Chapter 4

Findings and Results

4.1 Preparation of LDL in various degrees of oxidation and oxidative assessment

Human native LDL isolated from healthy volunteer blood and induced oxidation by using $CuSO_4$ to be mildly, moderately and fully oxLDL as described in chapter 3. We assessed lipid peroxidation by malondialdehyde (MDA) in thiobarbituric reactive substance (TBAR) assay and conjugated diene formation by continuously monitored diene kinetic test. Together, we assessed protein oxidation by running on 1 % agarose gel electrophoresis and calculated the distance migration of LDL band compared with bovine serum albumin (BSA) standard and measured relative electrophoretic mobility (REM). Moreover, we determined apolipoprotein B (apo B) fragmentation during oxidation by running on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) at 10 % polyacrylamide of separate gel and 4 % polyacrylamide of stacking gel. We used the assessment criteria from various degrees of oxLDL as cited by Anthonsen and Kennedy as following shown data in table 4.1(Anthonsen, Stengel, Hourton, Ninio, & Johansen, 2000; Kennedy, et al., 2003).

Table 4.1

Criteria of various degree oxidation of oxLDL

Degrees of oxLDL	TBARs (nmol/mg)	REM *	Lag phase (min)
Native LDL	< 1.3	0.3	> 90
Minimally oxLDL - mildly oxLDL	1.3-8	0.56 – 0.7	60– 90
Moderately oxLDL	13.1-25	> 0.7	< 60
Fully oxLDL	> 26	0.9	< 45

* REM; measure the distance of oxLDL in cm./standard BSA distance (cm)

Our data as shown in table 4.2 represented our oxLDL preparation and we obtained three different batches: mildly, moderately and fully oxLDs.

Table 4.2

Showing the characterization of oxLDL in three different degrees of oxidation, lag phase is determined in the initial step of diene formation curve.

Degrees of oxLDL	TBARs (nmol/mg)	REM*	Lag phase (min)
Mildly oxLDL	9 - 11	0.45 - 0.48	> 60
Moderately oxLDL	12 - 24.2	0.48-0.56	32 – 55
Fully oxLDL	> 25	0.8 – 0.9	< 20

* REM; measure the distance of oxLDL in cm./standard BSA distance (cm)

Each of oxidative characterization of LDL is presented below.

4.1.1. Conjugated diene test

Figure 4.1

Showing the lag phase (min) of mildly degree oxLDL group

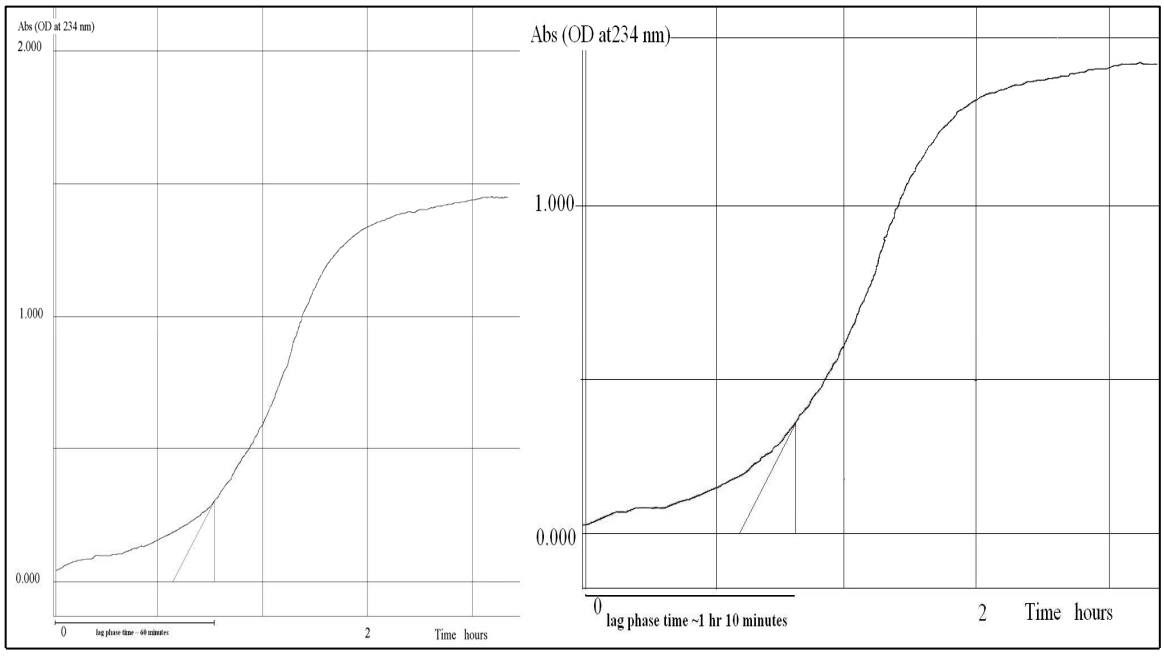


Figure 4.2

Showing the lag phase (min) of moderately degree oxLDL group

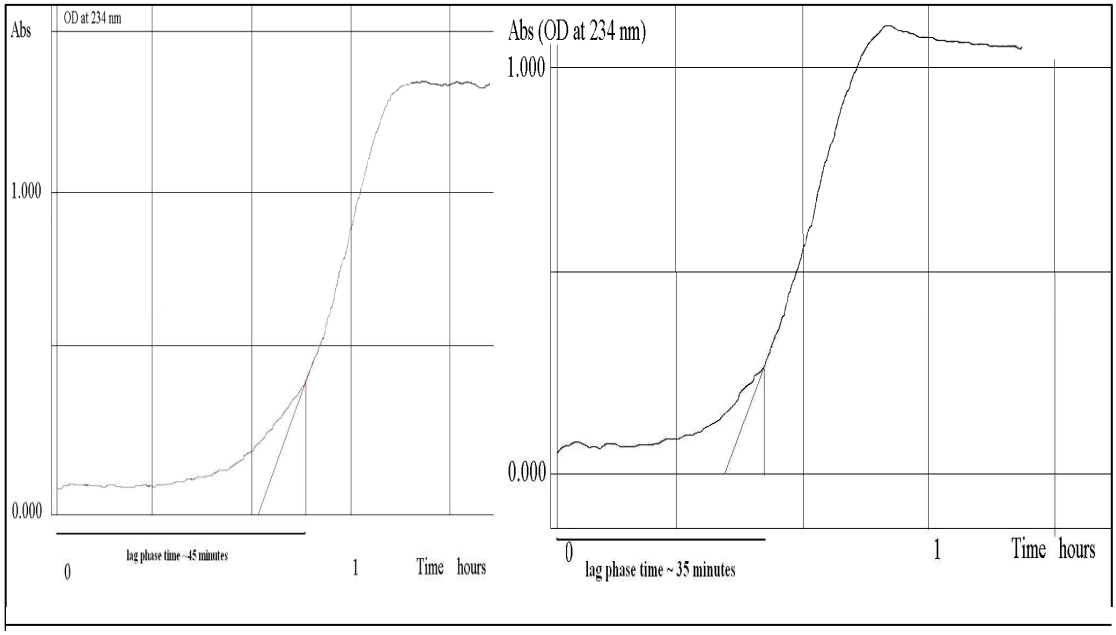
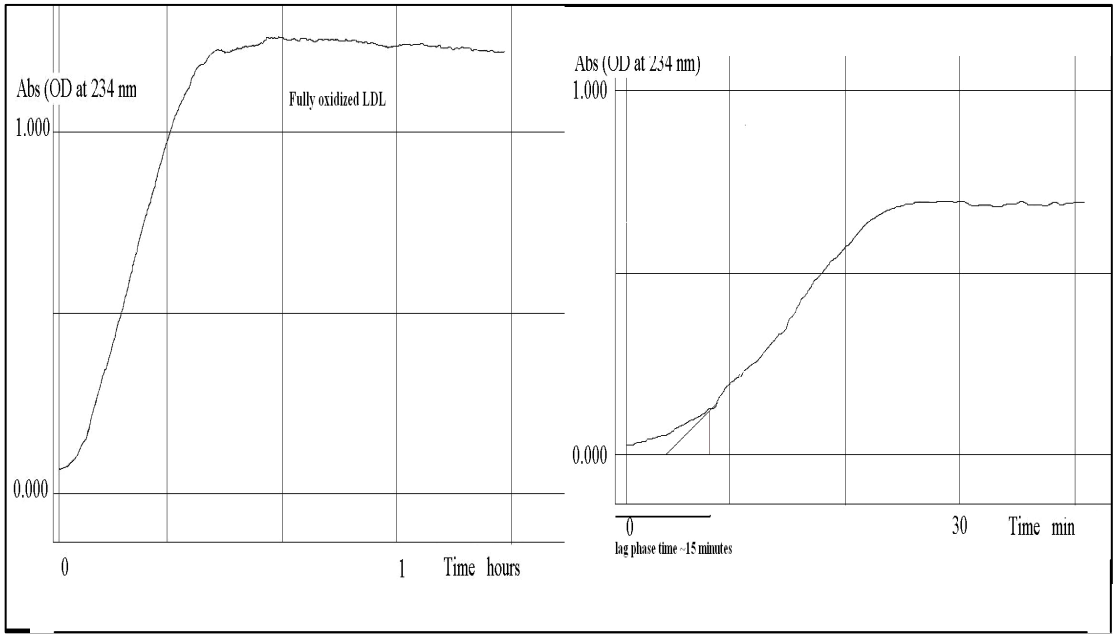


Figure 4.3

Showing the lag phase (min) of fully degree oxLDL group

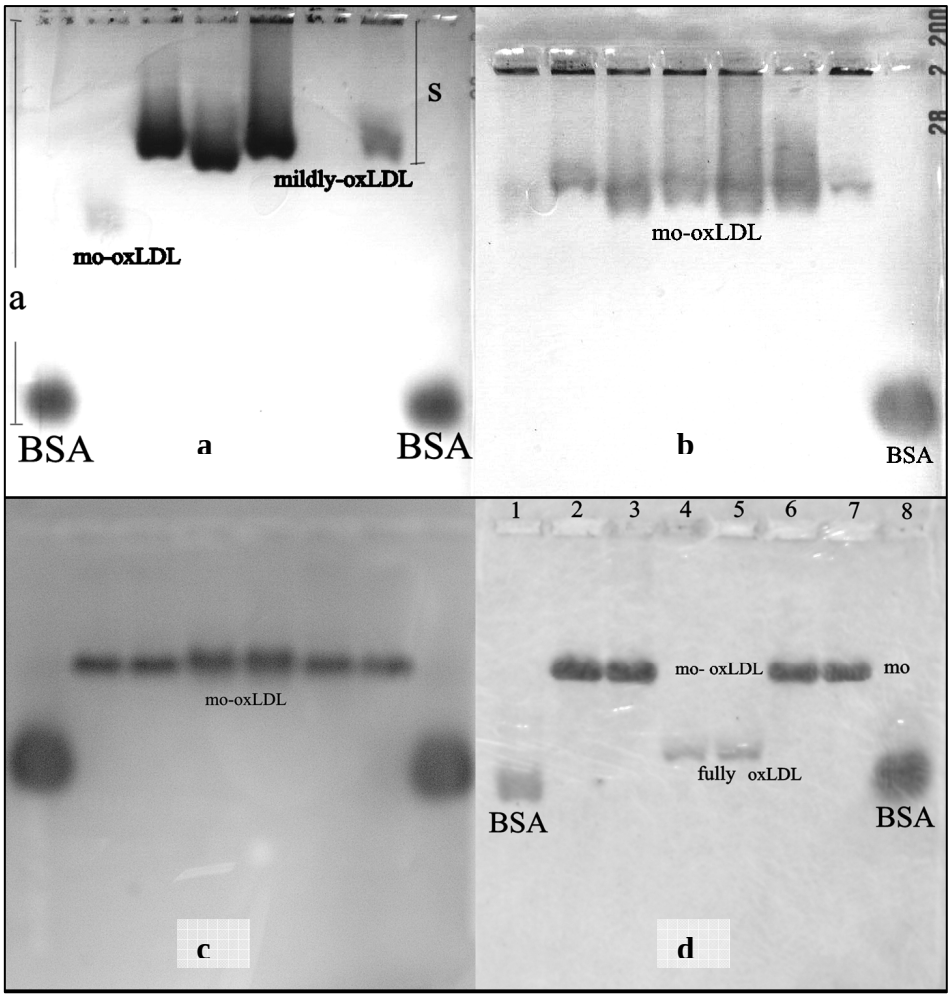


Conjugated diene test of each various degree of oxLDL was presented. Lag phase indicated oxidative susceptibility and was determined in an initial step of diene formation curve in minute.

4.1.2. Relative electrophoretic mobility (REM) of oxLDL on 1% agarose gel electrohoresis

Figure 4.4

Relative electrophoretic mobility (REM) of both moderately and fully oxLDL was shown REM was calculated from the distance of oxLDL band and BSA band (s/a)



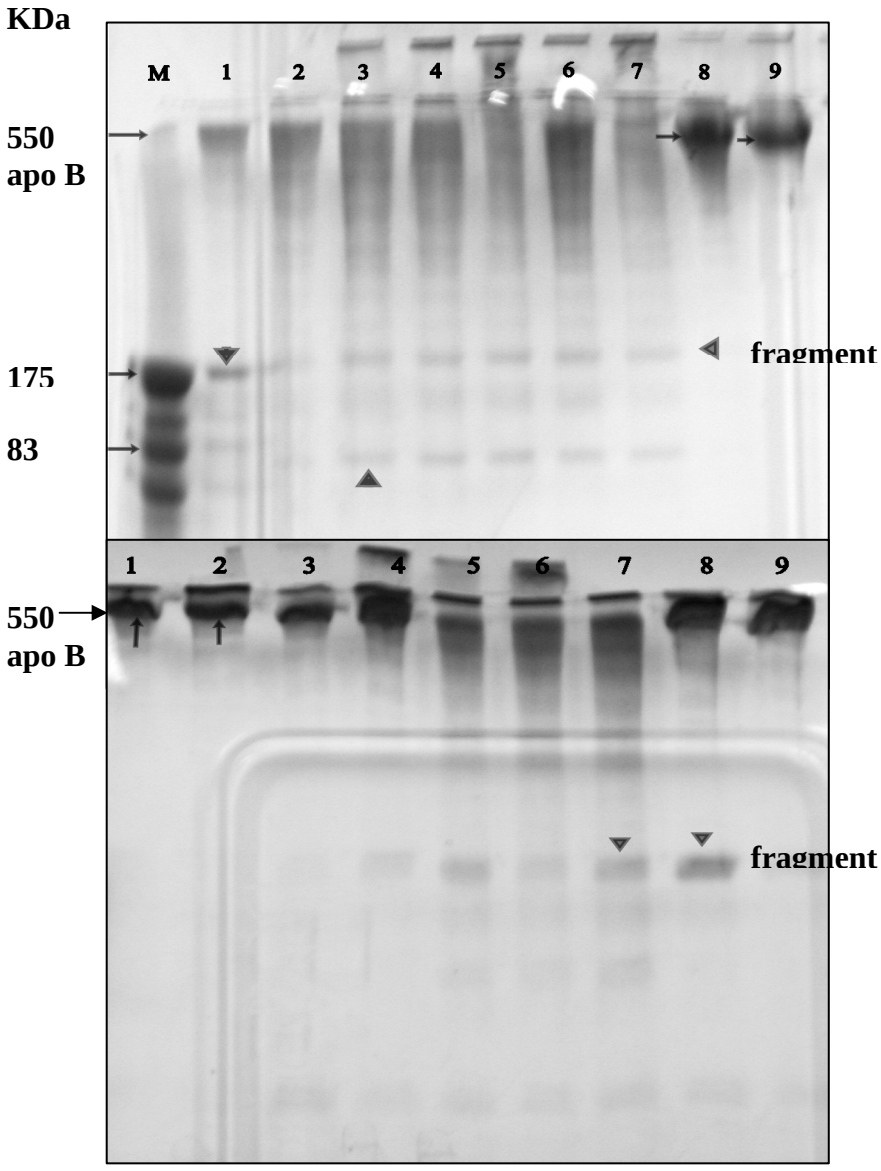
Lane (d) 1, BSA; 2 and 3, moderately oxLDL; 4 and 5, fully oxLDL; 6 and 7 moderately oxLDL; 8, BSA

4.1.3 Apolipoprotein B – 100 (apo-B) of oxidized LDL

Lipid peroxidation of oxLDL causes aldehyde compound that is readily forms Schiff bases with the lysine groups of apo B (Haberland, Olch, & Folgelman, 1984; Steinbrecher, 1987). Running of oxLDL on SDS–PAGE showed apo B fragmentation. Molecular weight of apo B is approximately 550,000 Da. Figure 4.5 showed Apo B fragmentation.

Figure 4.5

SDS-PAGE of apo B fragmentation



lane 1-7 (upper figure) and lane 4-8 (lower figure)

4.2 Various degrees of oxLDL influence LOX-1 expression in cultured human umbilical artery

Several lines of evidence indicate that oxLDL found at atherosclerotic lesion and in plasma which played a crucial role in pathophysiology of atherosclerosis. We hypothesized that various degrees of oxLDL as mildly, moderately and fully oxidation as described in chapter 2 influence the expression of LOX-1 receptor. An increased uptake of oxLDL has been reported and also upregulated LOX-1 expression in endothelium cell (Li & Mehta, 2000). We used a powerful cultured human umbilical artery that represents to complete structure of endothelium cell, smooth muscle cell, extracellular matrix and adventitia fibroblast to be definite model study. Moreover, we used three different degrees of oxidation as mildly, moderately and fully oxLDL represent to those oxLDL entrapped in the vascular wall that seen in early, intermediate and late steps of atherosclerosis.

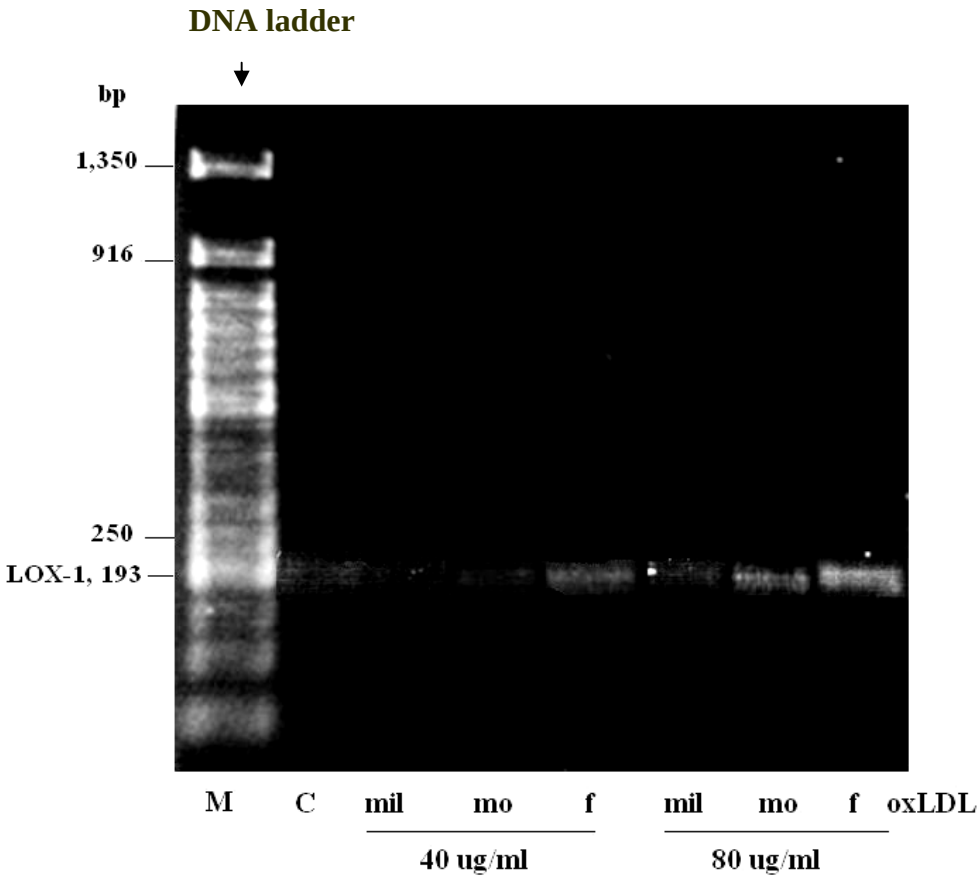
Result

Various degrees of oxLDL as mildly, moderately and fully oxidation in dose of 40 μg/ml increase the percentage of LOX-1 expression as 26.1, 51.8, 40.3 (p value < 0.05), respectively. Significant difference of moderately and fully oxidation was found when compared with control. In addition, 80 μg/ml oxLDL resulted gradually increased percentage of LOX-1 expression in mildly, moderately and fully oxidation at 40.3, 61.3 and 76.4, respectively. Significant level was also shown when compared with control. An increased LOX-1 expression through mildly, moderately and fully oxLDL in 80 μg/ml was significantly differed when compared among those groups but no significant difference in 40 μg/ml.

