



## Normal and reconstituted high-density lipoprotein protects differentiated monocytes from oxidized low-density lipoprotein-induced apoptosis

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### Original Article

#### Abstract

**BACKGROUND:** The progression of atherosclerosis is an ongoing struggle between cell division and cell death. Lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1), a novel receptor for oxidized low-density lipoprotein (ox-LDL), mediates ox-LDL-induced apoptosis of monocytes. The anti-atherogenic function of plasma high-density lipoprotein (HDL) includes the ability to inhibit apoptosis of macrophage, although the exact mechanism and consequences of apoptosis in the development and progression of this disease are still controversial. Thus, in the present study, the effect of normal HDL (nHDL) and reconstituted HDL (rHDL) on ox-LDL-induced cellular responses in differentiated monocytes in view of apoptosis and LOX-1 receptor expression was investigated.

**METHODS:** The expression of B-cell lymphoma-2 (Bcl-2), B-cell lymphoma-extra large (Bcl-xL), caspase-3, and cytochrome c (cyt c) was assessed and substantiated in 30 hyper-LDL and control subjects. To assess the expression of LOX-1 on differentiated THP-1 cells, western blotting was carried out, followed by statistical analysis in 30 patients and control subjects.

**RESULTS:** nHDL/rHDL inhibited the ox-LDL-induced apoptosis in the differentiated human monocytic cells, THP-1 cells, and differentiated monocytes of patient and control subjects. Enhanced expression of scavenger receptor, LOX-1, in the differentiated monocytes was also downregulated in presence of nHDL/rHDL. nHDL/rHDL could inhibit the ox-LDL-induced mitochondrial apoptotic pathway and aberrant expression of LOX-1 in patients. Double immunostaining using fluorescein isothiocyanate (FITC)-conjugated ox-LDL and LOX-1 in apoptotic cells indicates its significant role in the differentiated monocytes.

**CONCLUSION:** It was observed that nHDL/rHDL could promote macrophage survival by preserving mitochondrial integrity from ox-LDL-induced apoptosis.

**Keywords:** Atherosclerosis; Lipoproteins; Macrophages; Apoptosis

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#### Introduction

Atherosclerosis involves the deposition of fatty substances inside the artery walls, which causes thickening and hardening of the arteries. Such conditions affect circulation, causing high blood pressure, ultimately leading to angina, heart attack, stroke, and/or sudden cardiac death.<sup>1</sup> The initial phase of atherogenic process involves low-density lipoprotein cholesterol (LDL-C) accumulation, generation of reactive oxygen species (ROS), and oxidative modification of LDL-C. Exposure of macrophages to oxidized LDL (ox-LDL), a major component of atherosclerotic plaques, appears to be a key event in this process, promoting both

inflammation and intracellular cholesterol deposition with the formation of lipid-laden foam cells.<sup>2</sup>

Apoptotic macrophages have been found to be concentrated in the areas of plaque rupture. The death of macrophages in atherosclerotic lesions is multifactorial and strongly correlated to the developmental stage of the atherosclerotic plaques.<sup>3</sup>

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Loss of macrophages can also lead to the accumulation of apoptotic bodies, which can result in the activation of inflammatory mediators.<sup>4</sup>

Serum high-density lipoprotein cholesterol (HDL-C) level is an independent predictor of risk for cardiovascular events in both men and women. HDL particles have been reported to exhibit various anti-atherogenic and cardioprotective effects by modulating the function of various cells including the cells of the artery wall.<sup>5</sup> Thus, the biological role of such potentially important molecules in scavenger receptor-mediated ox-LDL-induced apoptosis of differentiated monocytes needs further evaluation in view of cellular modulation.

### Materials and Methods

Blood samples (15 ml) were obtained from 30 cases of age- (30-60 years old) and sex-matched hyper-LDL patients with hypertension (HTN) and subjects with normal LDL (nLDL) (control) diagnosed and managed by Department of Internal Medicine, Post Graduate Institute of Medical Education and Research, Chandigarh, India, in 2010. Patients having diabetes mellitus (DM), smoking habit, renal insufficiency, hyper/hypothyroidism, nephritic syndrome, and acute myocardial infarction (MI) within one year (single/combination) were excluded from this study. Peripheral blood mononuclear cells (PBMC) were separated from blood of each patient and control subject by Ficoll-Histopaque density gradient centrifugation.<sup>6</sup> A formal written informed consent for this study was obtained from each patient. The study followed all ethical guidelines and was approved by the institutional ethical committee (No.:5/4/1-8/2004-NCD-II).

Human monocytic leukemic cell line THP-1 [National Centre for Cell Science (NCCS), Pune, India] and total PBMCs (obtained from blood samples of patients/control subjects) were cultured separately in RPMI-1640 (ICN, USA) supplemented with 2 mM glutamine, 50 U/ml penicillin, 50 µg/ml streptomycin, and 10% heat-inactivated fetal calf serum at 37 °C in a humidified atmosphere of 5% carbon dioxide (CO<sub>2</sub>)-95% air. Cell viability was assessed by the exclusion of 0.02% trypan blue dye.<sup>7</sup> The monocytes were differentiated in the presence of phorbol-12 myristate-13-acetate (PMA).<sup>8</sup>

**Preparation of lipoproteins:** Normal HDL (nHDL) (d = 1.125-1.210 g/ml) and nLDL (d = 1.019-1.250 g/ml) were isolated from human plasma obtained from blood bank of Post Graduate Institute of Medical Education and Research.<sup>9</sup> For purification of apolipoprotein A1 (apo A-I), nHDL was delipidified.<sup>10</sup>

