Pulpitis Induced Carotid Atherosclerosis

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Abstract

Atherosclerosis on head or neck artery is the primary cause of ischemic stroke which is the leading cause of death at any age in Indonesia. In the last few years, chronic inflammatory due to bacteria, such as pulpitis, has been known to play an important role in the onset of atheroschlerotic pathogenesis. Pulpitis causes bacteremia which affects systemic inflammation. This condition may lead to endothelial cells defunct, elevated oxidation, and lipid deposition which increases the risk of aterosclerosis.

This research aimed to identify the formation of carotid atherosclerotic lesions in pulpitis rat models.

Ten rat samples are divided into 2 groups: a control group (K) without treatment and a pulpitis group (PU). The pulpitis group was established by inducing pulp perforation to the occlusal surface of mandibular first molar teeth. The pulp cavity was then induced with 0.05 ml (0.5 McF) of Streptococcus mutant, 3 times a week for 4 weeks. On the 29th day, rats were sacrified, their carotid arteries were extracted and cross-sectionally cut. Histological preparations were performed and colored by Picrosirius Red and Sudan IV. Histomorphometric analysis began with morphological observation of carotid artery wall thickness, then statistically tested using Independent T-test. Histomorphology analysis began with endhotelial disintegration, lipid deposition and atheroma, then statistically tested using Mann-Whitney method.

The arterial walls of pulpitis group were significantly thicker 102,85 ± 3,37 µm than those of control group ($p<0.05$). There is no significant difference in endothelial disintegration in each group.

The presence of lipid deposition and atheroma are seen at all samples in pulpitis group (100%). Pulpitis increases the risk of carotid atherosclerosis.

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Introduction

Carotid Atherosclerosis is the major cause of ischemic stroke.[1] Stroke, as one of non-contagious diseases, is the third leading cause of death in industrial countries after heart disease and cancer.[2] Atherosclerosis is the hardening and thickening of arterial walls that occurs due to the deposition of fat, complex carbohydrates, blood products, connective tissue and calcium. It is preceded by endothelial injury. Atherosclerosis is characterized by the protrusion of the local blood vessels intima called atheroma plaque.[3]

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In the last few years, many studies proved the role of inflammation which is believed to be an important stimuli to cause atherosclerosis. Observational studies have shown that higher risk of atherosclerosis occurs in patients with pulp infection.[4] *Streptococcus mutants* are the major cause of bacterial caries which, if not treated, can destroy enamel and dentine; they will eventually reach the pulp and cause pulp infection. Pulpitis causes bacteremia which affects systemic inflammation.[5,6,7]

Numerous studies have demonstrated the existence of genomic DNA of some types of oral bacteria. *Streptococcus mutants* bacteria was found in atheroma plaque when analyzed using *Polymerase Chain Reaction* (PCR) through sequencing and alignment of the nucleotide (DNA). From the 27 atheroma plaques specimens analyzed using PCR methods, the detectable *S. mutants* was 74% whereas other

bacterial species, including those related to the cause of periodontitis, was detected with a much lower frequency of occurrence.[8] Earlier report showed that *S. mutants* were detected with high prevalence in the atheroma plaque: 22.5% in younger patients (aged up to 27 years) and 44.4% in older patients (aged up to 67 years).[9]

Carotid artery bifurcation is susceptible to atherosclerosis. The blood flow located near the center of the carotid artery is turbulent and slow. The arterial wall receiving lower blood pressure (<4dyn / cm²) has been proven to be easily induced by endothelial injury due to increased intracellular permeability and prolonged existence of blood atherogenic particles in the area.[10]

Although it has been known that pulpitis is related to atherosclerosis, the causal relationship between pulpitis and atherosclerosis occurring in carotid arteries has not been widely studied. This prompted the authors to perform an experimental research to identify atherosclerotic lessions in carotid arteries pulpitis rat models.

Materials and methods

Ten healthy male Wistar rats aged 3-4 months were divided into 2 groups: control group (K) and the pulpitis group (PU) with 5 samples in each group.[11] Pulpitis rats were prepared by first administering them with anesthesia (IM) ketamine (KTM 1000) dose of 1 ml / KgBW.[12] Cavity was prepared by perforating the occlusal surface of mandibular first molar teeth. The resulting pulp cavity was then induced with 0.05 mL (0.5 McF) of *Streptococcus mutants*, 3 times a week for 4 weeks. The protocol applied to this animal experiment has been approved by the ethics committee of the Faculty of Medicine, Universitas Jember No. 771/H.25.1.11/KE/2016.