These findings suggest that LOX-1 are expressed as dose-degree dependent effect of oxLDL.

Figure 4.6

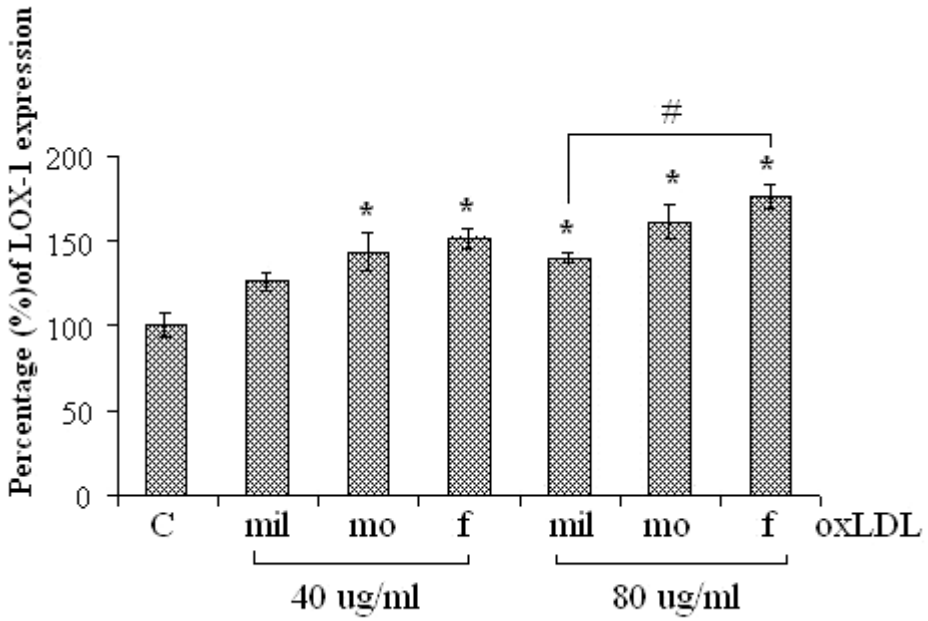
LOX-1 expression activated by 40 and 80 µg/ml oxLDL in various doses and degree oxidation: mildly, moderately and fully oxidation on 1.5 % gel electrophoresis.



mil = mildly; mo = moderately; f = fully; C =control, oxLDL = oxidized LDL

Figure 4.7

Quantitation of LOX-1 expression in percentage of expression was presented with significant difference when compared with control (*) and among groups (#)



Data are mean ± SEM and *, # significantly differed when $p < 0.05$.

mil = mildly; mo = moderately; f = fully; C =control, oxLDL = oxidized LDL

4.3 Influence of iron chelator and peroxyl radical scavenger pretreatments on LOX-1 expression

4.3.1 Effect of desferoxamine (DFO), a specific ferric chelator of Haber-Weiss reaction, on LOX-1 expression induced by oxLDL

Since oxLDL in various degrees oxidation and doses activate the expression of LOX-1, we postulated that upregulation of LOX-1 expression mediates intracellular reactive oxygen species (ROS). We used desferoxamine (DFO) as a specific ferric (Fe^{3+}) chelator of Haber-Weiss reaction inhibits H_2O_2 and highly reactive $^{\bullet}OH$ formation (Steinbrecher, Parthasarathy, Leake, Witztum, & Steinberg,

1984) that driven from $O_2^{\bullet-}$. As we known *in vivo,* highly reactive $^{\bullet}OH$ radical has very short half-life, approximately 10^{-9} second, but it has more potentiation than other radicals (Pastor & Hadengue, 2000). We added 10 μM DFO (Mollnau, et al., 2002) as pretreatment for 45 min in the culture system in order to limit or reduce metal transition elements that will catalyst ROS formation. We speculated the expression of LOX-1 might be increased or decreased is depend on vascular source ROS production and predictable peroxynitrite ($ONOO^{-}$) formation from $O_2^{\bullet-}$ and NO in the system.

Result

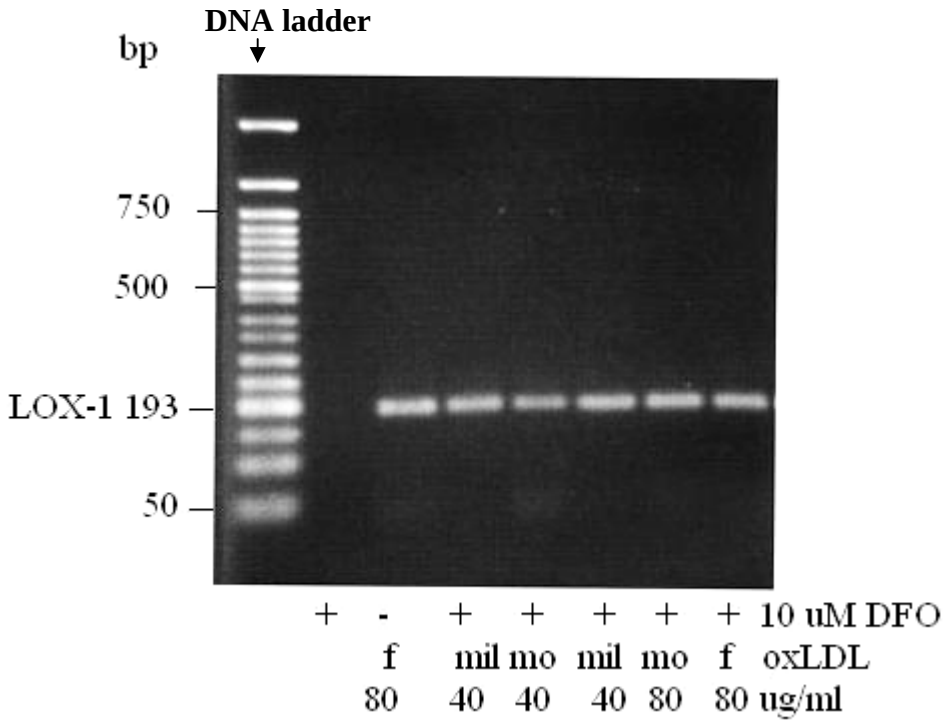
Overexpression of LOX-1, approximately 101.5, 89.6 and 68.5 % compared with control was presented by mildly, moderately and fully oxLDLs in 40 μg/ml of 10μM DFO pretreatment group, respectively. Moreover, significant higher expression was seen clearly in mildly and moderately oxLDL in DFO pretreatment group compared with oxLDL treatment group only (figure 4.9a).

In figure 4.9b, upregulation of LOX-1 expression was shown in DFO pretreatment group with 80 μg/ml oxLDL in moderately and fully oxidation. Significant difference between 80 μg/ml oxLDL group and those with 10μM DFO pretreatment group in fully oxidation was shown.

This findings suggest that upregulation of LOX-1 expression activated by oxLDL in various degree and doses mediated intracellular reactive oxygen species (ROS) which is further increasing expression when pretreatment with 10μM DFO. DFO, a specific ferric chelator, will reduce subsequent radical, H_2O_2 and highly reactive $^{\bullet}OH$. If so, LOX-1 expression should be reduced in DFO pretreatment group and might be result from ROS available in the system and additional peroxynitrite which generated from combination of $O_2^{\bullet-}$ and NO. Rate of dismutation by SOD is usually slower than rate of $ONOO^{-}$ formation. Our data indicate major ROS source generated in vasculature are from activated enzymatic ROS production such as NADPH oxidase rather than metal-transition driven ROS path.

Figure 4.8

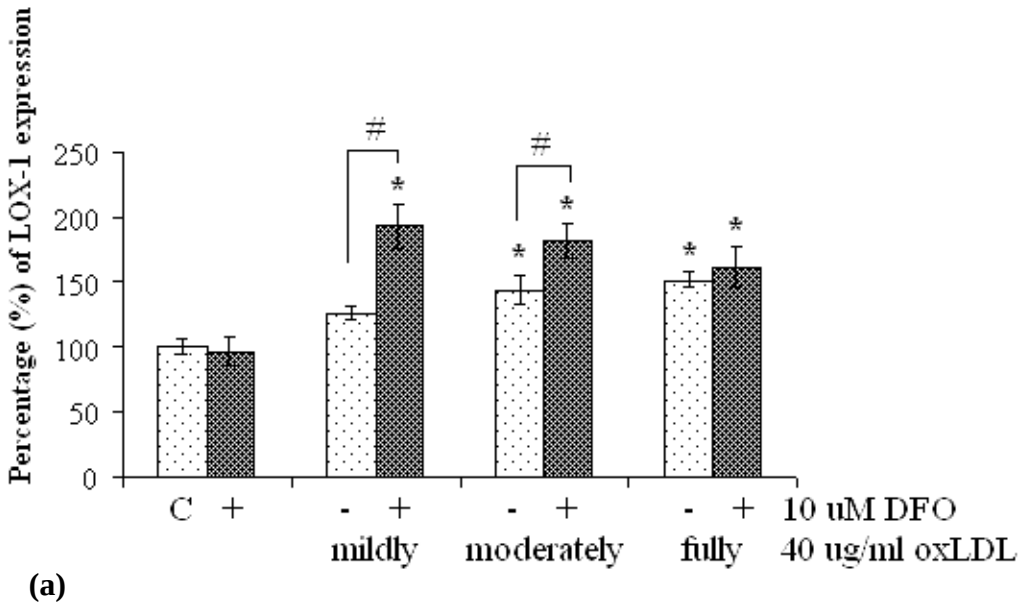
Showing LOX-1 expression induced by 10 μM DFO pretreatment and various degree oxidation. Doses of oxLDL as indicated in each lane.



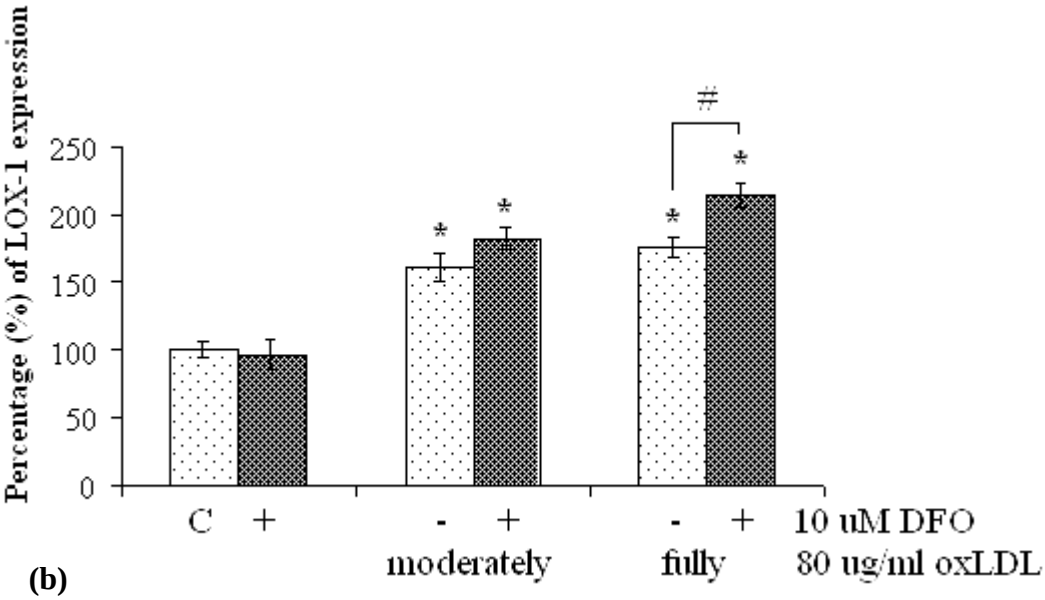
mil = mildly; mo = moderately; f = fully; C =control, oxLDL = oxidized LDL

Figure 4.9

(a) Percentage of LOX-1 expression of 40 μg/ml oxLDL (b) and 80 μg/ml oxLDL in 10 μM DFO pretreatment when compared with control (*) and among groups (#).



(a)



(b)

Data are mean ± SEM. *, # Significantly differed when $p < 0.05$

4.3.2 Effect of ethylenediaminetetraacetic acid (EDTA), a ferrous (Fe^{2+}) chelator of Fenton's reaction, on LOX-1 expression

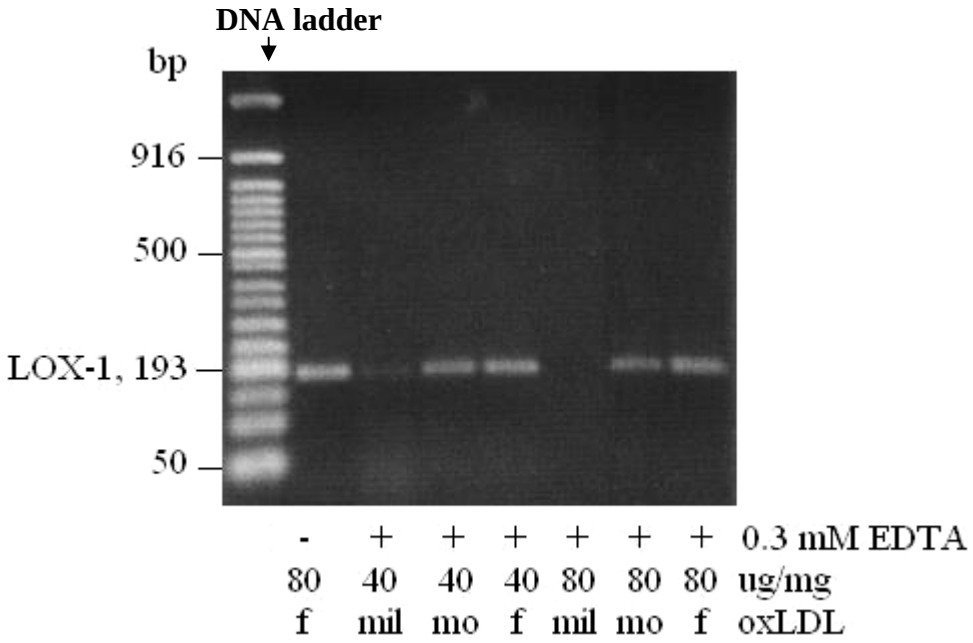
We confirmed intracellular ROS formation in the vascular wall involved in the activation of oxLDL through LOX-1 receptor by using EDTA as ferrous (Fe^{2+}) chelator in Fenton's reaction step. 0.3 mM EDTA pretreatment provided limitation of metal transition catalyst in the system and also inhibited highly reactive $^{\bullet}OH$ formation driven from H_2O_2. Hence, in the system, there is amount of H_2O_2 available and cell membrane, lipid peroxidation and other inflammatory response (Khouw, Parthasarathy, & Witztum, 1993). We anticipated LOX-1 expression activated by oxLDL in EDTA pretreatment group should be reduced if highly reactive $^{\bullet}OH$ was limited definitety in the system. Another possibility was the effect of $ONOO^-$ would promote greater LOX-1 expression.

Result

Inducible LOX-1 expression of 0.3 mM EDTA pretreatment group remained greater expression when compared with 40 μg/ml oxLDL group. Those of moderately and fully degree reached significant difference compared with the control group. Notably, LOX-1 expression of 0.3 mM EDTA pretreatment group was reduced remarkably when compared with 80 μg/ml oxLDL group in mildly, moderately and fully degree. Furthermore, gradual increase in LOX-1 expression at 30.9 %, 50.1 % and 61.3 % was seen clearly in 0.3 mM EDTA pretreatment group in degree of mildly, moderately and fully, respectively (Figure 4.10, 4.11). It indicated that enhanced inducible LOX-1 expression involved ROS mainly. Contrast to DFO pretreatment group, lowered LOX-1 expression compared with those activated by 80 μg/ml oxLDL only might indicate the role of highly reactive $^{\bullet}OH$ which strongly potentiated than $O_2^{\bullet-}$ and H_2O_2 (Biondi, et al., 2006).

Figure 4.10

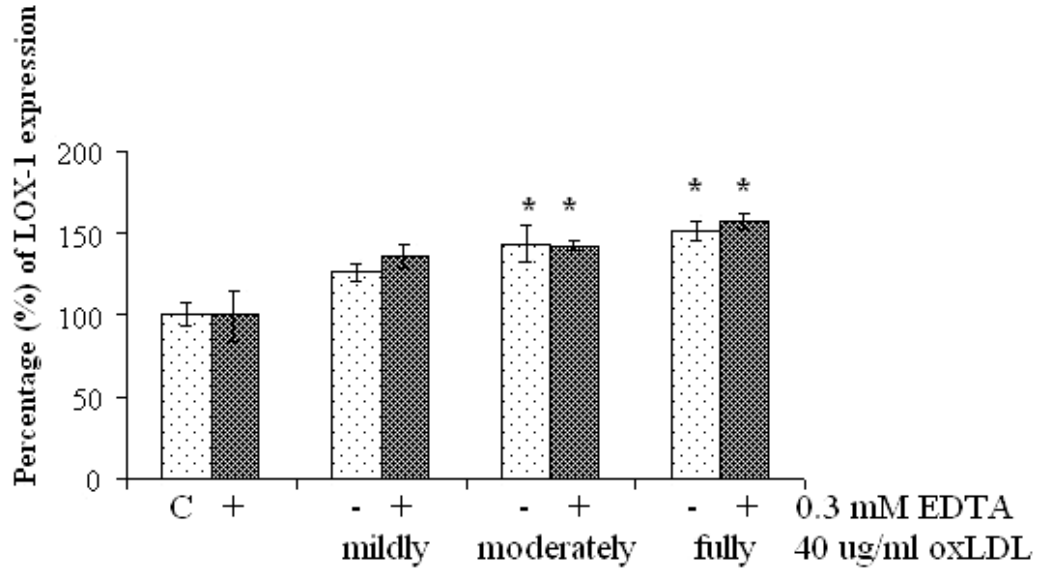
Inducible expression of LOX-1 pretreatmented with 0.3 mM EDTA and oxLDL in various doses and degrees.



