoid arthritis: Implications for therapy," *Arthritis Rheum*, vol. 48, pp. 1901–1912, 2003.

[8] M. I. Cybulsky, K. Iiyama, and H. Li, "A major role for VCAM-1, but not ICAM-1, in early atherosclerosis," *J. Clin Invest*, vol. 107, pp. 1255–1262, 2001.

[9] T. L. Clanton, "Hypoxia-induced reactive oxygen species formation in skeletal muscle," *J. Appl Physiol*, vol. 102, pp. 2379–2388, 2007.

[10] J. Wang, L. Weigand, W. Lu, J. T. Sylvester, G. L. Semenza, and L. A. Shimoda, "Hypoxia-inducible factor 1 mediates hypoxia-induced TRPC expression and elevated intracellular Ca2+ in pulmonary arterial smooth muscle cells," *Circ Res*, vol. 98, pp. 1528–1537, 2006.

[11] M. Mayr, A. Sidibe, and A. Zampetaki, "The paradox of hypoxic and oxidative stress in atherosklerosis," *J American College of Cardiology*, vol. 51, no. 13, pp. 1266-1267, 2008.

[12] F. L. Celletti, J. M. Waugh, P. G. Amabile, A. Brendolan, P. R. Hilfiker, and M. D. Dake, "Vascular endothelial growth factor enhances atherosclerotic plaque progression," *Nat Med*, vol. 7, pp. 425–429, 2001.

[13] G. L. Semenza, "Targeting hif-1 for cancer therapy," *Nat Rev Cancer*, vol. 3, pp. 721–732, 2003.

[14] G. Pasterkamp, A. H. Schoneveld, and D. J. Hijnen, "Atherosclerotic arterial remodeling and the localization of macrophages and matrix metalloproteases 1, 2 and 9 in the human coronary artery," *Atherosclerosis*, vol. 150, pp. 245–253, 2000.

[15] R. Virmani, F. D. Kolodgie, and A. P. Burke, "Atherosclerotic plaque progression and vulnerability to rupture angiogenesis as a source of intraplaque hemorrhage," *Arterioscler Thromb Vasc Biol*, vol. 25, pp. 2054–2061, 2005.

[16] P. R. Moreno, K. R. Purushothaman, M. Sirol, A. P. Levy, and V. Fuster, "Neovascularization in human atherosclerosis," *Circulation*, vol. 113, pp. 2245–2252, 2006.

[17] A. Luttun, M. Tjwa, L. Moons, *et al*., "Revascularization ofischemic tissues by PlGF treatment, and inhibition oftumor angiogenesis, arthritis and atherosclerosis byanti-Flt1," *Nat Med*, vol. 8, pp. 831–840, 2002.

[18] C. Maes, I. Stockmans, K. Moermans, *et al*., "Soluble VEGFisoforms are essential for establishing epiphyseal vascularizationand regulating chondrocyte development andSurvival," *J. Clin Invest, vol.* 113, pp. 188–199, 2004.

[19] H. Sies, "Oxidative stress: Oxidants and antioxidants," *J. Academic*, vol. 1, pp. 10, 1991.

[20] P. A. Mayes, "Lipid yang memiliki makna fisiologis," *EGChal*, pp. 54-56, 2003

[21] P. J. Barnes and M. Karin, "NFκB: A pivotal transcription factor in chronicinflammatory diseases," *N Engl J. Med*, vol. 336, pp. 1066–1071, 1997.

[22] R. T. Lee, C. Yamamoto, and Y. Feng, "Mechanical strain induces specific changes in the synthesis and organization of proteoglycans by vascular smooth muscle cells," *J. Biol Chem*, vol. 276, pp. 13847–13851, 2001.

[23] S. Murwani and M. Ali, "Diet aterogenik pada tikus (rattus novergicus strain wistar) sebagai model hewan aterosklerosis," *Jurnal Kedokteran Brawijaya*, vol. 22, 2006.

[24] T. Bourcier, G. Sukhova, and P. Libby, "The nuclear factor kappa beta signalling pathway participates in disregulation of vascular smooth muscle cells in vitro and human atherosclerosis," *J. Biol Chem*, vol. 272, pp. 15817-1584, 1997.

[25] N. Y. Ben, "Regulatory functions of ubiquitination in the immune system," *Nat Immunol*, vol. 3, pp. 20–26, 2002.

[26] S. Ghosh, "Karin missing pieces in the NF-KB puzzle," *Cell*, vol. 109, pp. 81–96, 2002.