Histologic Sample Preparation On day 29, the rats were sacrified. Surgery was performed on the neck to extract the carotid arteries. The arteries were then fixated by using the mixture solution of PBS and formalin 10% (9:1). The incision was performed using *Frozen Section* method producing 10 µm of thickness, stainned with *Picrosirius Red* and *Sudan* IV and *counter Mayer's Hematoxilin*.[13] Rats' lower jaws including the perforated teeth were removed to observe the signs of pulpitis.

Atherosclerosis Parameter The specimens were observed for atherosclerotic lesions morphology, which consisted of the thickness of the arterial wall, the disintegration of the endothelium, lipid deposition and atheroma. Carotid artery wall thickness (µm) was measured from the intima to media on the histological specimens which had been painted with *Picrosirius* Red, under microscope with a 400x magnification. The analysis of atheroma was carried out by observing the same specimens with a magnification of 400x. Atheroma was characterized by the bulging of the inner the blood vessel wall (the intima).

The analysis of lipid deposition was performed using a light microscope with 1000x magnification. The carotid artery where lipid deposition occurred was marked by red color on the medial layer of subendothelial or on the endothelial specimens painted with *Sudan.* Endothelial disintegration was examined in the same specimens that have been painted with *Sudan* using *counter stain Mayer's Hematoxilin* which revealed the nucleus of endothelial cells, visible as purple-colored. Observations were performed using a light microscope with a magnification of 1000x. Endothelial disintegration was characterized by discontinuities or peeling-off of carotid artery endothelial cells (denudation).

Data Analysis Quantitative data from the arterial wall thickness measurements were tested for normality using Kolmogorov-Smirnov test, for homogeneity using Levene test and then analyzed with T-test. Disintegration of endothelial morphology, lipid deposition and atheroma were analyzed with *Mann-Whitney U* test.[14]

Results

The results showed the occurrence of pulpitis on the mandibular first molars in animals which had undergone pulp perforation and *Streptococcus mutants* injection. Pulpitis is characterized by the occurrence of deep cavities which led to pulp perforation. Inflammatory cells were also found in the pulp cavity which outnumbered those in the control group (Figure 1).

Clinical observation shows that *miller* needle can penetrate the cavity with a depth of ± 2mm. Radiology imaging results support this clinical conditions, revealing that *miller* needle penetrates down to the pulp chamber of the tooth (Figure 2).

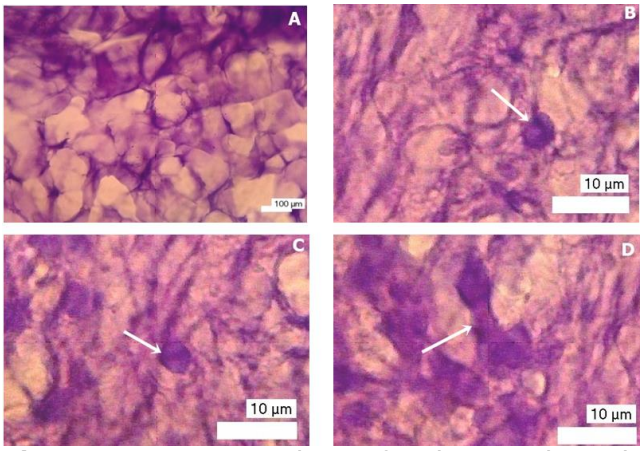

Figure 1. An overview of microscopic pulp chamber, with 1000X magnification. There were no inflammatory cells in control group **(A)**. In pulpitis group, inflammatory cells (arrows) were found as PMN **(B)**, lymphocytes **(C)**, and macrophages **(D)**.