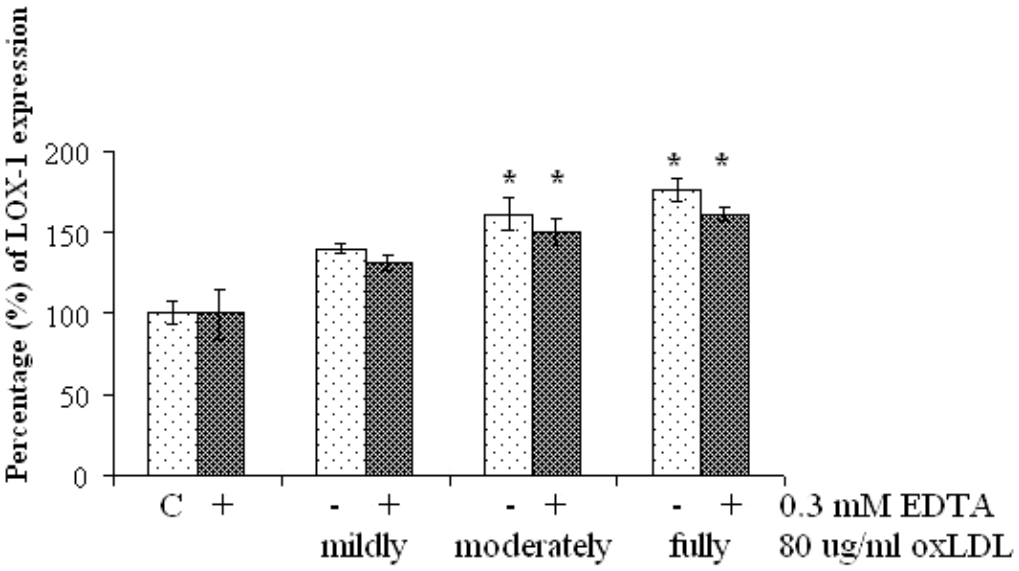
mil = mildly; mo = moderately; f = fully; C =control, oxLDL = oxidized LDL

Figure 4.11

(a) Quantitation of LOX-1 expression of 0.3 mM EDTA pretreatment group compared with 40 μg/ml oxLDL group. (b) LOX-1 expression activated by 80 μg/ml oxLDL compared with those of 0.3 mM EDTA pretreatment group.



(a)



(b)

Significantly difference of those groups compared with control (*). Data are mean ± SEM. Significantly differed when $p < 0.05$. Each band is indicated: mil; mildly, mo; moderately, f; fully

4.3.3 Effect of butylated hydroxytoluene (BHT), a peroxyl radical scavenger of oxLDL on LOX-1 expression

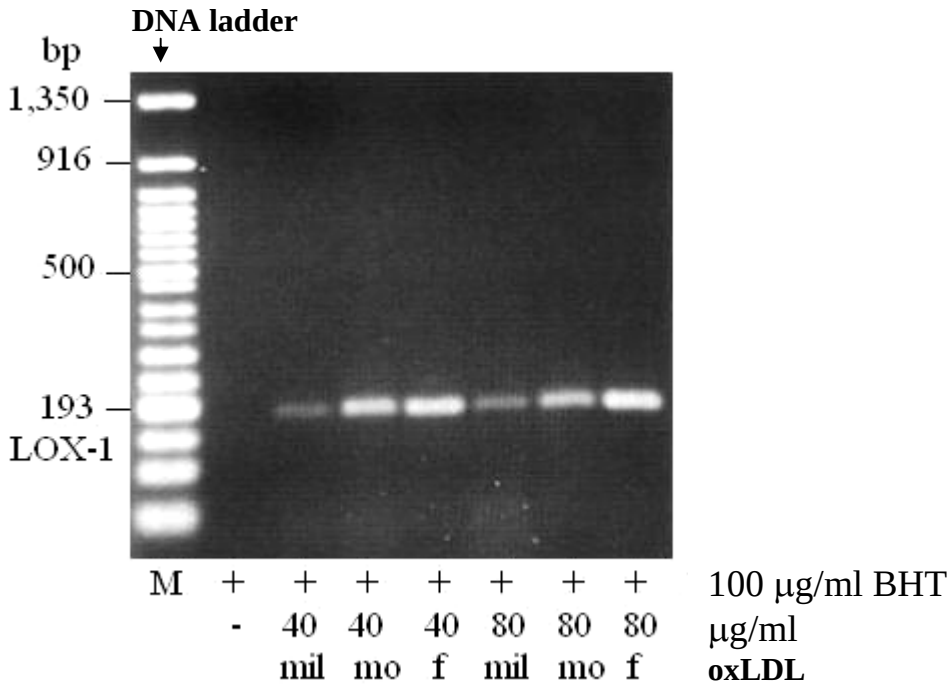
We hypothesized that upregulation of LOX-1 expression induced by oxLDL mediated by intracellular ROS and once it was activated, it might overwhelm and attack many enzymes, proteins, and lipids or even, smooth muscle cell, extracellular matrix and oxLDL itself. We limited auto-oxidation of oxLDL in the culture system by using 10% FCS that containing antioxidant in serum. Meanwhile, we used BHT for peroxyl radical scavenger breaking lipid chain peroxidation of oxLDL and converted peroxyl radical to hydroperoxide. BHT acts as antioxidant and did not affect any step in ROS formation as DFO or EDTA did. We expected that BHT had little effect on LOX-1 expression since it did not mediate ROS pathway.

Result

Inducible LOX-1 expression was still greater in 100 μg/ml BHT pretreatment group than those of 40 and 80 μg /ml oxLDL in various degree. Increasing LOX-1 expression of BHT pretreatment group was significant higher than those of 40 and 80 μg /ml oxLDL in fully degree when compared with control. Those LOX-1 expression of BHT pretreatment and oxLDL group were significant greater. As we known, BHT acted as antioxidant and protected oxLDL to further oxidation if there was ROS/RNS available in the system and caused more oxidation of oxLDL. Inducible LOX-1 expression was gradally increased in BHT pretreatment group with increasing degree and doses of oxLDL might indicate pro-oxidant of BHT as the same as vitamin E or vitamin C did. Obviously, inducible LOX-1 expression in BHT pretreatment group was greater and these oxLDL in various doses and degree may support the pro-oxidant of BHT (Figure 4.12, 4.13).

Figure 4.12

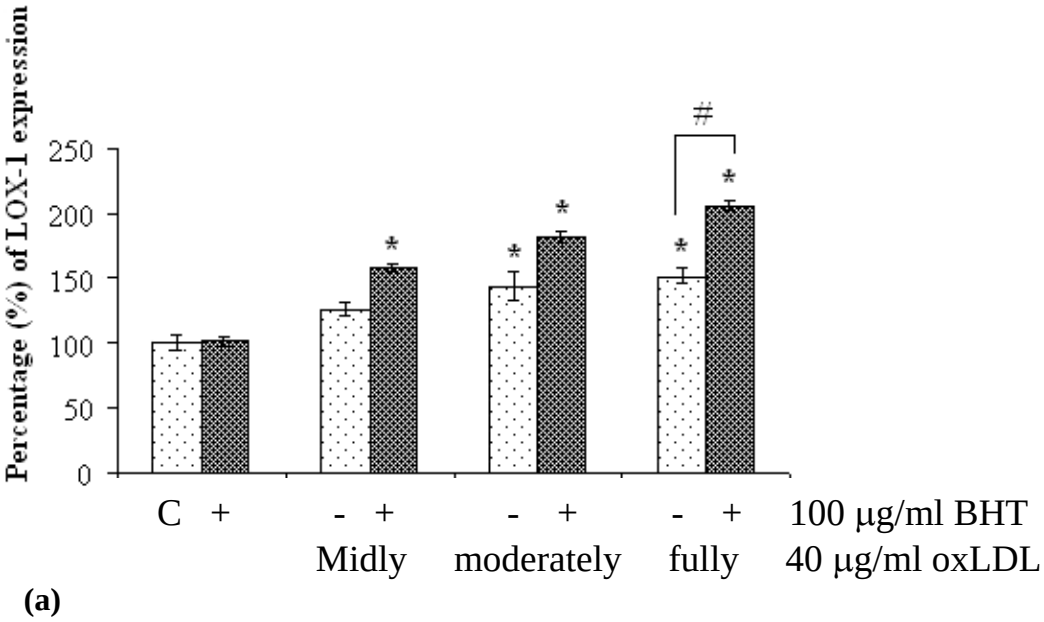
Inducible expression of LOX-1 pretreatmented with 100 μg/ml BHT and oxLDL in various doses and degrees.



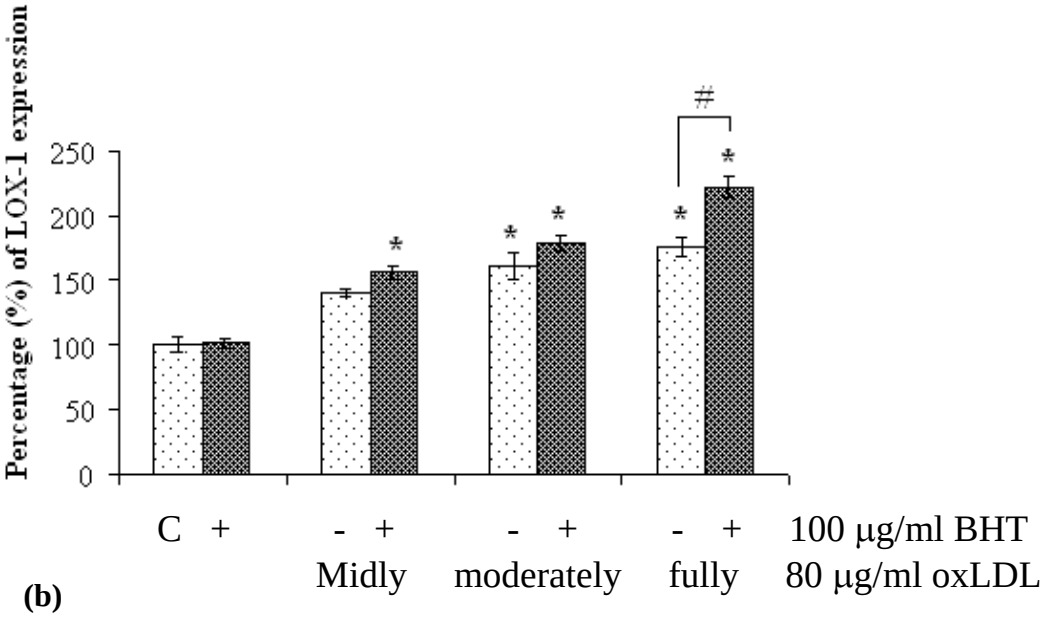
mil = mildly; mo = moderately; f = fully; C =control, oxLDL = oxidized LDL

Figure 4.13

(a) LOX-1 expression activated by 40 μg/ml oxLDL and 100 μg/ml BHT pretreatment. (b) LOX-1 expression activated by 80 μg/ml oxLDL and 100 μg/ml BHT pretreatment.



(a)



(b)

Significantly different compared with control (*) and among groups (#). Data are mean ± SEM. mil; mildly, mo; moderately, f; fully

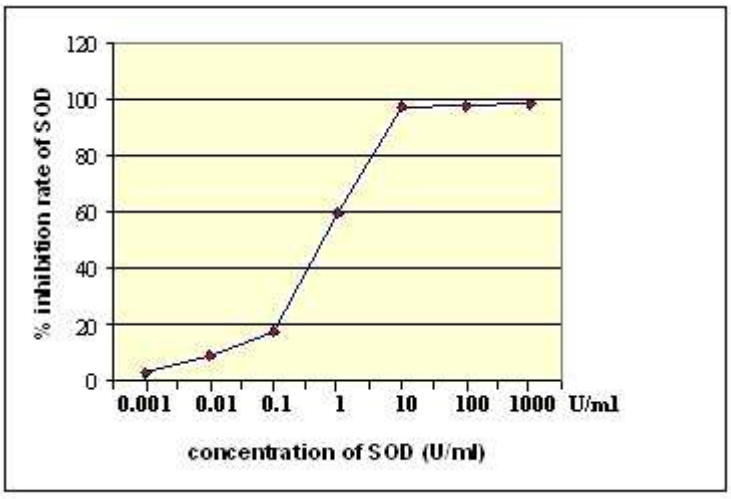
4.4 Effect of oxLDL in various doses and degrees activated LOX-1 expression mediated intracellular ROS and changes in superoxide dismutase (SOD) activity

We determined changes in antioxidant enzymes such as superoxide dismutase (SOD) activity during perturbation of culture system with various doses and degrees of oxLDL in order to look insight the role of $O_2^{\bullet-}$, one of free radical. Once $O_2^{\bullet-}$ is activated and formed, it was scavenged by SOD rapidly. Thus, changes in SOD activity will provide the context of $O_2^{\bullet-}$ in the system. Interestingly, the rate for the reaction of $O_2^{\bullet-}$ with NO to produce $ONOO^-$ is very high [6.7×10^9 $(mol/L)^{-1}$ s^{-1}] and exceeds the rate of dismutation by SOD [2×10^9 $(mol/L)^{-1}$ s^{-1}]. Exploring through both ROS and RNS, we investigated changes in both of SOD activity and eNOS mRNA expression with real-time NO release. Results are shown in figure 4.14 - 4.18 and table 4.3

We constructed standard SOD activity curve and expressed in U/ml. Quantitation of SOD activity from standard curve presented as U/ml/mg protein.

Figure 4.14

Showing the standard curve of SOD activity (U/ml)

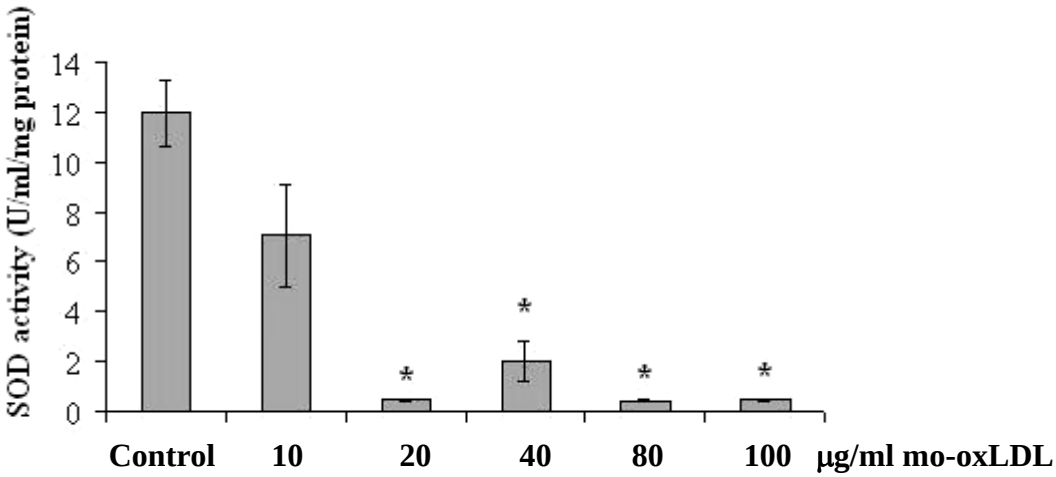


4.4.1 Effect of various doses and degree of oxLDL on SOD activity

SOD activity was declined gradually as advancing higher doses of moderately oxLDL (10 – 100 μg/ml). SOD activity decreased significantly compared with control by approximately 94.6%, 68.3%, 96.9%, and 96.6% in 20, 40, 80, and 100 μg/ml moderately oxLDL, respectively, meanwhile, at 10 μg/ml it showed no significant difference. These results indicate advancing decline in $O_2^{\bullet -}$ as substrate for SOD which might combine NO to be peroxynitrite. Furthermore, SOD might be damaged from ROS/RNS as well.

Figure 4.15

SOD activity in various doses of moderately oxLDL (U/ml/mg protein)



* p value < 0.05 compared with control

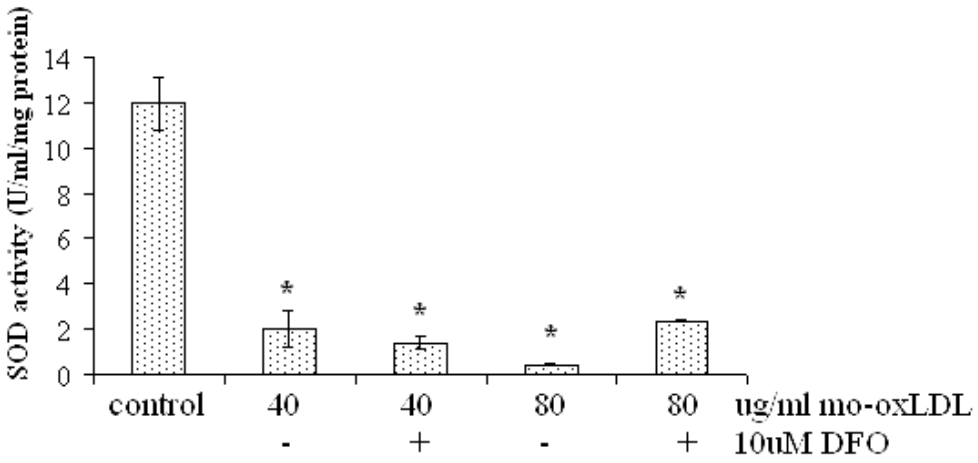
4.4.2 Effect of 10 μM DFO pretreatment and various doses of oxLDL in moderate degree on SOD activity

Result

SOD activity was reduced remarkably with advancing higher doses of moderately oxLDL (40, 80 μg/ml) as shown in figure 4.16. In 10 μM DFO pretreatment group, there were still lowered SOD activity. Compared with control, those of 10 μM DFO pretreatment and 40, 80 μg/ml oxLDL were significantly differed.

Figure 4.16

Showing the SOD activity in 10 μM DFO pretreatment group and doses of 40 and 80 μg/ml moderately oxLDL.



Data are presented as mean ± SEM. * p value < 0.05 compared with control

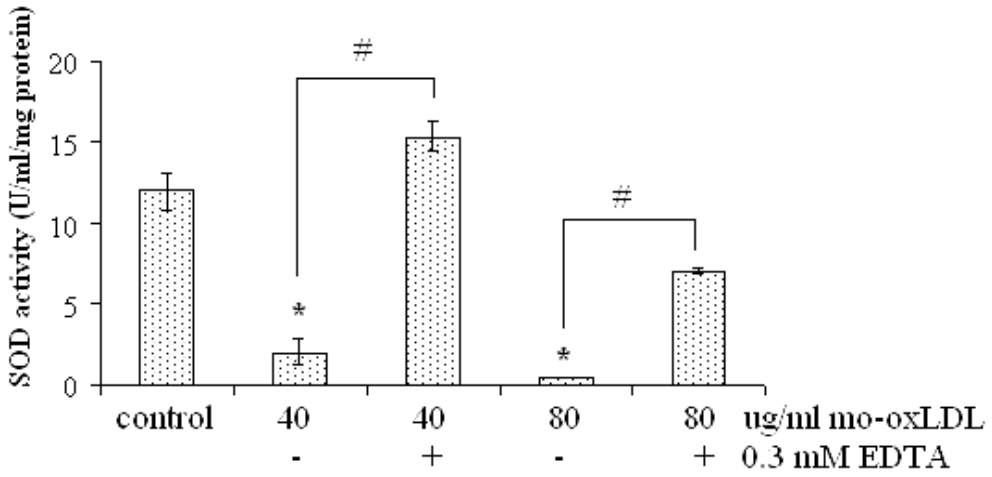
4.4.3 Effect of 0.3 mM EDTA pretreatment and various doses of oxLDL in moderate degree on SOD activity

Result

SOD activity was reduced significantly compared with control in doses of 40 and 80 μg/ml oxLDL in moderate degree. Interestingly, increasing SOD activity was seen clearly in 0.3 mM EDTA pretreatment group and reached the significant difference in doses of 40 and 80 μg/ml oxLDL. These findings indicate accumulation of H_2O_2 in the system resulting from EDTA effect at Fenton's reaction. Higher H_2O_2, perhaps, feedbacks SOD to increase the activity in order to reduce $O_2^{\bullet -}$ and eventually reduces the amount of H_2O_2.