[27] L. G. Chen, L. L. Yang, and C. C. Wang, "Anti-inflammatory activity of mangostins from garcinia mangostana," *Food Chem. Toxicol*, vol. 46, pp. 688–693, 2007.

[28] N. Li and M. Karin, "Is NFκB the sensor of oxidative stress," *FASEB J*., vol. 13, pp. 1137–1143, 1999.

[29] R. Schreck, P. Rieber, and P. A. Baeuerle, "Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1," *EMBO J*., vol. 10, pp. 2247–2258, 1991.

[30] G. Bonizzi and M. Karin, "The Two NFκB activation pathway and their role in innate and adaptive immunity," *Trends Immunol*, vol. 25, pp. 280-288, 2004.

[31] C. Murdoch, M. Muthana, and C. E. Lewis, "Hypoxia regulates macrophage functions in inflammation," *J Immunol*, vol. 175, pp. 6257– 6263, 2005.

[32] I. P. Torres, M. Leunig, F. Yuan, M. Intaglietta, and R. K. Jain, "Noninvasive measurement of microvascular and interstitial oxygen profiles in a human tumor in scid mice," *Proc Natl Acad SciUSA*, vol. 91, pp. 2081–2085, 1994.

[33] N. R. Madamanchi, A. Vendrov, and M. S. Runge, "Oxidative stress and vascular disease," *Arterioscler Thromb Vasc Biol*, *vol.* 25, pp. 29–38, 2005.

 