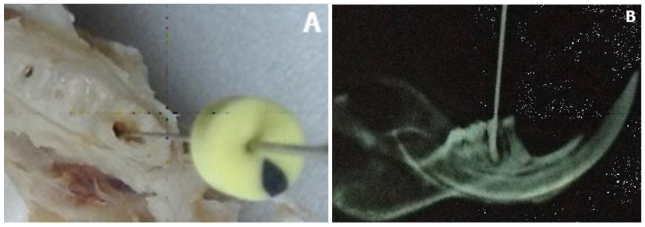

Figure 2. A. The clinical features of the lower left jaw cavity of pulpitis group. **B.** Radiology of the lower left jaw cavity of pulpitis group. There were deep cavities perforating the pulp as indicated by the entry of the *miller* needle.

Atherosclerosis Symptoms	Percentage (%)		Sig. (p)
	Control (n = 5)	Pulpitis (n = 5)	
Disintegration endothelial	100	100	1,000
lipid deposition	0	100	0,008*
Atheroma	0	100	0,008*

Table 1. Results of Mann -Whitney histomorfometric carotid atherosclerotic lesions.

Description:
n : the number of samples in one group
* : significant difference (p <0.05)

The signs of atherosclerotic lesions histomorphology in the carotid artery of the pulpitis group are more prevalent than those of the control group. The observed signs of atherosclerotic lesions consisted of disintegration of the endothelium, lipid deposition and atheroma. Mann-Whitney test analysis (Table 1) showed that lipid deposition and atheroma differed significantly (p <0.05) between pulpitis and control groups. There was no significant difference in the endothelial disintegration (p> 0.05) between the groups.

Identification results of endothelial disintegration showed that the formation of endothelial disintegration occurred on all samples (100%) in both control and pulpitis groups. It is characterized by discontinuities or the peeling off of carotid artery endothelial cells (denudation) (Figure 3).

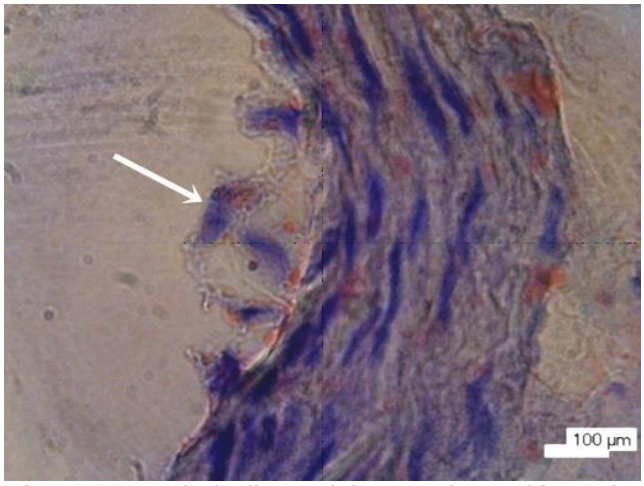

Figure 3. Endothelium Disintegration, with Sudan staining (1000X magnification). Endothelium disintegration (arrows) in the form of discontinuities and endothelial cells detachment from the walls of the carotid arteries.

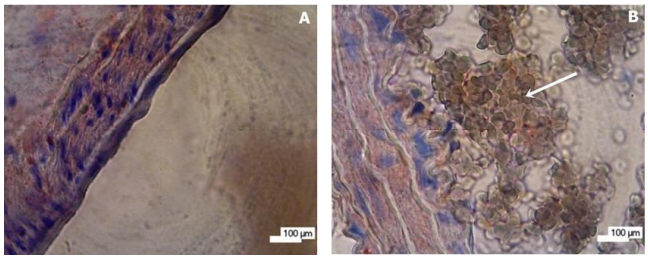

Figure 4. Lipid Deposition, with *sudan* staining (1000X magnification). No lipid deposition in the control group (A). Lipid deposition (arrow) in the sub-endothelial layer in pulpitis group (B).

The observation of lipid deposition showed that the formation of lipid deposition occurred in all samples (100%) in the pulpitis group, however it was not found in the control group (0%). Lipid deposition with *sudan staining* is characterized by reddish color around the layer of the intima (Figure 4).

With *Picrosirius Red* staining, atheroma formation was found in the whole sample of pulpitis group (100%), while none was found (0%) in the control group. Atheroma is characterized by artery wall (the intima) bulging toward the luminal (Figure 5).