Figure 4.17

Showing the SOD activity in 0.3 mM EDTA pretreatment group and doses of 40 and 80 μg/ml moderately oxLDL.



Data are presented as mean ± SEM. * p value < 0.05 compared with control and # p value < 0.05 compared among groups

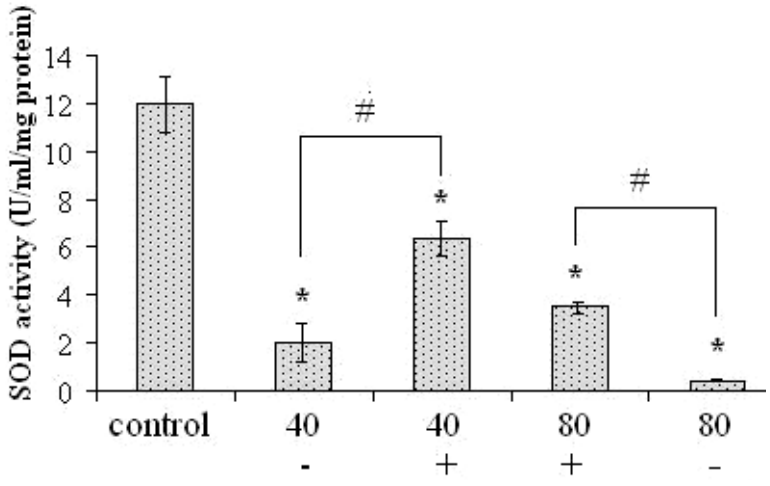
4.4.4 Effect of 100 μg BHT pretreatment and various doses of oxLDL in moderate degree on SOD activity

Result

SOD activity was declined significantly compared with control in doses of 40 and 80 μg/ml oxLDL in moderate degree. At dose of 40 and 80 μg/ml oxLDL with 100 μg BHT pretreatment, increasing SOD activity was showed significantly compared with those of the same dose.

Figure 4.18

SOD activity in pretreatment group with 100 μg BHT
and doses of 40 and 80 μg/ml moderately oxLDL.



Data are mean ± SEM. * p value< 0.05 compared with control group and # p-value < 0.05 compared among groups

Table 4.3

Summarized data of SOD activity

Group of moderately oxLDL	mean ± SEM (U/ml/mg protein)			
	OxLDL	10μM DFO and oxLDL	0.3 mM EDTA and oxLDL	100 μg/ml BHT and oxLDL
Control	11.960 ± 1.290	11.960 ± 1.290	11.960 ± 1.290	11.960 ± 1.290
10 μg/ml	7.050 ± 2.050	-	-	-
20 μg/ml	0.428 ± 0.060	-	-	-
40 μg/ml	3.79 ± 1.701	1.362 ± 0.269	15.34 ± 0.912	6.323 ± 0.701
80 μg/ml	0.37 ± 0.072	2.351 ± 0.034	7.04 ± 0.216	3.455 ± 0.236

These findings are strongly mention that ROS produced from vascular oxidant enzymes such as NADPH oxidase etc, involved in endothelial dysfunction caused by oxLDL through LOX-1 receptor. $O_2^{\bullet-}$ and H_2O_2 are generated widely in an initiation of endothelial dysfunction. Meanwhile, fluctuation of $O_2^{\bullet-}$ caused by uncoupling eNOS will be affect the SOD activity and moreover, SOD might be damaged from ROS/RNS as well.

4.5 eNOS expression activated by oxLDL in various doses and degree of oxidation

We determined the endothelial function in term of eNOS expression and NO production during the treatment of oxLDL in various doses and degree and also with pretreatment groups. OxLDL binds to LOX-1 receptor on endothelial surface and enter cell as receptor-mediated endocytosis, then causes activation of many enzymes such as NAD(P)H oxidase releasing ROS to the system. Abundant ROS will back to endothelial cell and causes cell damage and injury. For this reason, the assessment of endothelial function through eNOS expression and real-time NO measurement is necessary and being main key to determine endothelial dysfunction. Another reason is the reaction of $O_2^{\bullet -}$ with NO to produce $ONOO^-$, one of RNS radical, which in turn attack cell and tissue as well.

4.5.1 Effect of various doses and degree oxidation of LDL on eNOS expression and NO release

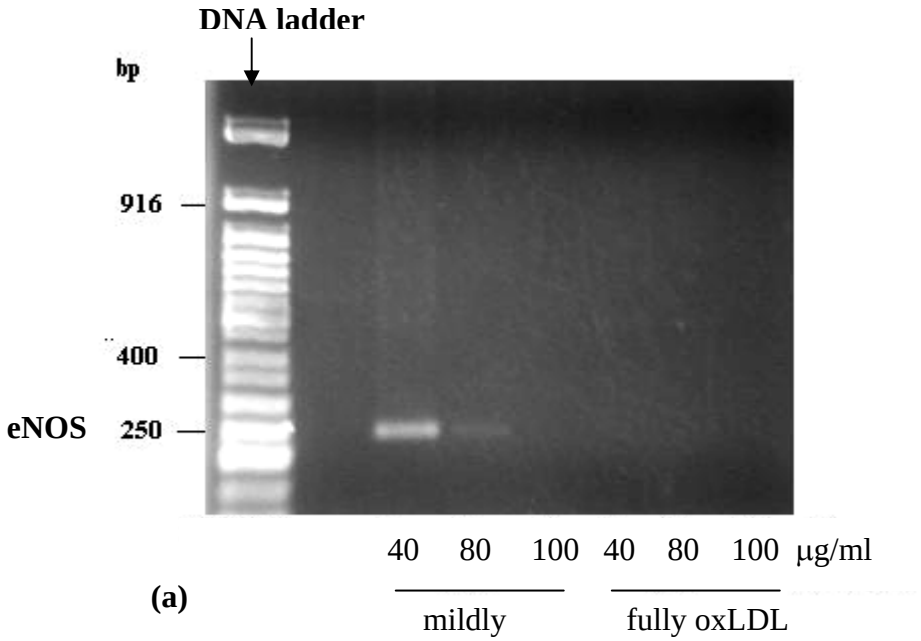
Result

We induced eNOS expression through 40, 80 and 100 μg/ml oxLDL in mildly to fully oxidation groups as shown in figure 4.19. Both doses of mildly; 40 μg/ml oxLDL and moderately; 10 μg/ml oxLDL caused upregulation of eNOS expression about 149 % and 97.3 %, respectively. Downreguration of eNOS was found in mildly, 80 and 100 μg/ml oxLDL. In fully, 40-100 μg/ml oxLDL resulted non-detectable band. Suppression of eNOS mRNA expression in dose of 20 μg/ml oxLDL in moderate degree was followed by those upregulation at dose of 10 μg/ml and then recovered at doses 40 and 80 μg/ml indicates the early response effect of uncoupling eNOS, but eventually reduced subsequently. Upregulation of eNOS expression in dose of 40 μg/ml mildly oxLDL and 10 μg/ml moderately oxLDL suggestes that the endothelium is capable of producing NO, indicates the normal endothelial function. Suppression of eNOS expression as revealed in a higher dose of

mildly and moderately oxLDL indicates to endothelial dysfunction and reducing NO production, which might be caused by ROS overwhelmed in vasculature.

Figure 4.19

Showing the expression of eNOS induced by oxLDL in various doses and degree oxidation on 1.5 % agarose gel electrophoresis: mildly and fully oxLDL (a); moderately oxLDL (b)



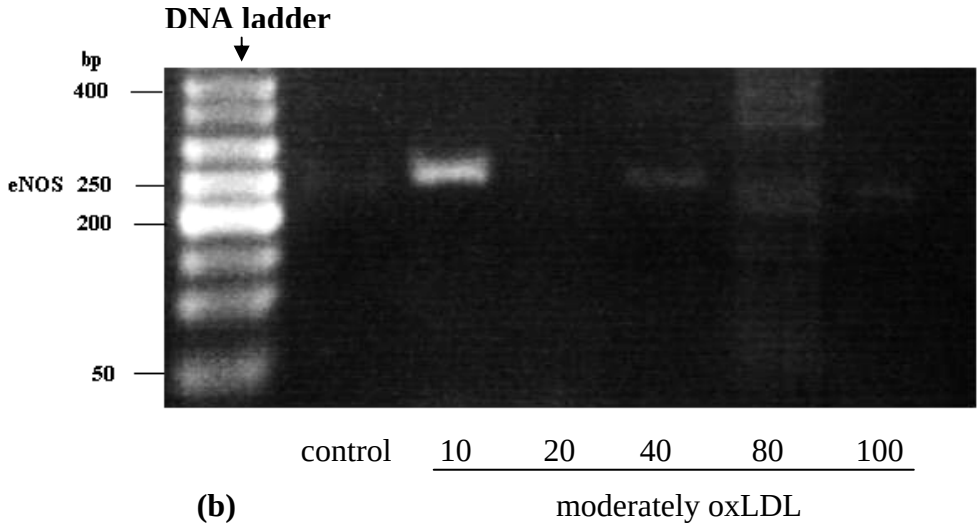
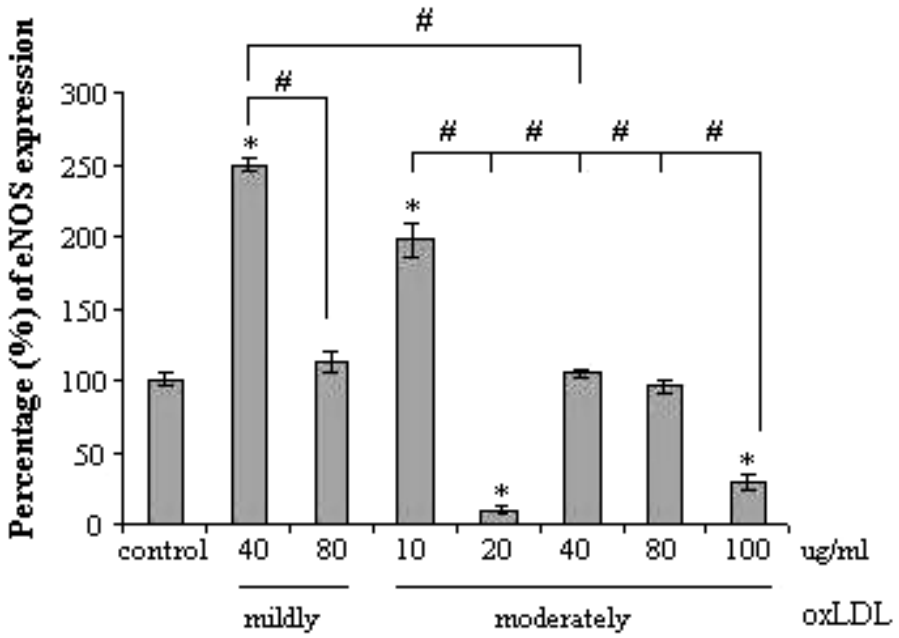


Figure 4.20

Significant difference of eNOS expression induced by oxLDL in various degree oxidation and doses are shown.



Data are presented to percentage of mean ± SEM.

* p value < 0.05 compared with control and

p value < 0.05 compared between groups

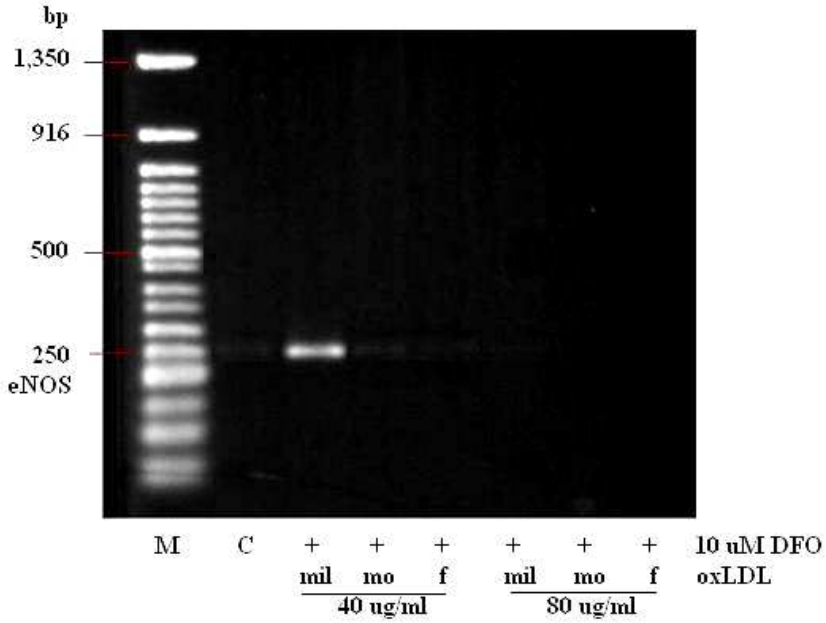
4.5.2 Expression of eNOS induced by 10 μM DFO pretreatment and oxLDL in various doses and degree oxidation

Result

We induced eNOS expression by 10 μM DFO pretreatment and oxLDL in various doses and degree oxidation. We expected the expression of eNOS might be increased or decreased depending on the turn over rate production of ROS, $O_2^{\bullet -}$, and predictable $ONOO^-$ formation that related to eNOS expression and NO released in the system. Upregulation of eNOS was seen in doses of 40 μg/ml mildly oxLDL and significantly differed the control. In 10 μM DFO protreatment group, only dose of 40 μg/ml mildly oxLDL, eNOS expression was increased and showed significantly. In moderately and fully oxidation, eNOS were downregulated subsequently. These findings indicate endothelial function producing NO in mildly degree but it rather was damaged in moderately to fully oxidation leading to dysfunction.

Figure 4.21

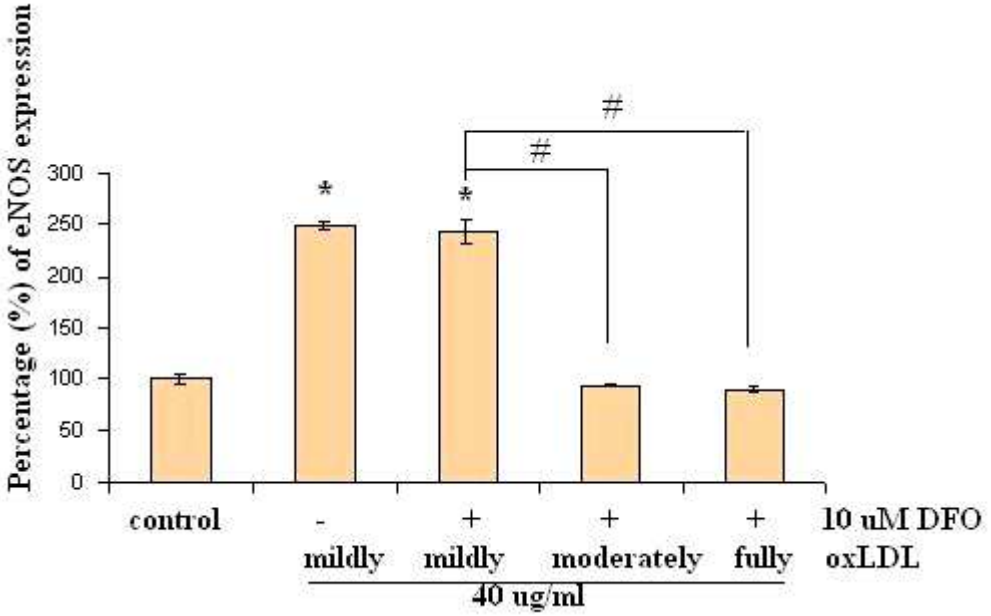
Showing the inducible eNOS expression of each 10 μM DFO treatment group



C = control; mil = mildly; mo = moderately; f = fully

Figure 4.22

Significant difference of eNOS expression induced by oxLDL in 10 μM DFO pretreatment group is shown.



Data are presented to percentage of mean ± SEM.

* p value < 0.05 compared with control

p value < 0.05 compared between groups

4.5.3 eNOS expression induced by 0.3 mM EDTA pretreatment and oxLDL in various doses and degree oxidation

Result

Upregulation of eNOS expression was clearly seen in 0.3 mM EDTA pretreatment with 80 μg/ml oxLDL in mildly degrees and showed significantly with those of the same dose. In 0.3 mM EDTA pretreatment of doses 40 μg/ml mildly and moderately oxLDL including of dose 80 μg/ml moderatelyoxLDL, eNOS expression was also suppressed and showed significance when compared with those of the same dose. These finding indicates the endothelial dysfunction and uncoupling eNOS involved.

Figure 4.23

Inducible eNOS expression of each treatment group

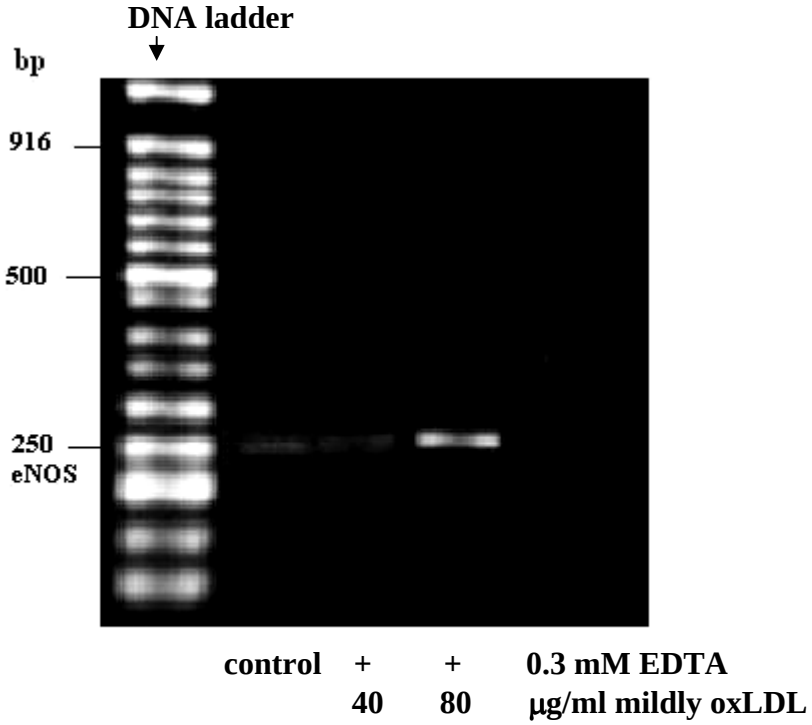
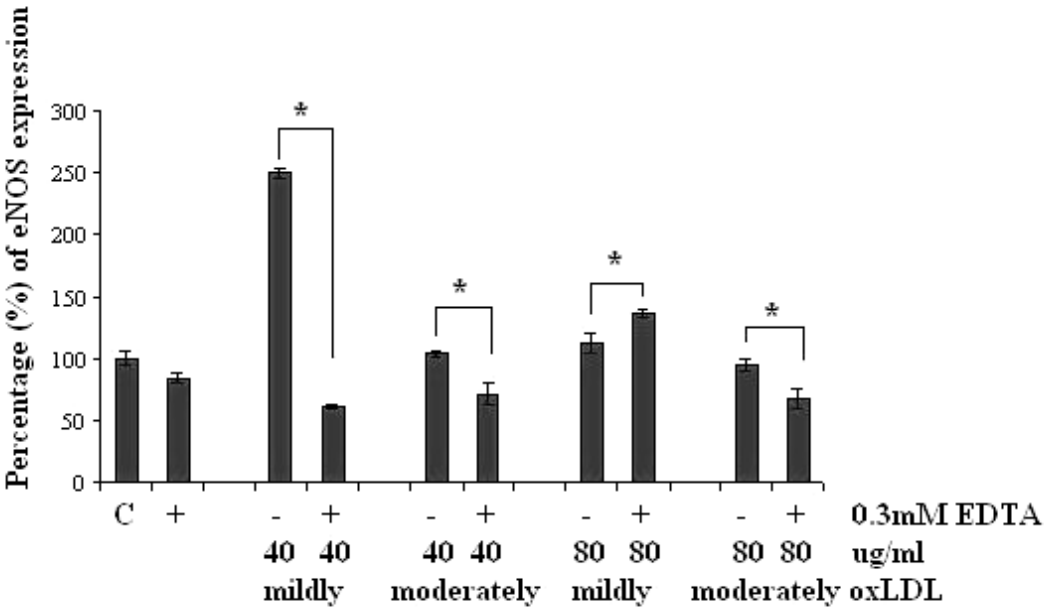


