Vasa Vasorum Angiogenesis through Increased Levels of H$_2$O$_2$, HIF-1$\alpha$, 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$_2$O$_2$, HIF-1$\alpha$, NfκB and iNOS pathways in Wistar strain of Rattus norvegicus given HFD. This research is a true experimental laboratory, with an in vivo approach to post-test with control group, using 24 males wistar strain of Rattus norvegicus, 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$_2$O$_2$ measured by colorymetric; NFkB, iNOS and HIF-1$\alpha$ were measured using double staining immunofluoresence which was observed with CLSM (Confocal Lasser Scaning Microscope) in aortic smooth muscle cells; vasa vasorum 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$_2$O$_2$, NfκB and iNOS in wistar strain of Rattus norvegicus (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-1$\alpha$. The conclusion of this study is the vasa vasorum angiogenesis is more dominantly influenced by inflammatory conditions.

Index Terms—vasa vasorum angiogenesis, H$_2$O$_2$, HIF-1$\alpha$, NF-κB, 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$_2$O$_2$ 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 expression 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-1$\alpha$) in the process of atherosclerosis. HIF-1$\alpha$ is a transcription factor that regulates glycolysis, angiogenesis and cell survival [10]. HIF-1$\alpha$ in normal oxygen conditions (unstable state) can’t be detected due to hydroxylation. In hypoxic conditions, HIF-1$\alpha$ 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].

Abstract — This study aims to determine the increased of vasa vasorum angiogenesis through H$_2$O$_2$, HIF-1$\alpha$, NFκB and iNOS pathways in Wistar strain of Rattus norvegicus given HFD. This research is a true experimental laboratory, with an in vivo approach to post-test with control group, using 24 males wistar strain of Rattus norvegicus, 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$_2$O$_2$ measured by colorimetric; NFkB, iNOS and HIF-1$\alpha$ were measured using double staining immunofluorescence which was observed with CLSM (Confocal Laser Scanning Microscope) in aortic smooth muscle cells; vasa vasorum 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$_2$O$_2$, NFκB and iNOS in wistar strain of Rattus norvegicus (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-1$\alpha$. The conclusion of this study is the vasa vasorum angiogenesis is more dominantly influenced by inflammatory conditions.

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I. INTRODUCTION

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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 norvegicus 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 Biomedical 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) \( \text{H}_2\text{O}_2 \) measurement

\( \text{H}_2\text{O}_2 \) 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 MgCl₂, 10% [v/v] dimethyl sulfoxide (DMSO) containing 3.7% formaldehyde and 0.05% glutaraldehyde) buffer and were processed by immunofluorescence double labeling with anti-α-tubulin antibody, HIF-1α, NF-κB using fluorescein isothiocyanate (FITC) 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 600x 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.
In the ND group s, the expression of NF-κ will increase the expression NF-κ whereas in the HCD groups NF-κ 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.

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.

H₂O₂ 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 H₂O₂ 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 H₂O₂, especially in the groups with administration of HCD.

Table I. The average level and standard deviation of VEGFR-1, Vasa vasorum, H₂O₂, HIF-1α, NFkB, and iNOS in each group.

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. 4.

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 5. Immunofluorescence staining results of INOS at smooth muscle cell (SMC) labeled α-actin (A: α-actin FITC; B: iNOS rhodamine; C: double staining of NF-κB FITC and HIF-1α 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 H2O2, HIF-1α, NF-κB and iNOS in animal models Wistar strain of Micetus norvegicus, 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 duration of treatment will increase the number of vasa vasorum, especially in the HCD group. VEGFR-1 levels in this study were influenced by the duration of HCD admission, dietary factors and the type of interaction between the two. The difference in VEGFR-1 levels influenced by duration 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 dimumiclon 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 dimumiclon of VEGFR-1 in each groups is significantly different from one another. The interaction effect of dietary factors and dimumiclon 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 dimumiclon 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 H2O2, HIF-1α, NF-κB and iNOS in Wistar strain of Micetus norvegicus 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 H2O2. H2O2 is continuously produced in the lipid peroxidation process that occurs in the dyslipidemia conditions [20].

The increased levels of H2O2 in this study are due to the provision of hypercholesterol diet that increase LDL cholesterol levels in mice. 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 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 H2O2 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 (H2O2) increases the phosphorilation of tyrosine kinase, which causes neutrophils’ bond to endothelial cells become stronger and changes the vascular permeability [24]. Phosphorilation also activate NF-κB [6]. NF-κB activation occurs through inhibitors of kappa beta (IKB) phosphorilation and ubiquinination [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 H2O2 activates NF-κB through those mechanism [29]. IκB contain methion residues that are sensitive to redox reactions. H2O2 increase phosphorilation 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 inflammation, 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 normoksa condition is undetected due to the hydroxylation by prolyl-hydroxylase, so that HIF-1α experienced ubiquinination 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 O2 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 CO2 [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 O2-dependent hydroxylation.

When cells acutely undergo hypoxia, hydroxylation reaction is inhibited due to loss of substrat (O2) 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-1α. HIF-
them have the pro-atherosclerotic effect [9]. In accordance
massive production of prostanoid and NO, which both of
Inflammation showed a large role in the development of
macrophages and vascular smooth muscle cells in the
process [53]. The iNOS expression increase in
produce large amounts of NO during the inflammatory
α cytokines such as IL-1 and TNF-
exposure of macrophage, SMC and endothelial cells to
activation of NF-
κ product, cytokines and lymphocytes also leads to the
modification, metabolic factors, microbial agents and
stimuli, like local factors (vascular injury), LDL
macrophage on human atherosclerotic lesions [52] where
identified in endothelial cells, smooth muscle cells and
inflammation processes associated with the development of
LDL oxidation [59] and lipid [60] in vitro, which all the
illustrated by an increase in cytokine production [31] ,
of hypoxia pro-angiogenic on macrophage. This is
progression of atherosclerosis is supported by the effects
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 microvessel 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 H2O2, HIF-1α, NF-κB and iNOS in Animal Models Wistar strain of Rattus norvegicus. 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 H2O2 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.

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