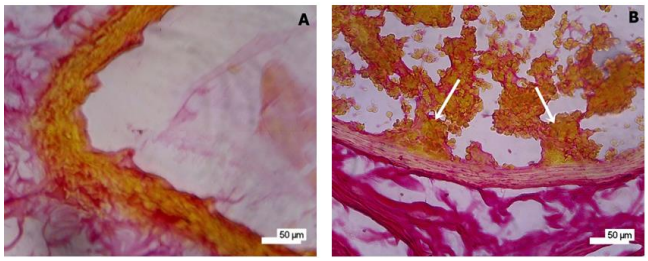

Figure 5. Atheroma, with *Picrosirius Red* staining (400X magnification). (A) Control group, the arterial wall appears regular, with no evidence of atheroma. (B) Pulpitis group, evidence of atheroma (arrows) as characterized by the protrusion of the wall toward the lumen and the irregular features of the wall.

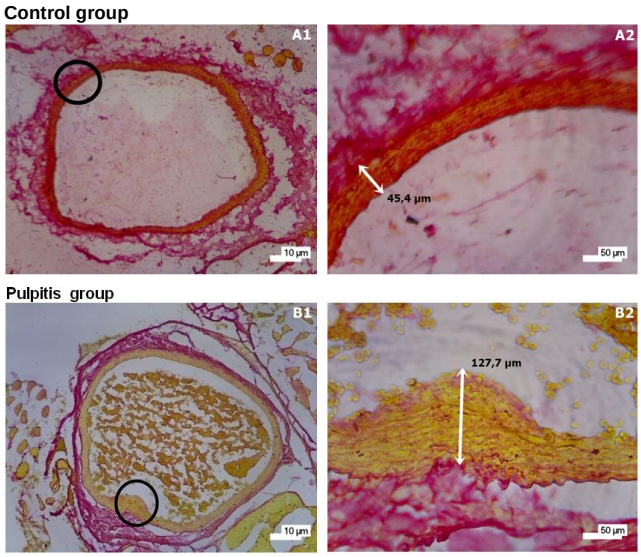

Figure 6. Wall thickness of Carotid Artery, with *Picrosirius* Red staining (100X and 400X magnification, respectively). The control group, no thickening of the arterial walls (A2) and regular shape of the walls (A1). Pulpitis group, thickened arterial walls (B2) and irregular walls (B1).

The measurements of the carotid artery wall thickness showed that the average thickness of carotid artery wall in pulpitis rat model was thicker than of the control group. (Figure 6). The test for normality and homogeneity yielded normal and homogeneous data (p> 0.05). *Independent*-T test (Table 2) showed that the wall of the carotid artery in pulpitis groups were significantly thicker (p <0.05) than the control group. The average thickness in the control group was 67.67 ± 9.26 µm, whereas 102.85 ± 3.37 µm in pulpitis group.

Group	N	Wall Thickness X ± SD (µm)
Control	4	67.67 ± 9.26
Pulpitis	4	102.85 ± 3.37
T test Sig. (p)		0,000 *

Table 2. Test results: *Independent*-T carotid artery wall thickness (intima-media).
Description:
n: number of specimens
X: Average
SD: standard deviation
*: significant difference (p <0.05)

Based on the histological features (Figure 6), carotid artery wall thickening and asymmetric / irregular luminal surface of the artery wall were found in pulpitis group, whereas the luminal wall surface of the control group appeared flat and without thickening. The thickening of the walls in the pulpitis group occurs in the layer of elastin fiber, which appeared yellow due to *Picrosirius Red* staining.

Discussion

The results showed that the carotid atherosclerotic lesions are significantly higher in the pulpitis group than those in the control group. They were analyzed by observing the symptoms of carotid atherosclerotic lesions which included histomorphometric in the form of arterial wall thickening and histomorphology in the form of disintegrating endothelium, lipid deposition, and atheroma. These results are supported by observational study which showed that patients with pulpitis had greater risk of atherosclerosis.[4] *Streptococcus mutans,* the main bacteria that causes caries, are found in the atheroma plaques of blood vessel wall.[8,18]