Figure 4.24

Significant difference of eNOS expression in 0.3 mM EDTA



Data are percentage of mean ± SEM. * p value < 0.05

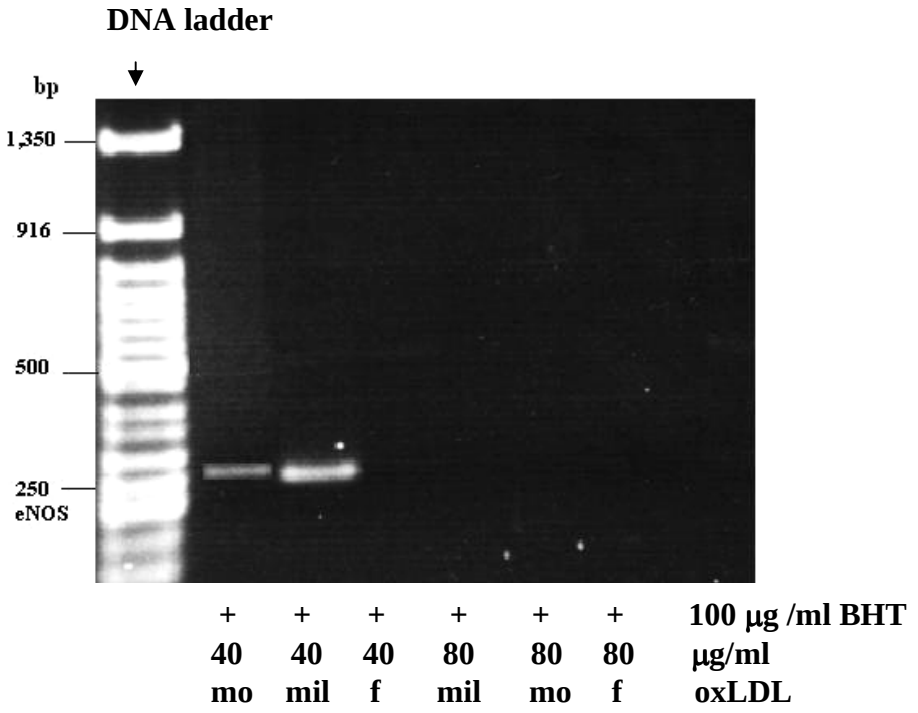
4.5.4 eNOS expression induced by 100 μg/ml BHTpretreatment and oxLDL in various doses and degree oxidation

Result

Upregulation of eNOS was presented in dose of 40 ug/ml mildly oxLDL with 100 ug/ml BHT pretreatment group. In moderately oxLDL group, downregulation of eNOS was seen. Suppression of eNOS at moderately degree was differed significantly from mildly degree group.

Figure 4.25

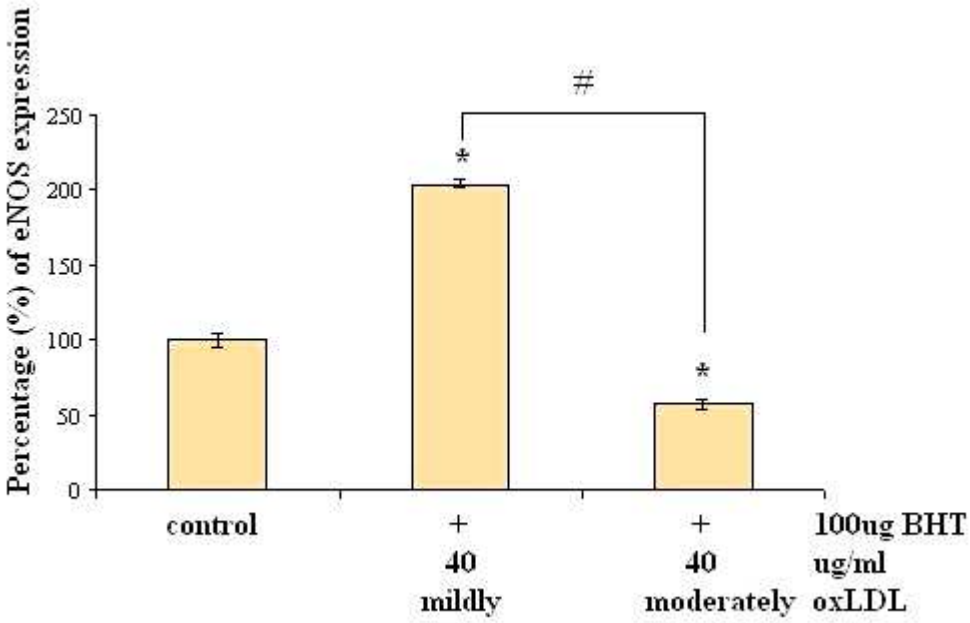
Inducible eNOS expression of each treatment group in 100 μg/ml BHT



mil = mildly; mo = moderately; f = fully

Figure 4.26

Significant difference of eNOS expression in 100 μg/ml BHT



Data are presented to mean ± SEM.

* p value < 0.05 compared with control

p value < 0.05 compared with among group

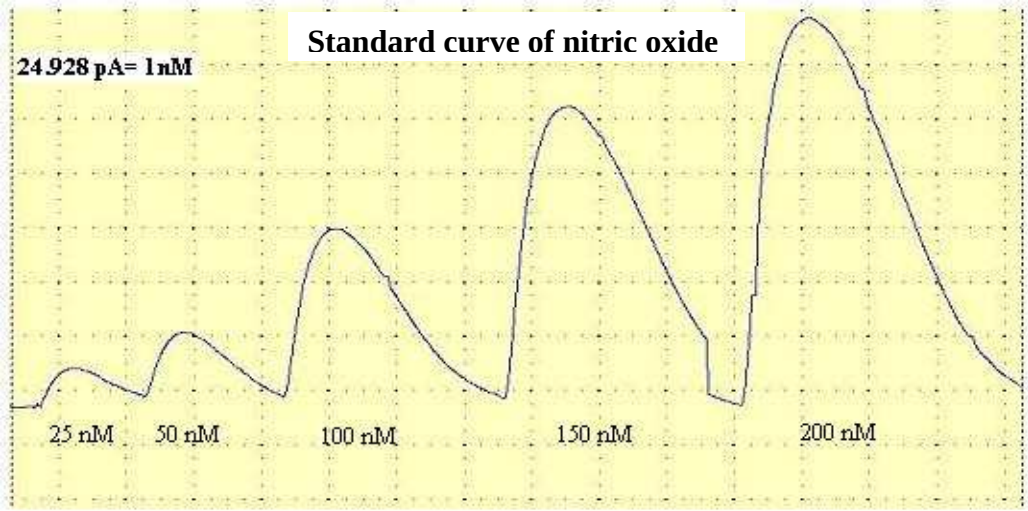
4.6 Nitric oxide production and endothelial dysfunction mediated by intracellular ROS and RNS

As previous mentioned, abundant ROS/RNS resulted from oxLDL induced LOX-1 expression may cause endothelial dysfunction. Real-time NO measurement by biosensor probe allows us explore

1) endothelium might loss the function
2) peroxynitrite ($ONOO^-$) might be produced from the reaction of $O_2^{\bullet -}$ and NO. If it was produced, NO should be reduced.

In this experiment, the standard curve of NO was constructed base on conversion of current (pA) and concentration (nM)

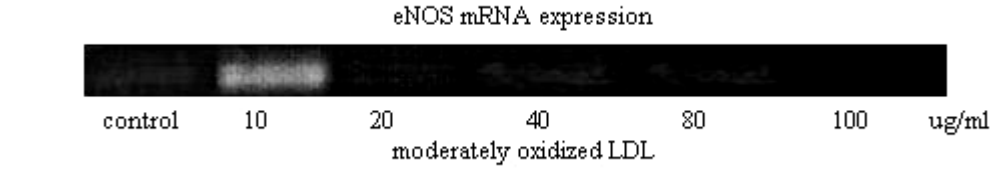
Figure 4.27
Showing the standard curve of NO at 25, 50, 100, 150 and 200 nM
when 24.928 pA = 1nM



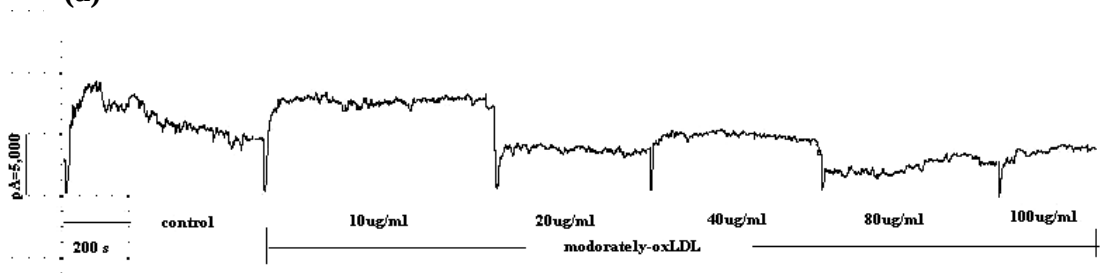
4.6.1 NO production induced by doses and degrees oxidation of LDL

Figure 4.28

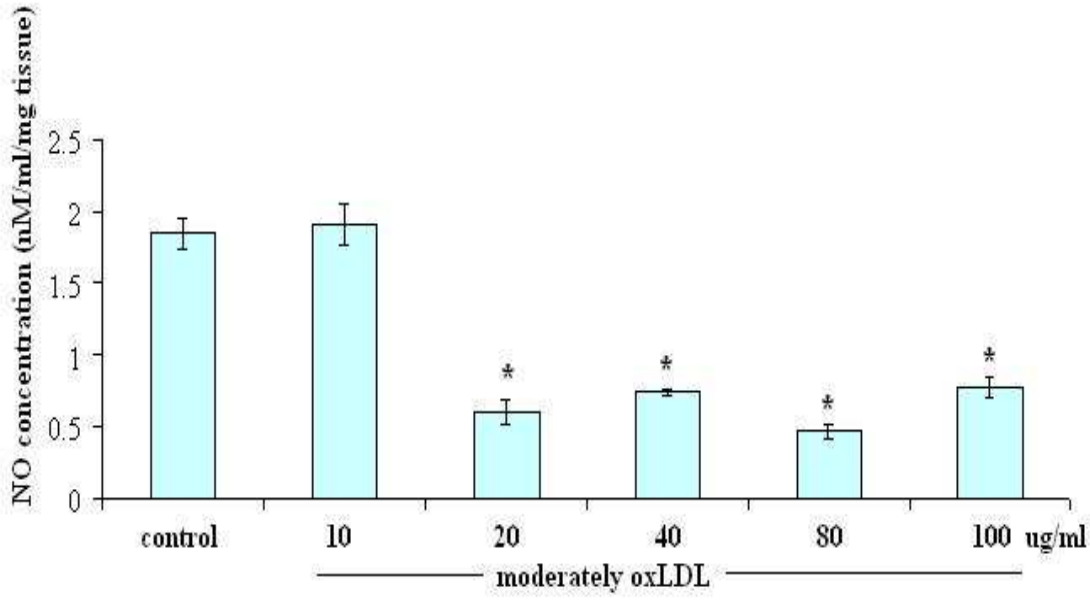
NO production induced by various doses of moderately oxLDL (b, c) is correspondent with eNOS expression (a)



(a)



(b)



(c)

* p-value < 0.05 compared with control

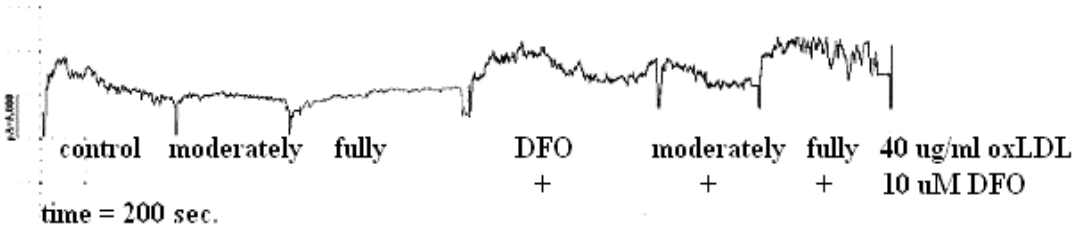
Upregulation of eNOS expression induced by 10 μg/ml oxLDL in moderately oxidative degree is corresponded with a higher production of NO as shown in figure 4.28. Meanwhile, downregulation of eNOS expression as seen in varied doses of 20, 40, 80 and 100 μg/ml oxLDL is also correspondent with lower levels of NO production and showed differed significantly when compared with control. Interestingly, a higher dose and degree of oxLDL reduced eNOS activity through more generated intracellular ROS especially $O_2^{\bullet-}$ will forward "uncoupling state of eNOS" resulting reduced NO level. Turn over rate of $O_2^{\bullet-}$ is rapidly when compares with those of H_2O_2 production. Hence, $O_2^{\bullet-}$ is driven fastly to the system and leads to combination with NO promoting $ONOO^-$ formation.

4.6.2 NO production induced by doses and degrees oxidation of oxLDL of10 μM DFO pretreatment group

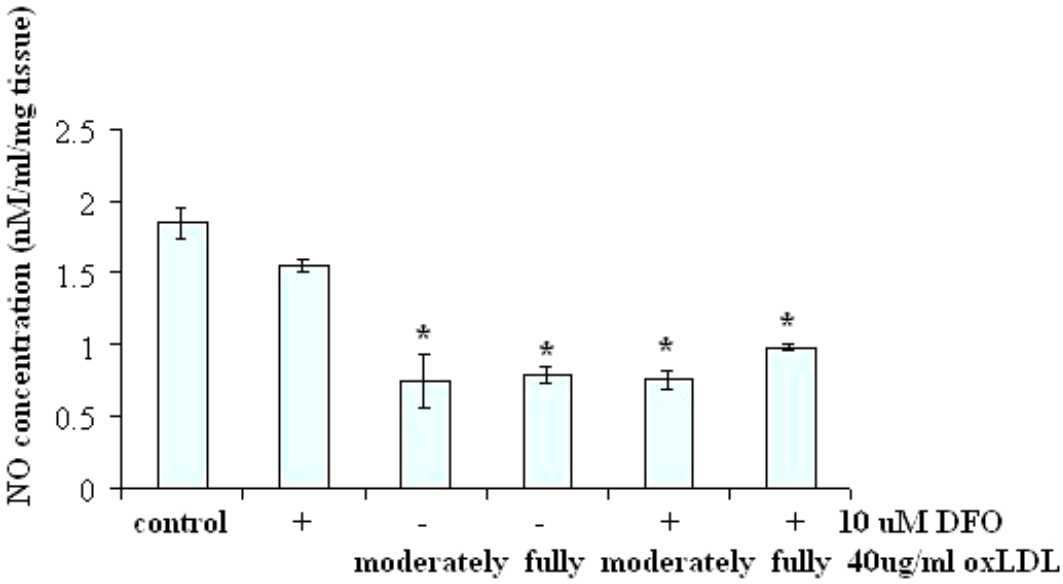
NO was reduced significantly in 40 μg/ml oxLDL of both moderately and fully degrees. In 10 μM DFO pretreatment group, slight increase in NO concentration was presented especially in fully degree when compared with those of the same degree of oxLDL. However, level of NO in DFO pretreatment group remained low indicates the endothelial dysfunction.

Figure 4.29

Real-time measurement of NO released and recorded as current in pA of 40 μg/ml in moderately and fully oxidation of LDL with 10 μM DFO pretreatment (a). Statistically significant difference of each pretreatment group (b).



(a)



(b)

Data are mean ± SEM.

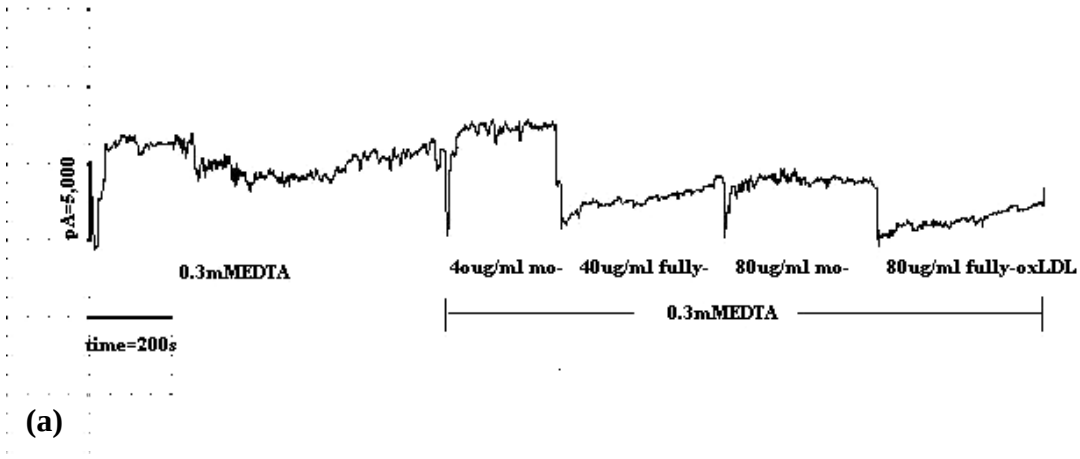
* indicated p value < 0.05 compared with control and negative control

4.6.3 NO production induced by doses and degrees oxidation of LDL and 0.3 mM EDTA pretreatment

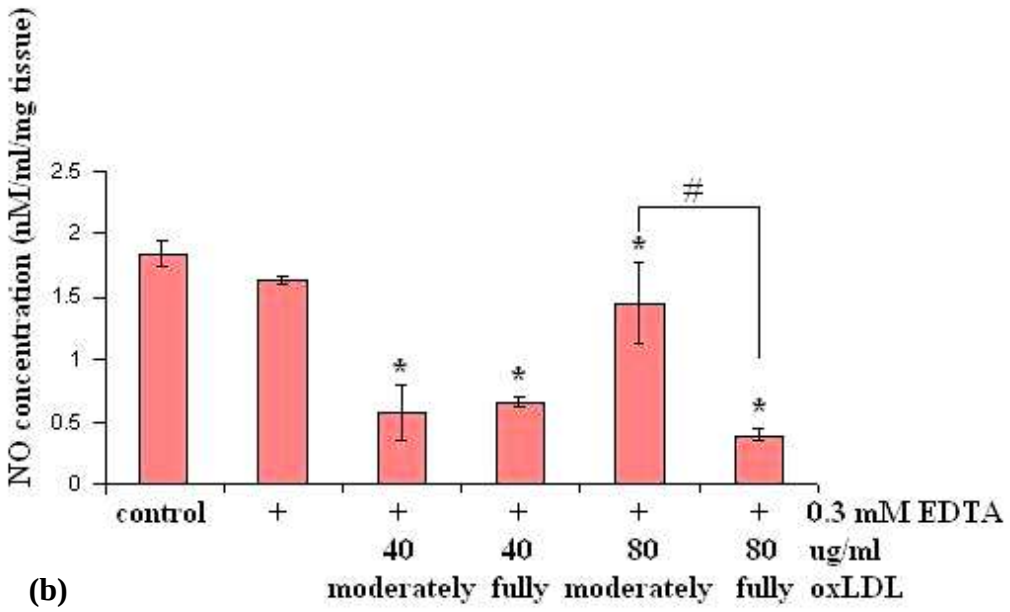
Figure 4.30

Real-time measurement of NO release and was recorded as current in pA of various doses and degree oxLDL with 10 μM DFO pretreatment (a).