[34] B. Lassegue and K. K. Griendling, "Reactive oxygen species in hypertension an update," *Am J. Hypertens*, vol. 17, pp. 852– 860, 2004.
[35] D. Steinberg, "Low density lipoprotein oxidation and its pathobiological significance" *J. Biol Chem*, vol. 272, pp. 20963-20966, 1997.
[36] K. Tanimoto, Y. Makino, T. Pereira, and L. Poellinger, "Mechanism of regulation of the hypoxia inducible factor alpha by the von Hippel Lindau tumor suppressor protein," *Embo J.*, vol. 19, pp. 4298-4309, 2000.
[37] V. Imbert, R. A. Rupec, and A. Livolsi, "Tyrosine phosphorylation of I kappa B-alpha activates NFκB without proteolytic degradation of I kappa B-alpha," *Cell*, vol. 86, pp. 787–798, 1996.
[38] G. L. Semenza, "HIF-1 and human disease: One highly involved factor," *Genes Dev*, vol. 14, vol. 1983–1991, 2000.
[39] A. Costanzo, F. Moretti, and V. L. Burgio, "Endothelial activation by angiotensin II through NFkappaB and p38 pathways: Involvement of NFkappaB-inducible kinase (NIK), free oxygen radicals, and selective inhibition by aspirin," *J. Cell Physiol*, vol. 195, pp. 402– 410, 2003
[40] H. Kai, F. Kuwahara, and K. Tokuda, "Coexistence of hypercholesterolemia and hypertension impairs adventitial vascularization," *Hypertension*, vol. 39, pp. 455-459, 2002.
[41] R. Khurana, L. Moons, S. Shafi, A. Luttun, D. Collen, J. F. Martin, *et al.*, "Placental growth factor (PlGF) promotes atherosclerotic intimal thickening and macrophage accumulation," *Circulation*, vol. 111, pp. 2828 –2836, 2005.
[42] A. Scalbert and G. Williamson, "Dietary intake and bioavailability of polyphenols," *Chocolate: Modern Science Investigates an Ancient Medicine*, 2000.
[43] W. Mahabusarakam, P. Iriyachitra, and W. C. Taylor, "Chemical constituentsof garcinia mangostana," *J. Nat Prod*, vol. 50, pp. 474–478, 1987.
[44] M. Levin, T. Bjornheden, M. Evaldsson, S. Walenta, and O. Wiklund, "A bioluminescence method for the mapping of local ATP concentrations within the arterial wall, with potential to assess the in vivo situation," *Arterioscler Thromb Vasc Biol*, vol. 19, pp. 950–958, 1999.
[45] L. Gu and Y. Okada, "Absence of monocyte chemmoattractant protein-1 reduces atherosclerosis in low density lipoprotein deficient mice," *Mol Cel*, vol. 19, pp. 98, 1998.
[46] G. L. Semenza, "HIF-1, $O_2$, and the 3 PHDs: how animal cells signal hypoxia to the nucleus," *Cell*, vol. 107, no. 1, pp. 1–3, 2001.
[47] R. Fukuda, K. Hirota, F. Fan, Y. D. Jung, L. M. Ellis, and G. L. Semenza, "Insulin-likegrowth factor 1 induces hypoxia-inducible factor 1-mediated vascularendothelial growth factor expression, which is dependent on MAP kinaseand phosphatidylINOSitol 3-kinase signaling in colon cancer cells," *J BiolChem*, vol. 277, pp. 38205–38211, 2002.
[48] E. Laughner, P. Taghavi, K. Chiles, P. C. Mahon, and G. L. Semenza, "HER2 (neu) signaling increases the rate of hypoxia-inducible factor 1_ (HIF-1_) synthesis: novel mechanism for HIF-1-mediated vascular endothelial growth factor expression," *MolCell Biol*, vol. 21, pp. 3995–4004, 2001.
[49] C. Treins, S. Giorgetti-Peraldi, J. Murdaca, G. L. Semenza, and E. Van Obberghen, "Insulin stimulates hypoxia inducible factor 1 through a phosphatidylINOSitol 3-kinase/target of rapamycin-dependent signaling pathway," *J. Biol Chem*, vol. 277, pp. 27975–27981, 2002.
[50] M. H. Zou, C. Shi, and R. A. Cohen, "Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite," *J. Clin Invest*, vol. 109, pp. 817–826, 2002.
[51] G. Valen, Z. Q. Yan, and G. K. Hansson, "Nuclear factor kappa-B and the heart," *J. Am Coll Cardiol*, vol. 38, pp. 307–314, 2001.
[52] K. Brand, S. Page, G. Rogler, A. Bartsch, R. Brandl, *et al.*, "Activatedtranscription nuclear factor-kappa B is present in atherosclerotic lesion," *J. Clin Invest*, vol. 97, pp. 1715–1722, 1996.
[53] J. S. Stamler, D. J. Singel, and J. Loscalso, "Biochemistry of nitric oxide and its redox-activated forms," *Science*, vol. 258, pp. 1898-1902, 1992.
[54] J. Herrmann, L. O. Lerman, M. Rodriguez, D. R. Holmes, *et al.*, "Coronary vasa vasorum neovascularization precedes epicardial endothelial dysfunction in experimental hypercholesterolemia," *Cardiovasc Res*, vol. 51, pp. 762–766, 2001.
[55] C. Ihling, T. Szombathy, B. Bohrmann, M. Brockhaus, H. E. Schaefer, and B. M. Loeffler, "Coexpression of endothelin-converting enzyme-1 and endothelin-1 in different stages of human atherosclerosis," *Circulation*, vol. 104, pp. 864 –869, 2001.
[56] J. C. Sluimer, J. M Gasc, and J. L. Van Wanroij, "Hypoxia, hypoxiainducible transcription factor, and macrophages in human atherosclerotic plaques are correlated with intraplaque angiogenesis," *J. Am Coll Cardiol*, vol. 51, pp. 1258–1265, 2008.
[57] C. C. Blouin, E. L. Page, G. M. Soucy, and D. E. Richard, "Hypoxic gene activation by lipopolysaccharide in macrophages: Implication of hypoxia-inducible factor 1α," *Blood*, vol. 103, pp. 1124 –1130, 2004.
[58] E. L. Page, G. A. Robitaille, J. Pouyssegur, and D. E. Richard, "Induction of hypoxia-inducible factor-1_ by transcriptional and translational mechanisms," *J Biol Chem*, vol. 277, pp. 48403–48409, 2002.
[59] E. K. Rydberg, A. Krettek, and C. Ullstrom, "Hypoxia increases ldl oxidation and expression of 15-lipoxygenase-2 in human macrophages," *Arterioscler Thromb Vasc Biol*, vol. 24, pp. 2040 – 2045, 2004.
[60] P. Bostrom, B. Magnusson, P. A. Svensson, *et al.*, "Hypoxia converts human macrophages into triglyceride-loaded foam cells," *Arterioscler Thromb Vasc Biol*, vol. 26, pp. 1871– 1876, 2006.
[61] M. Slevin, J. Krupinski, and L. Badimon, "Controling the angiogenic switch in developing atherosclerotic plaque: Posible targets for therapeutic interventions," *J. Angiogenesis Research*, vol. 1, pp. 2040-2384, 2009.

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