The spread of dental pulp infection to the blood circulation may occur immediately by the penetration through blood vessels contained within the pulp. *Metastatic infection*, the spread of bacteria to the blood circulation, occurred - hence causing bacteremia. *Metastatic inflammation*

associated with *host* immune system also occurred. The latter is when the antigen in the dental pulp spreads to the blood circulation and reacts with antibodies to form immune complexes that will trigger acute and chronic inflammatory reactions. This event is marked by phagocytes response, especially neutrophils that will become phagocyte which then destroy either the bacterial antigens or the whole bacteria. The bacteria destruction mechanism by neutrophils is performed by producing prooxidative toxic materials such as *Reactive Oxygen Species* (ROS) in the forms of oxidants and enzymes.[15,16,17,19] *Streptococcus mutans* has a protein antigen c (PAc) associated with systemic virulence of the blood flow. The PAc level of phagocytosis by *polymorphonuclear* leukocytes is low, causing an imbalance in the amount of ROS produced by neutrophils with anti-oxidants in the body. This is called oxidative stress.[8]

The observations of carotid atherosclerotic lesions histomorphologic revealed that there was endothelial disintegration in all samples of carotid artery of both control and pulpitis groups as indicated by the endothelial denudation. In the pulpitis group, the chronic pulp infection by the *S. mutans* bacterium can indirectly stimulate a systemic inflammatory response causing oxidative stress. Oxidative stress may lead to decreased bioavailability of nitric oxide (NO), resulting in endothelial dysfunction by ways of peroxynitrite (ONOO) formation and NO synthesis pathway inhibition. The decreased levels of NO causes the endothel to be more proaterogenic and proinflammatory thus it is prone to endothelial disintegration.[20,21,22]

In this study, all samples in the control group also indicated endothelial disintegration. The underlying factor causing endothelial disintegration is thought to be carotid artery anatomical factors. According to *response to injury* hypothesis, blood flow can cause endothelial denudation in a certain region. [23]

In this study, a part of the bifurcation of the carotid artery is used. In that particular region, there was a peculiar change to the typical blood flow: decreasing *shear stress* and increasing turbulence. The changes in blood flow will increase the gene expression of endothelial cells *(ICAM-1 gene, PDGF* and tissue factor) when there is a decrease in *shear stress*.[24]

The area with low *shear force* (<4dyn / cm²) may induce endothelial injury. Another suspected factor which could affect endothelial disintegration is age, specifically those mice in age group of 3-4 months. Disintegration of the endothelial walls of arteries begins early in life. Many of those factors affecting the initial lesion will progress to pathological or even just as symptomatic lesions, depending on individual hemostasis, metabolic, environmental, and genetic risk. Nevertheless, the main factor of the vulnerability of the atherosclerotic plaque formation is inflammation. In the control group, the lesions do not progress to more advanced atherosclerotic lesions because there is no inflammatory responses.[10]

The observation of lipid deposition showed that its formation was found in all pulpitis group samples but not in the control group. Oxidative stress occurring in the pulpitis group also causes blood vessels to become more permeable. Low-density lipoprotein (LDL) gains easier entry into blood vessel's autonomous muscles. The state of oxidative stress causes LDL to easily oxidize into LDL-ox. This LDL-ox will then be recognized by the *macrophage receptor Scavenge* that do not experience *down-regulation.* This leads to macropinositosis-the macrophages which constantly fagocytes LDL-ox which then form foam cells. These foam cells are those forming lipid deposition subendothelial.[25,26,27]

In the research, atheroma formation was discovered and there was thickening of the carotid artery walls in the whole pulpitis sample group (100%). Those were not the cases in the control group. The state of pulpitis in the continuously treated group causes sustained inflammation. The situation then becomes increasingly chronic resulting in cytokines release and growth factor.[28]

These outcomes can stimulate the proliferation and the migration of autonomous cells from the tunica media to the intima. The thickening of the arterial wall occurs when autonomous muscle cells migrate to the intima. The development of lesions which leads to the change of autonomous muscle cells function causes the formation of *fibrous* cap. When the *fibrous cap* is formed, the lesion is called atheroma. It then allows the protrusion of the arterial lumen causing the reduction of the lumen's diameter.[10]

Conclusions

Pulpitis increases the risk of formation of carotid atherosclerotic lesions which is characterized by the thickening of the carotid artery wall, the disintegration of the endothelium, lipid deposition and atheroma. Further research is needed to measure the degree of systemic inflammation, the degree of bacteremia and circulation antigen.

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Declaration of Interest

The author declare that there is no conflict of interest regarding the publication of this article.

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