Significant difference of each pretreatment group (b)



(a)



(b)

Data are mean ± SEM.

* indicated p value < 0.05 compared with control (b)

Result

Reduction of NO concentration of 0.3 mM EDTA pretreatment group in 40 and 80 μg/ml oxLDL with various degree was shown significantly in figure 4.30 (b) when compared with control. Among degrees of oxidation, moderately oxLDL in 80 μg/ml was slight increased and shown significantly to fully oxidation. These result finding was corresponded with eNOS expression of 0.3 mM EDTA pretreatment indicating the endothelial dysfunction through ROS/RNS.

4.6.4 NO production induced by doses and degrees oxidation of oxLDL and BHT pretreatment

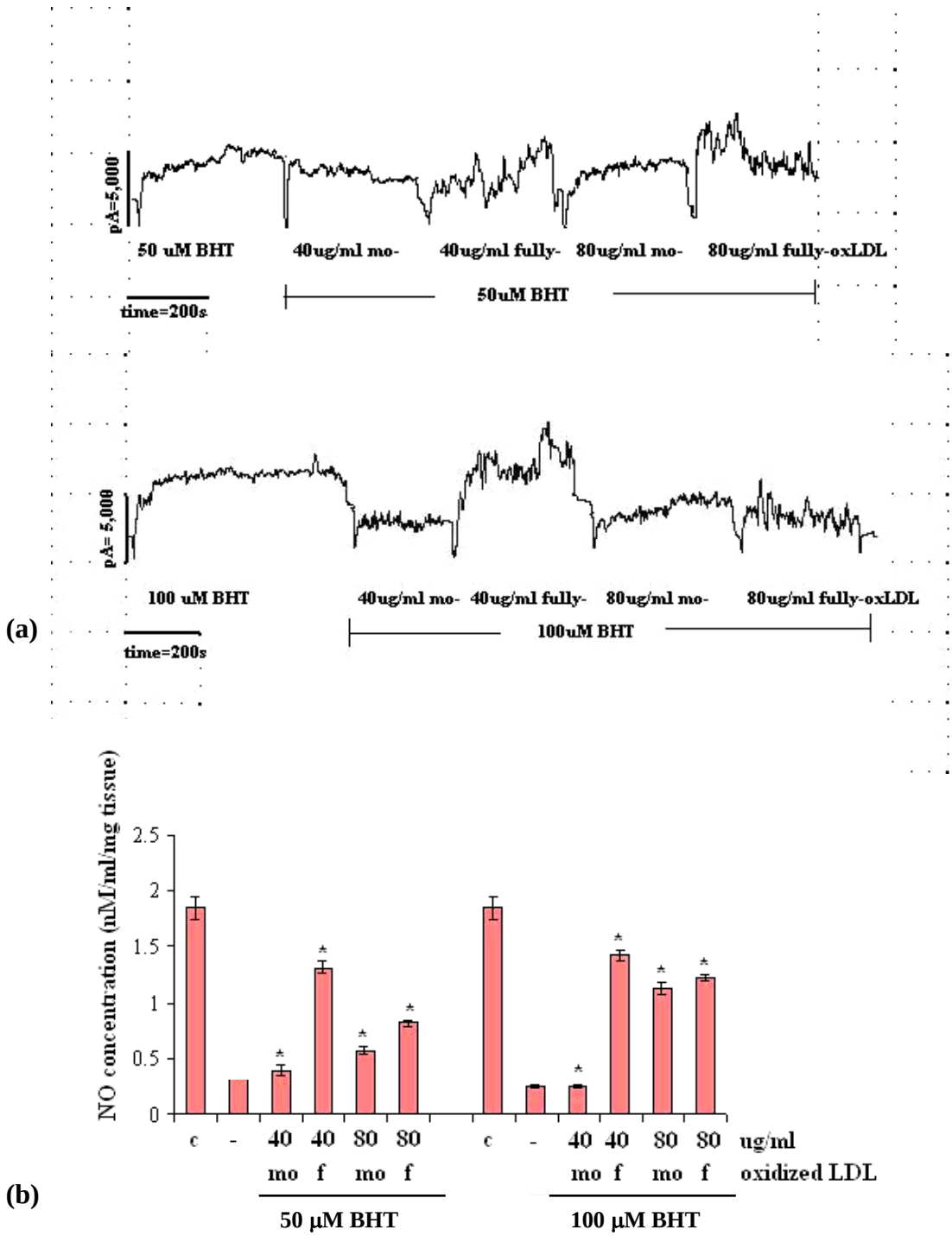
Eventhough BHT is peroxyl radical scavenger of oxLDL and acts as vitamin E, however, it has a prooxidant as well. We used BHT for pretreatment since we need mor data of ROS/RNS by it own pathway and BHT did not involved any step in ROS/RNS generation. We expected that endothelial dysfunction caused by ROS/RNS generated from activation of oxLDL through LOX-1 receptor.

Result

NO concentration levels were declined significantly in 50 μM and 100 μM BHT pretreatment group of various degree oxidation. Notably, redued NO levels were slightly in 100 μM BHT pretreatment but still lowered significantly when compared with control. These findings indicate its own ROS/RNS affect endothelium and might be involved uncoupling eNOS too.

Figure 4.31

NO release was detected in BHT pretreatment and various doses and degree oxidation of oxLDL by biosensor probe (a). Data are presented as mean ± SEM (b).



* p value < 0.05 compared with control group

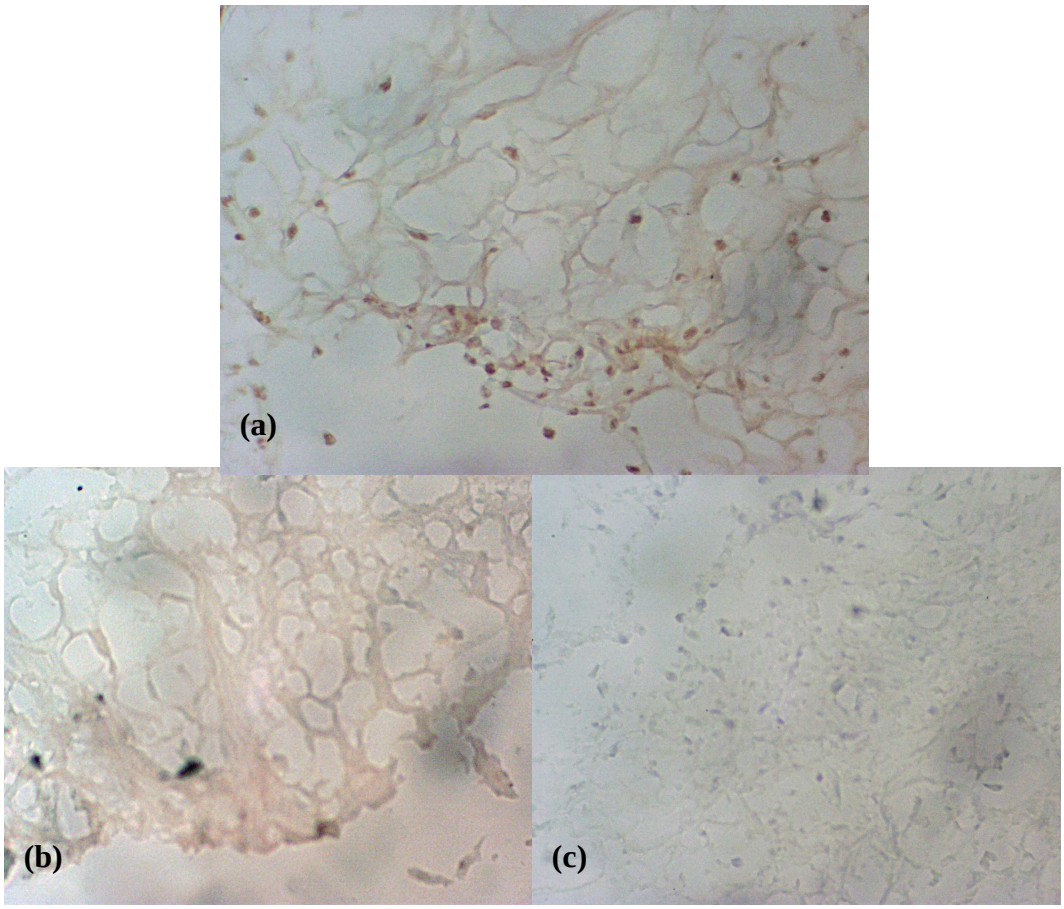
\# p value < 0.05 compared with among groups

4.7 Influence of oxLDL-mediated LOX-1 expression on p38 mitogen activated protein kinase (p38MAPK)

Intracellular ROS activated by oxLDL through LOX-1 reaching the pathophysiological state is able to trigger the specific signaling pathway, redox-sensitive gene and endothelial dysfunction. It serves as second messenger to activate multiple intracellular proteins and enzymes including p38 MAPK, resulting proliferation and growth of smooth muscle cell. We hypothesized that induced intracellular ROS and subsequent RNS led to increase p38 MAPK level and might be reduced if we limited ROS/RNS available with DFO, EDTA and BHT.

Figure 4.32

(a) Immunohistochemistry of anti-p38 MAPK activity and measured mean density by using image Proplus analysis program, (b) negative control; 3,3'- Diaminobenzidine (DAB), (c) negative control; Hematoxylin



4.7.1 Oxidized LDL-induced p38 MAPK activity

Figure 4.33

Immunohistochemistry of p38 MAPK which demonstrated in control group (upper) and 80 μg/ml fully oxidized LDL group (lower)

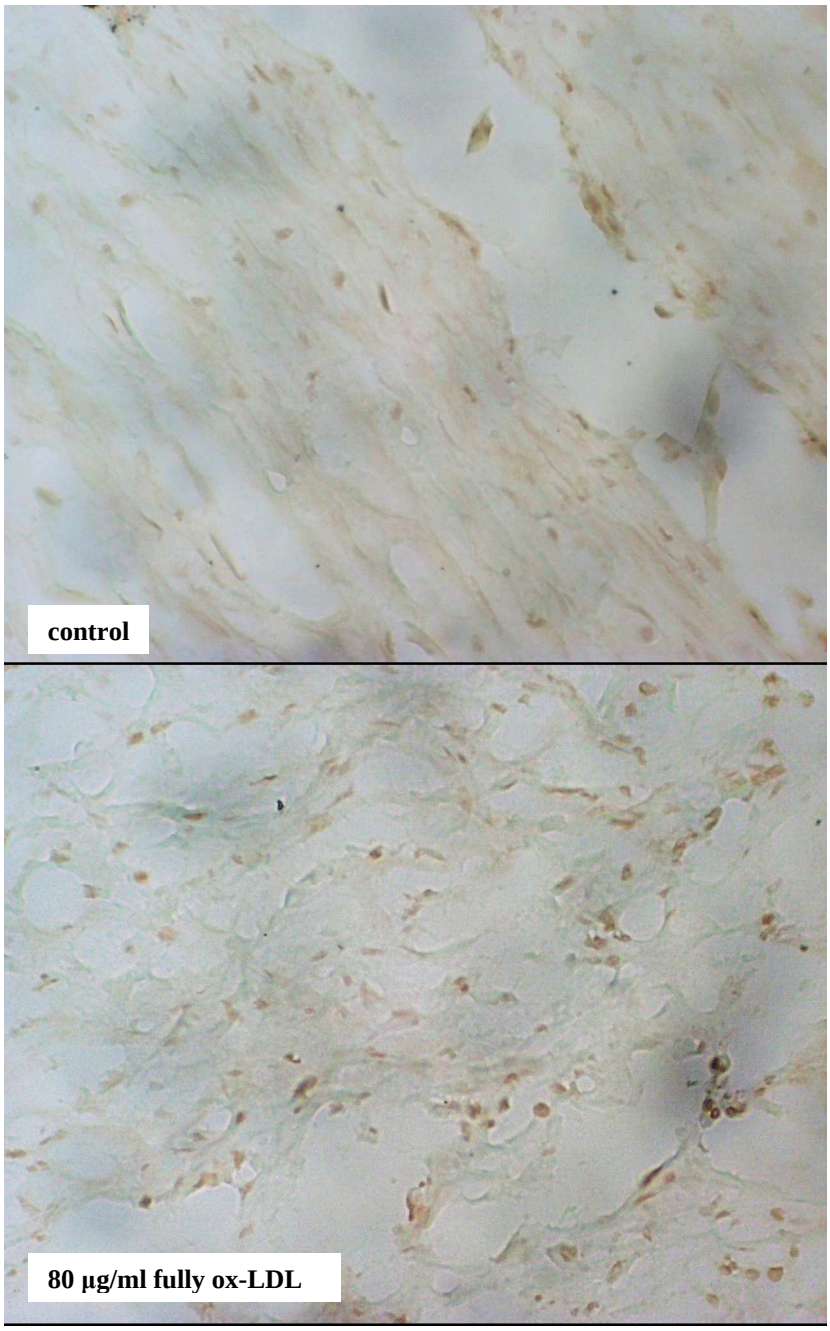
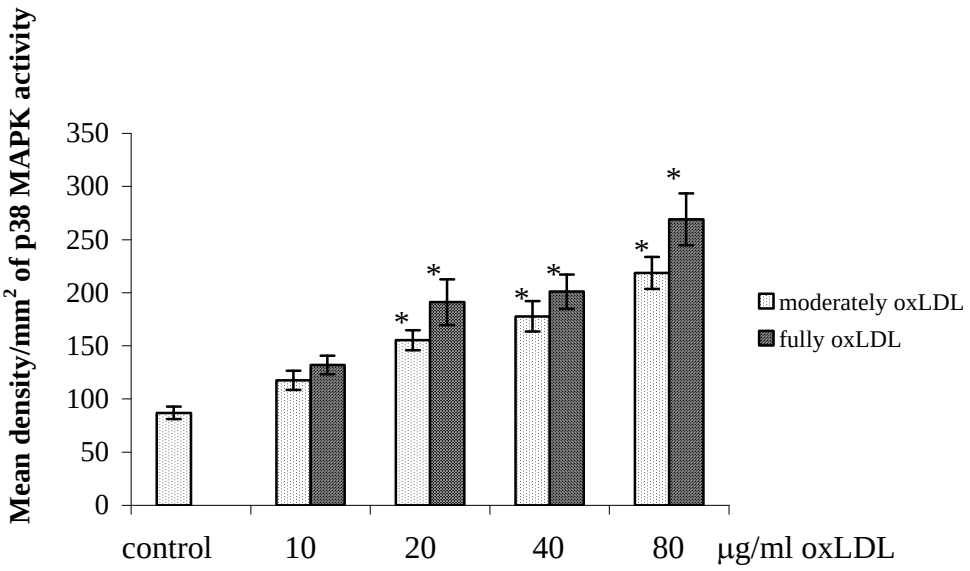


Figure 4.34

Mean density of p38 MAPK activity activated by various doses of oxLDL moderately and fully oxidation.



Data are presented as density mean ± SEM.

* p value < 0.05 compared with control group

Gradual increase in mean density of p38 MAPK activity of doses 10, 20, 40 and 80 μg/ml oxLDL in moderately and fully degrees are demonstrated. Difference of 20, 40 and 80 μg/ml oxLDL compared with control are significantly.

4.7.2 OxLDL induced p38 MAPK activity in EDTA pretreatment group

Figure 4.35

Immunohistochemistry of p38 MAPK of 0.3 mM EDTA pretreatment group with 40 μg/ml moderately oxidized LDL group (upper) and those with 40 μg/ml fully oxidized LDL group (lower)

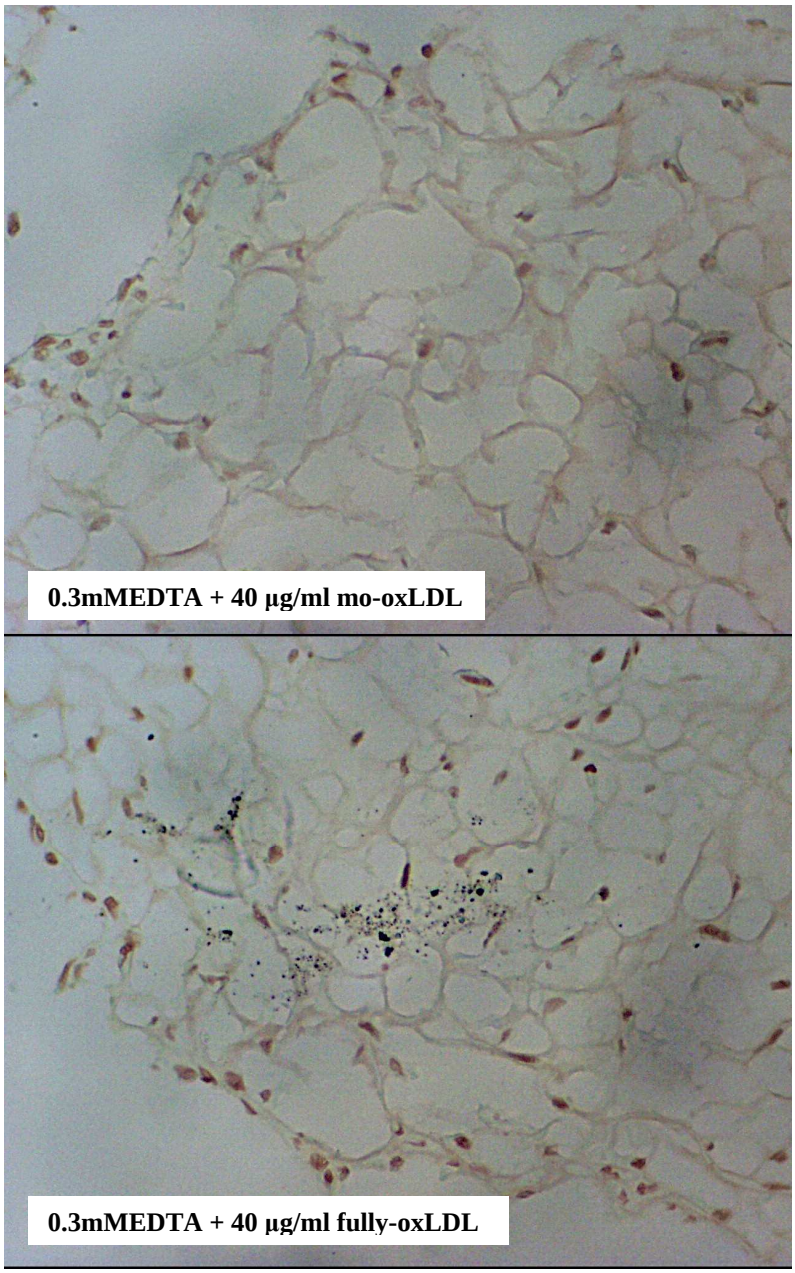
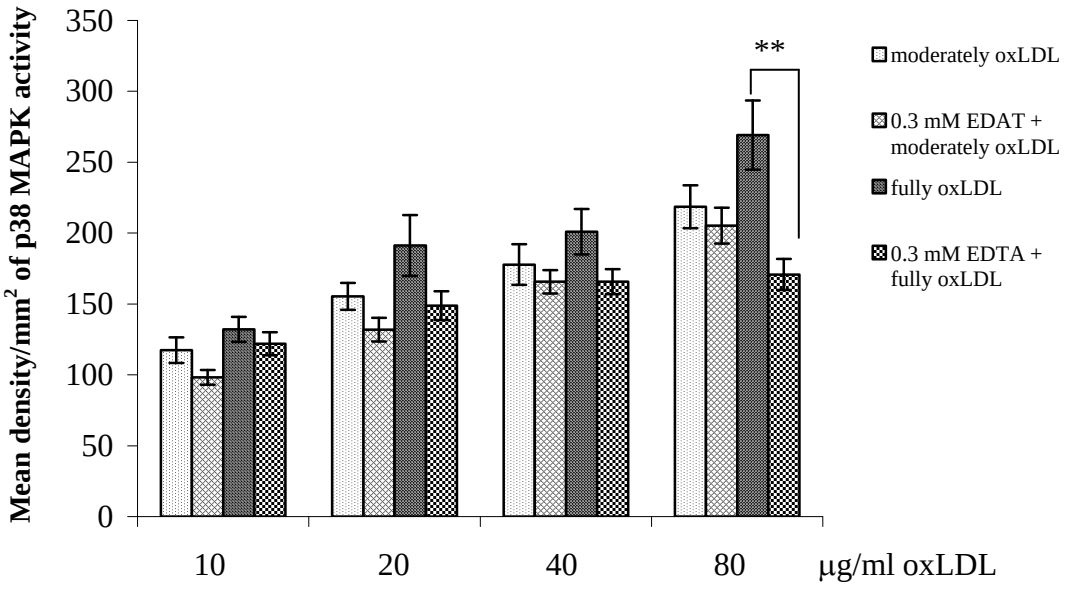


Figure 4.36

Mean density of p38 MAPK activity of 0.3 mM EDTA pretreatment group with various doses of oxLDL in moderately and fully degree oxidation.

Data are mean ± SEM.



Result

In mean density of p38 MAPK was gradually increased as dose-degree dependent oxLDL. In 0.3 mM EDTA pretreatmwnt group, decreased mean density was observed in each dose and degree oxidation. **Difference of fully, 80 μg/ml oxLDL and those with 0.3 mM EDTA was presented with $P < 0.10$.

4.7.3 OxLDL induced p38 MAPK activity in DFO pretreatment group

Figure 4.37

Immunohistochemistry of p38 MAPK in 80 μg/ml fully oxLDL group (upper) and 10 μM DFO with 80 μg/ml fully oxidized LDL group (lower)

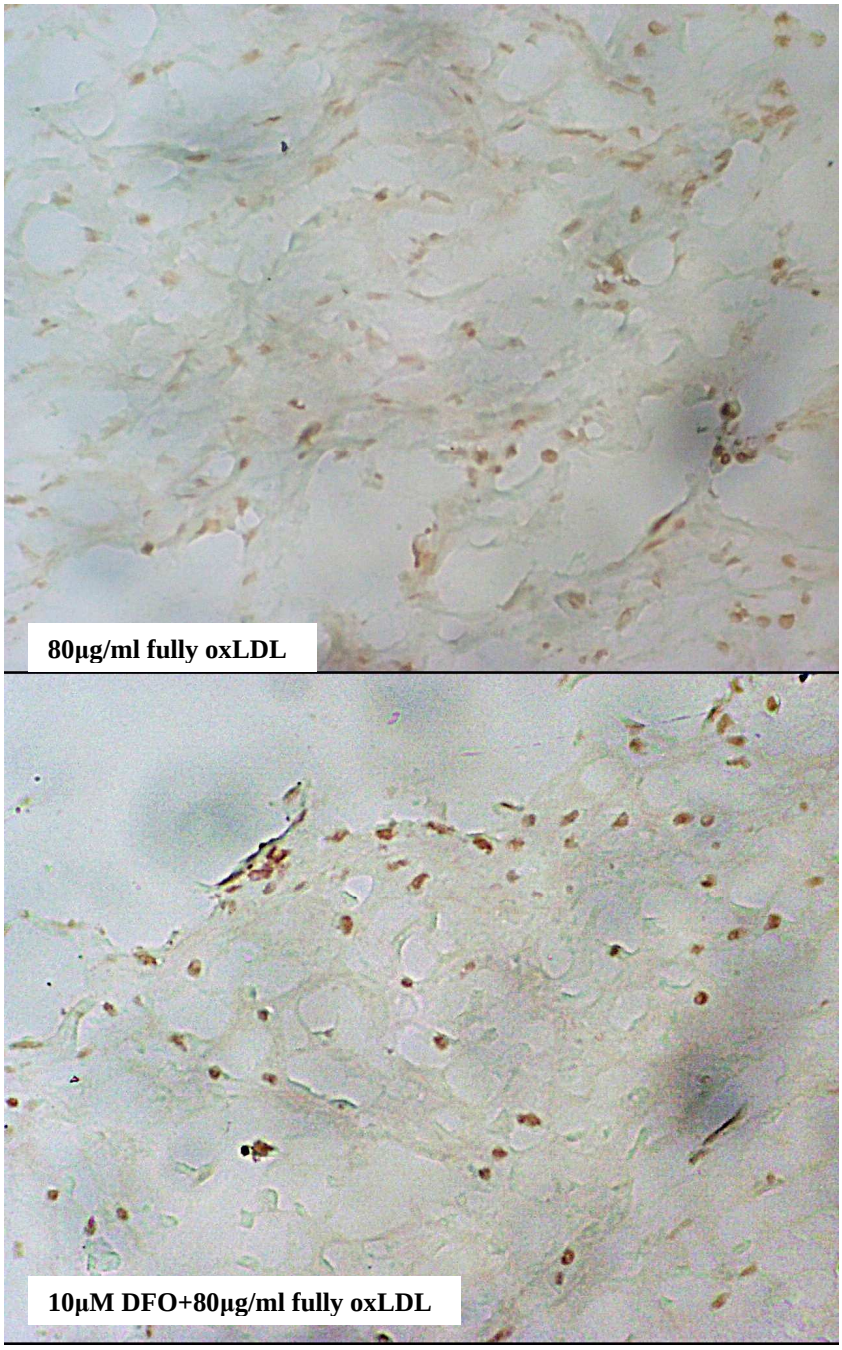
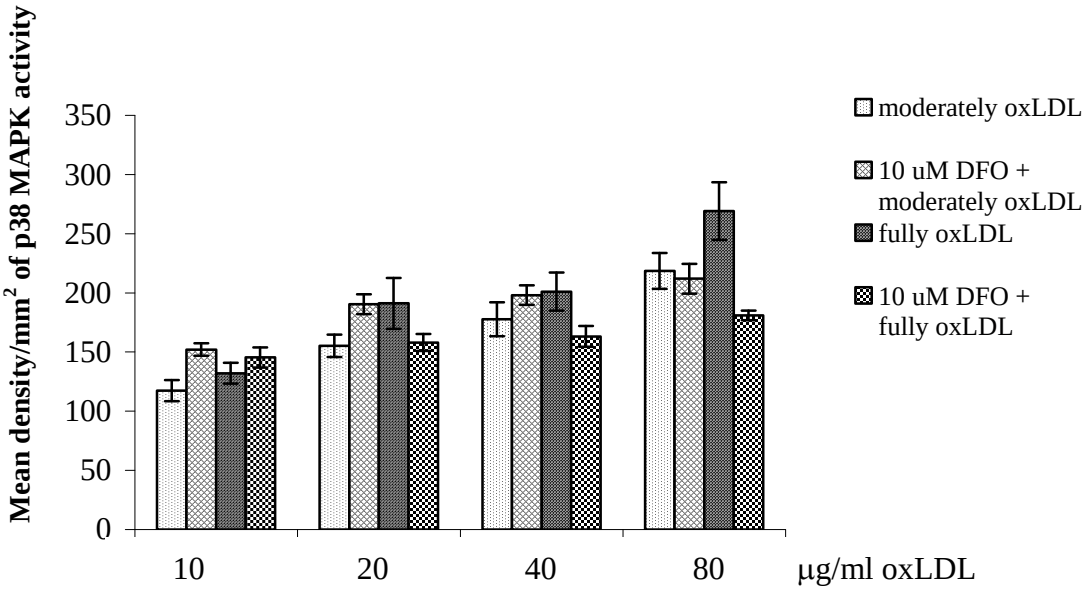


Figure 4.38

Mean density of p38 MAPK activity of 10 μM DFO with moderately and fully oxLDL in various doses. Data are mean ± SEM.



Mean density of p38 MAPK was gradually increased as dose-degree dependence of oxLDL. In 10 μM DFO pretreatment group, decreased mean density was shown remarkedly but not reached significance.

4.7.4 OxLDL induced p38 MAPK activity in BHT pretreatment group

Figure 4.39

Immunohistochemistry of p38 MAPK activity of 80 µg/ml fully oxidized LDL group (upper) and 100 µg/ml BHT with 20 µg/ml fully oxidized LDL group (lower)

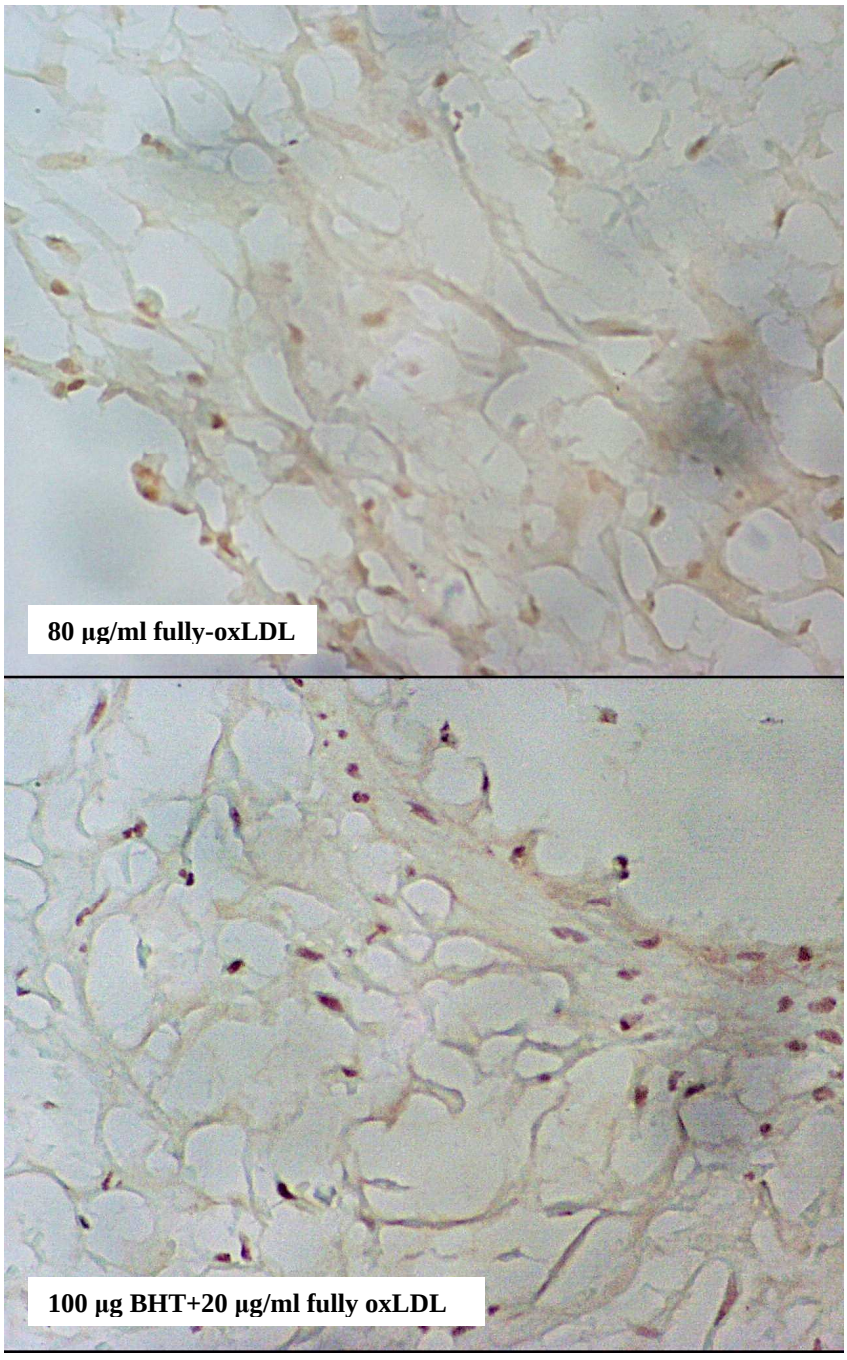
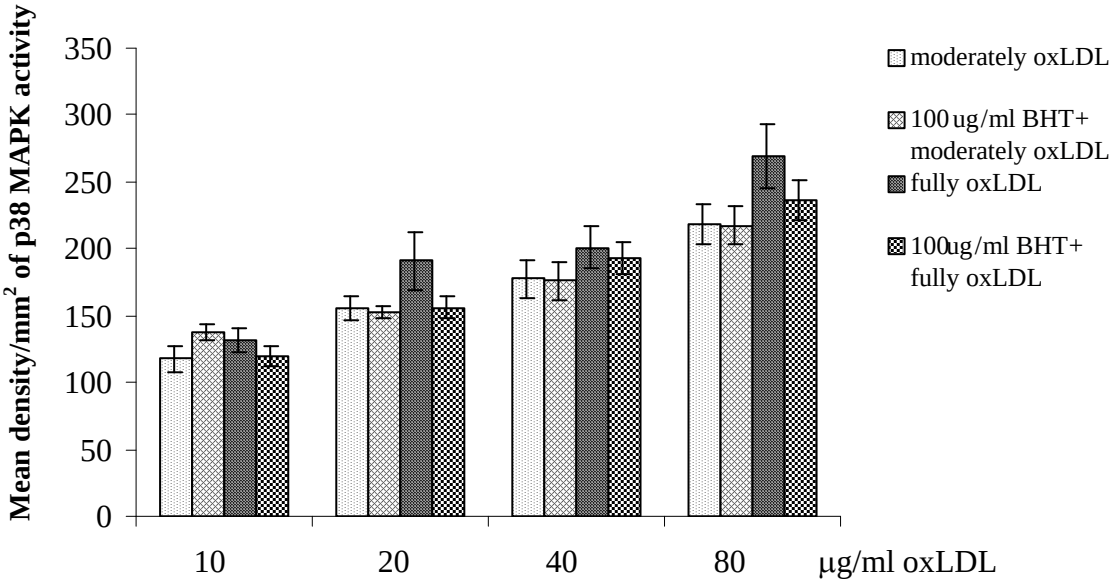


Figure 4.40

Mean density of p38 MAPK activity of 100 μg/ml BHT pretreatment with oxidized LDL in various doses and degrees. Data are mean ± SEM.



Mean density of p38 MAPK was gradually increased as dose-degree dependent oxLDL. In 100 μg/ml BHT pretreatment group, reduced mean density was observed especiaaly in fully, 20, 40 and 80 μg/ml oxLDL.

4.8 Morphological changes of cultured human umbilical artery

The morphology changes of control and treated umbilical arteries were demonstrated by frozen and paraffin embedded section of light microscope. In the control group, the lining of endothelial cells were intact and cleared dense nuclei. Clearly, fibroblast and collagen were observed. Ground substance of sub-endothelial cells was condensed sharply. Vascular smooth muscle cells were arranged in normal.

In the treatment groups, loose thickness and damaged endothelial cells were noticed. Less ground substance including fibroblast and collagen were observed as well. Invasion of vascular smooth muscle cells were demonstrated especially in

figure 4.42 of 40 µg/ml moderately oxLDL. In the pretreatment groups of DFO, EDTA and BHT, all were similar to dose of treatment group.

Figure 4.41

Intacted endothelium and normal vasculature of day 6 in control group; 10X (upper) and 40X (lower)

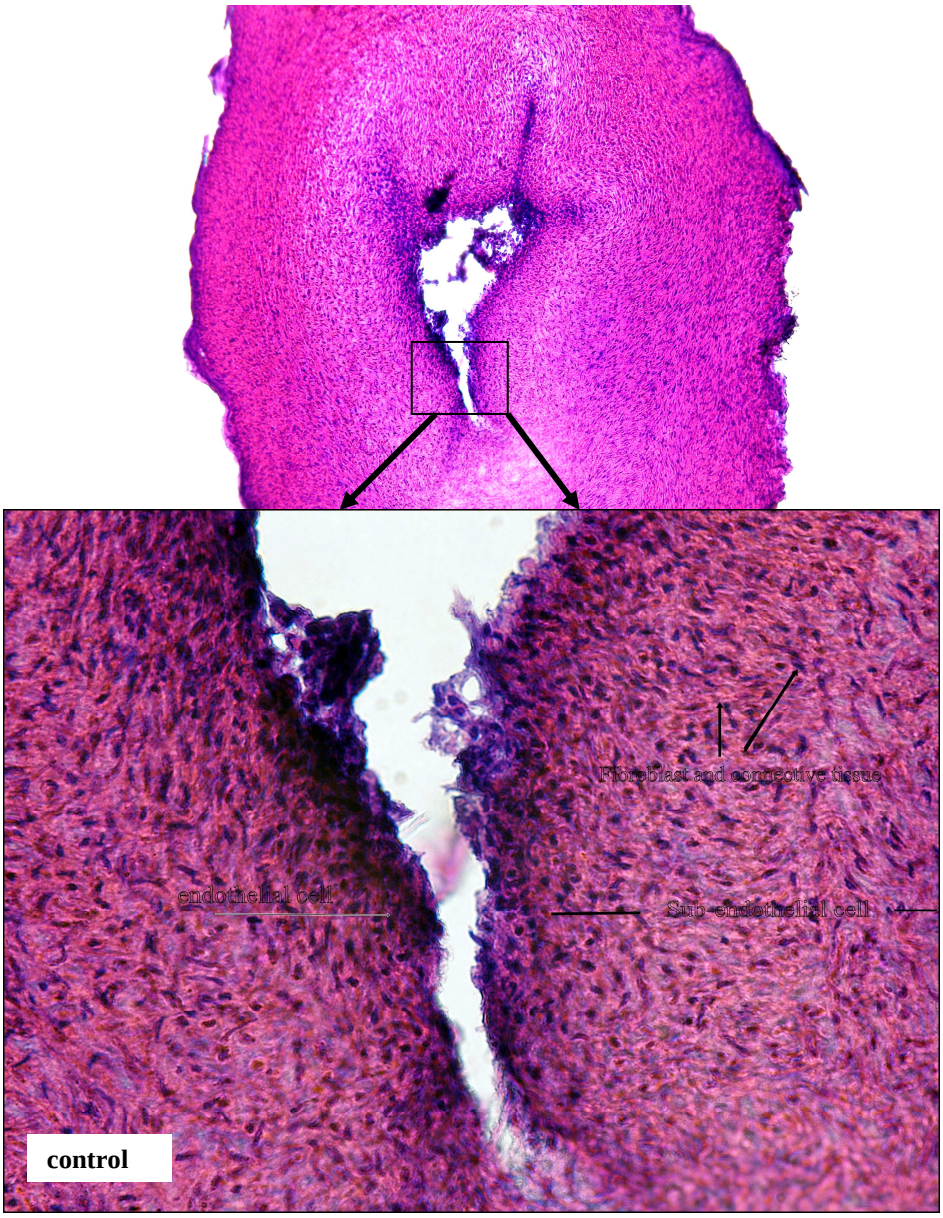


Figure 4.42

Damaged endothelium, loose ground substance, scantly fibroblast and invaded vascular smooth muscle cell (VSMC) were represented in various doses and degree of oxLDL on day 6; 40X

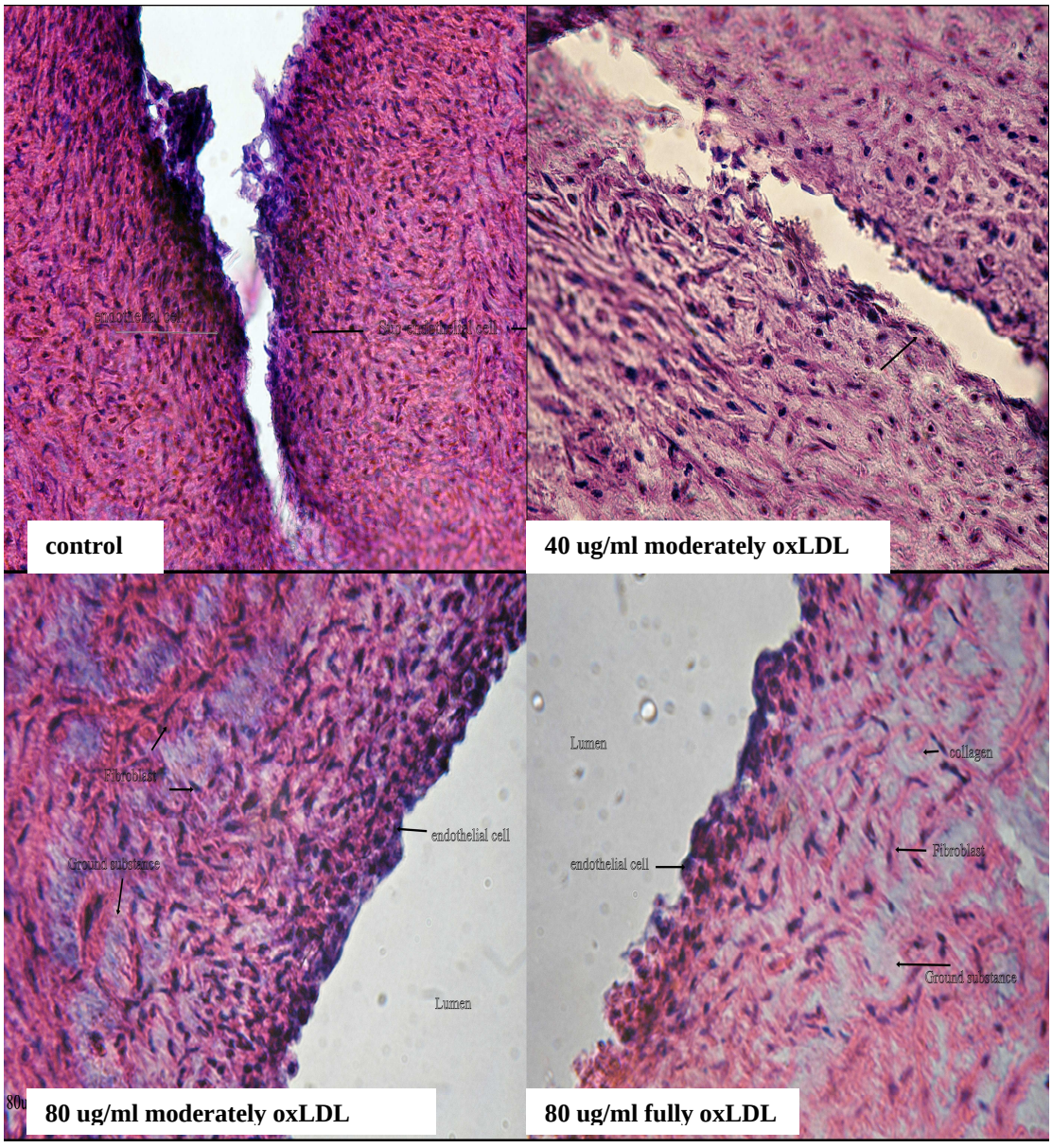


Figure 4.43

Morphology of 80 µg/ml moderately oxLDL with 10 µM DFO compared with 10 µM DFO and 80 µg/ml; 40X

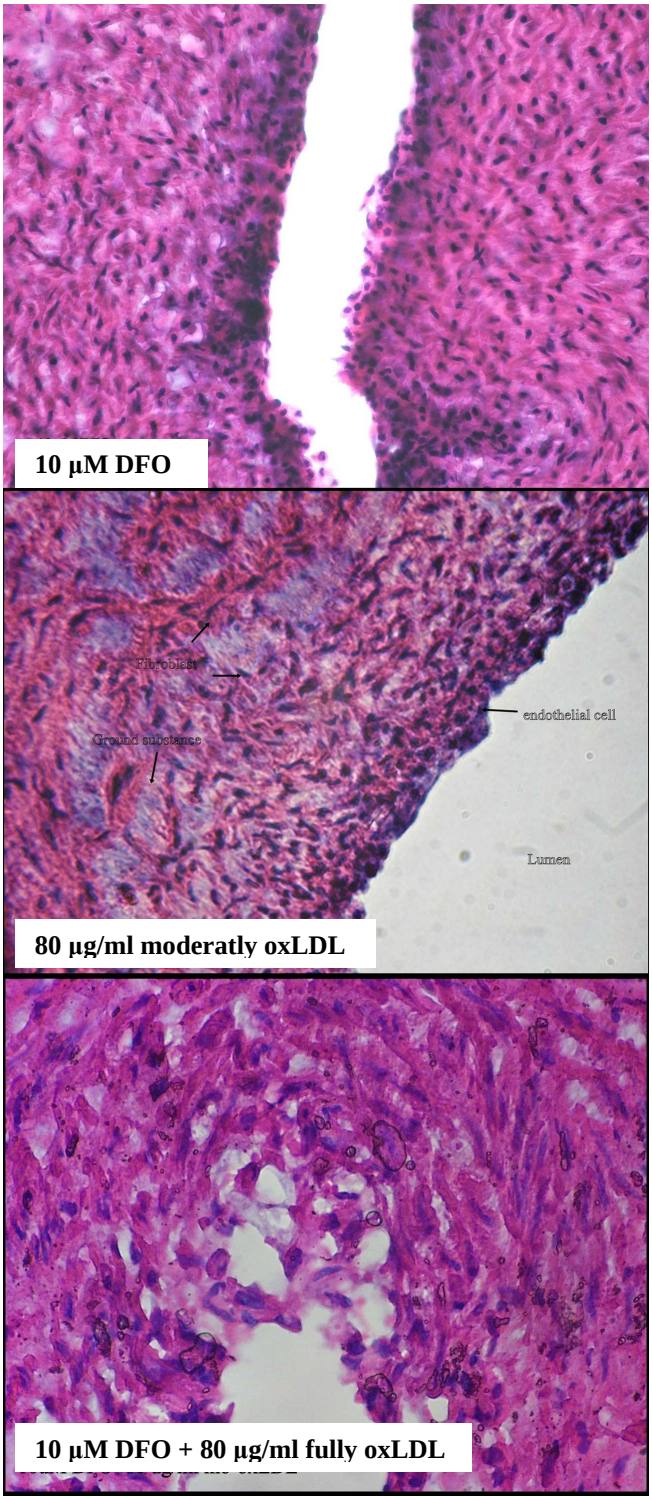


Figure 4.44

Morphology changes of 0.3 mM EDTA with 40 µg/ml and 80 µg/ml in moderately and fully oxidation of oxLDL compared with positive control 0.3 mM EDTA on day 6 of culture; 40X

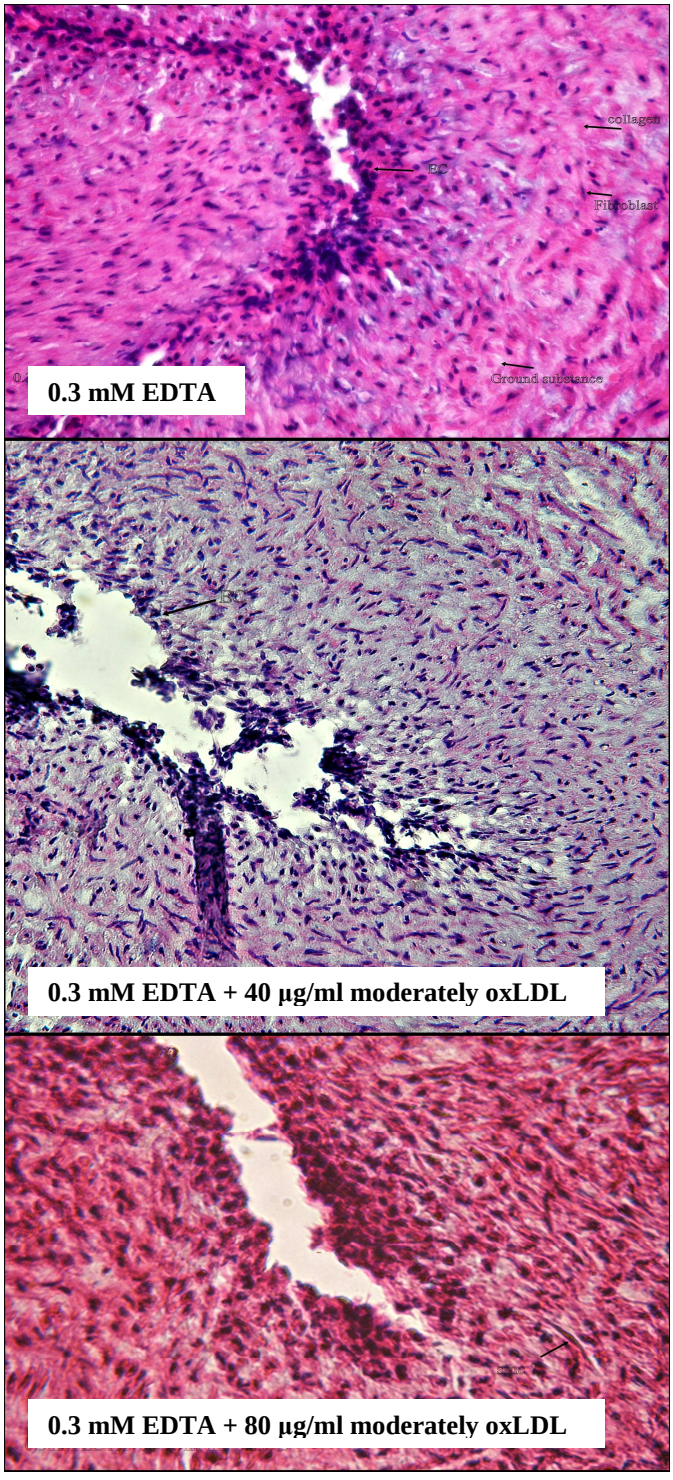
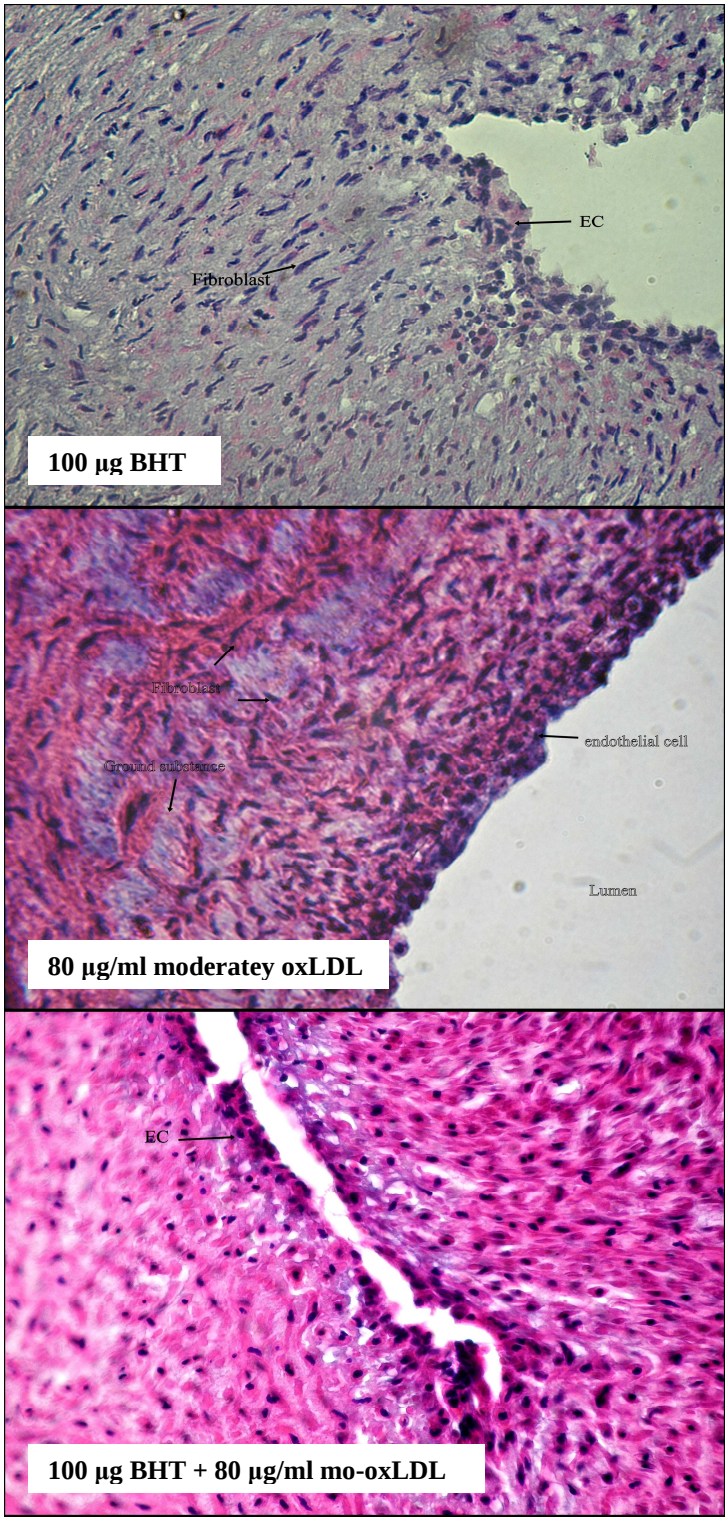


Figure 4.45

Morphology changes of 80 µg/ml moderately ox LDL with and without 100 µg/ml BHT compared with positive control 100 µg/ml BHT on day 6 of culture; 40X



Discussion

Oxidative modification of LDL is hypothesized that oxLDL plays a crucial role during development and plaque rupture of atherosclerosis. Failure of vitamin E protection in cardiovascular disease risks patients lead to revisit the mechanism of oxidative stress induced by oxLDL. Until now, it's still uncertain that how endothelium lost the protective vascular function resulting atherosclerosis.

The exposure of oxLDL at various doses (10, 20, 40 and 80 μg/ml) of mildly, moderately and fully oxidation causes gradually increased LOX-1 mRNA expression as dose-degree dependent fashion. It is the first report in vasculature culture that composes of complete vascular cells: endothelium, smooth muscle cell, adventitial fibroblast and extracellular matrix. Inducible expression and protein of LOX-1 was dependent in human endothelial cell activated by fully oxLDL (Mehta & Li, 1998). Moreover, increased uptake of oxLDL was also reported both in endothelial cell (Mehta & Li, 1998) and in vasculature (Chongcharoen, 1998). Interestingly, mildly oxLDL that represents any circulating oxLDL in early atherosclerosis is dose-degree dependent fashion as well. It is noteworthy that endothelium is still intact and functions as demonstrated by upregulation of eNOS expression and nitric oxide (NO) release. Meanwhile, downregulation of eNOS expression with reduced NO release is also seen in moderately (of dose 40 and 80 μg/ml) to fully oxidation of oxLDL. These finding suggests that endothelium is capable of producing NO indicating vascular tone function, in mildly degree and only low dose of moderately degree. Hence, activated LOX-1 expression of those treatments suggests the protective mechanism of endothelium. Furthermore, endothelial dysfunction is developed in high dose (40 and 80 μg/ml oxLDL) of moderately degree including in any doses of fully degree as we have seen the inhibition of NO and eNOS expression. Finding of morphological changes by light microscope is supported these observation.

We focus on intracellular ROS/RNS might involve in endothelial function because of it's paradox fashion of both physiological and pathophysiological states. Surprisely, DFO (ferric chelator) and EDTA (ferrous and copper chelator) promote the expression of LOX-1. DFO is iron chelator in Haber-Weiss reaction of ROS inhibiting $O_2^{\bullet -}$ -H_2O_2 driven, while as EDTA is iron chelator in Fenton's reaction

which inhibits $^{\bullet}OH$ formation driven from H_2O_2. Our findings suggest that $O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$ are involved in inducible expression of LOX-1 and produced from vascular enzymatic source such as NAD(P)H oxidase at endothelium rather than from metal transition catalyst source. Once oxLDL activated, ROS are produced through LOX-1 receptor and overwhelms the vascular cells with their ($O_2^{\bullet-}$, H_2O_2 and $^{\bullet}OH$) different diffusible rate and half-life. In a small dose and degree of oxLDL, ROS plays a role as physiological regulator for redox homeostasis of endothelium. Contrast data in a high dose and degree oxidation of LDL, ROS plays a role as pathophysiological regulator leading to endothelial dysfunction. Previous work done by other studied in cell (Cominacini, et al., 2000; Liao, 1994; Plane, Kerr, Bruckdorfer, & Jacobs, 1990) supported our findings and suggested the positive feed back loop of ROS. We used BHT as antioxidant substance protecting oxLDL in the system and is not involve in ROS pathway. Acts as vitamin E, BHT has pro-oxidant property as well (Black, 2002). Activated LOX-1 expression is also seen in BHT pretreatment group confirms ROS functions and points out to our oxLDL preparation is completely designed and limited further oxidation in the culture system.

During endothelial dysfunction development, uncoupling eNOS may be involved. Several lines of evidence reported that oxLDL through ROS is capable of decreasing BH_4 cofactors and eNOS-caveolin interaction including increasing NO metabolism (Behrendt & Ganz, 2002; Cominacini, et al., 2001; Harrison, 1997; Peterson, et al., 1999). Our finding of reducing real-time NO corresponds with downregulation of eNOS expression is documented. Further investigation of uncoupling eNOS should be speculated especially during the process of intact endothelial turn to dysfunction.

Remarkably increasing LOX-1 expression as seen in all treatment groups lead us to focus on RNS pathway and we hypothesized that upregulation of LOX-1 should be outcome of ROS and RNS regulation. One reason report is dismutation rate of $O_2^{\bullet-}$ by SOD which scavenges $O_2^{\bullet-}$ to be H_2O_2, stable and less active than $O_2^{\bullet-}$, goes slower than transformation rate of $O_2^{\bullet-}$ and NO to be peroxynitrite ($ONOO^-$). Activation of various doses and degree of oxLDL causes reducing activity of SOD. SOD activity remains low in pretreatment group too indicating low level of $O_2^{\bullet-}$.

According to reduced NO production, perhaps, $O_2^{\bullet-}$ combines with NO and peroxynitrite is generated. Lower SOD activity should be from low level of $O_2^{\bullet-}$, in the other hand, damaged from ROS/RNS in the system.

Activation of oxLDL via LOX-1 receptor causes ROS/RNS generated and subsequently activates redox-sensitive proteins and other in cell. We purpose that activation of oxLDL via LOX-1 receptor may trigger ROS/RNS signalings through activation of p38 MAPK. Mean density of p38 MAPK is increased gradually as dose-degree dependent fashion and then lowered in iron chelator and BHT pretreatment groups. These findings indicate the role of ROS and RNS signalings activate redox-sensitive protein / enzymes, in particularly, during endothelial dysfunction. Zhao (Zhao, et al., 2000) reported that activation of p38 MAPK is required for foam cell formation. Moreover, oxLDL induces p38 MAPK in 5 minutes and translocate smooth muscle cell in 15 minutes (Liaudet, Vassalli, & Pacher, 2009). Overview depicted mechanisms in endothelial dysfunction of this study is purposed in figure 4.46