The protein part of the delipidified HDL was resolubilised in 50 mM glycine/4 mM sodium hydroxide (NaOH) (pH = 8.8)/500 mM sodium chloride (NaCl)/6 M urea and was fractionated by gel filtration chromatography on Sephacryl S-200 column (1.7 × 82 cm).<sup>10</sup> The collected fractions (each 3 ml) were monitored for the presence of protein at 280 nm. The fraction containing apo A-I was further purified by gel filtration chromatography on Sephacryl S-300 column in the fast protein liquid chromatography (FPLC) (Pharmacia, Sweden). The protein was estimated by bicinchoninic acid (BCA) assay<sup>11</sup> and the molecular weight (M<sub>r</sub>) of apo A-I was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).<sup>12,13</sup> The specificity of apo A-I to anti-apo A-I was assessed by enzyme-linked immunosorbent assay (ELISA).<sup>14</sup> The purified apo A-I and phosphatidyl choline (PC) were used to prepare reconstituted HDL (rHDL).<sup>11</sup> The LDL fraction was oxidized<sup>15</sup> and oxidation of LDL was confirmed by the measurement of thiobarbituric acid reactive substances (TBARS).<sup>16</sup> Ox-LDL was conjugated separately to horseradish peroxidase (HRP) and fluorescein (FLUOS) using Peroxidase Labeling Kit and Fluorescein Labeling Kit (Roche Biochemicals, Germany), respectively, as per manufacturer's instructions.

**Apoptosis:** The optimum dose and time of PMA for differentiation of THP-1 cells were selected in view of deoxyribonucleic acid (DNA) fragmentation analysis using DNA Ladder Kit (Roche, Germany). Briefly, THP-1 cells (10<sup>6</sup>/ml media) were cultured in 6 well plates with different doses of PMA (5 nM-100 nM) for 24 hours. In a separate set of experiments, THP-1 cells were cultured with optimum dose of PMA for different time periods (6-24 hours). DNA was isolated as per manufacturer's instructions, analyzed by agarose gel (1.8%) electrophoresis and visualized under ultraviolet (UV) light in the gel documentation system (Bio-Rad Laboratories, USA). Cells cultured without PMA under the same conditions were taken as control.

For selection of optimum dose of ox-LDL, PMA-differentiated THP-1 cells (0.25 × 10<sup>6</sup>/500 µl serum free media/well) were cultured in presence of different doses of ox-LDL (25-100 µg/ml) for 24 hours. In another set of experiment, PMA-differentiated THP-1 cells were triggered in presence of optimum dose of ox-LDL for different time intervals (12 hours, 18 hours, and 24 hours). After washing, the cells were fixed in 1% paraformaldehyde solution and fluorescein isothiocyanate (FITC)-deoxyuridine triphosphate (dUTP) staining was performed using Apo-Direct

Kit (Roche, Germany). PMA-differentiated cells cultured under the same conditions without ox-LDL as well as controls supplied with the kit were run in parallel. The cells were analyzed by flow cytometry (FC) using CellQuest software program.

The optimum dose and time of nHDL/rHDL for treatment of PMA-differentiated THP-1 cells ( $0.25 \times 10^6$  cells/500  $\mu$ l media/well) cultured in absence and in presence of ox-LDL were selected in view of apoptotic index using the Cell Death Detection ELISA Kit (Roche, Germany).

**Experimental groups:** Based on the selection of optimum dose and time for different triggering agents used in the present study, all the parameters were studied in four different groups. Group a consisted of PMA-differentiated cells (THP-1 cells as well as cells from patients and control subjects), group b consisted of the differentiated cells cultured in presence of ox-LDL, and group c and group d consisted of the differentiated cells preincubated with nHDL and rHDL, respectively, followed by triggering with ox-LDL.

**Reverse transcription polymerase chain reaction (RT-PCR) analysis:** Total ribonucleic acid (RNA) was extracted from all groups of THP-1 cells using the Qiagen RNA/DNA Mini Kit (Qiagen, Germany). The quantitation of RNA/DNA was done spectrophotometrically. The level of expression of various pro- and anti-apoptotic transcripts [B-cell lymphoma-2 (Bcl-2)-associated X-protein (BAX), Bcl-2, Bcl-extra large (Bcl-xL), caspase 3, and cytochrome c (cyt c)] was assessed by RT-PCR using primer pair specific to respective transcripts and Titan One-Step RT-PCR Kit (Sigma Aldrich, United States). The amplified transcripts were analyzed by agarose gel electrophoresis (1.8%). The changes in band intensity in case of each parameter were quantified by scanning densitometry using Scion Image 4.3 software (Bio-Rad, USA). Variations in the complementary DNA (cDNA) concentration were normalized by co-amplification with  $\beta$ -actin in each set. The increase/decrease in the expression of each parameter in the cells of group b $\rightarrow$ d was calculated by taking the normalized value of the same transcript as in cells of group a.

**Western blot analysis:** THP-1 cells ( $10^6$ /ml) from each group were lysed with lysis buffer 10 mM HEPES (pH = 7.5)/150 mM NaCl/10% glycerol/10 mM sodium orthovanadate ( $\text{NaVO}_4$ )/0.2% Triton X-100/cocktail protease inhibitors (1:10, Roche, Germany). The debris was removed by centrifugation (10000 rpm, 10 minutes). After estimation of the protein content, equal amount (60  $\mu$ g) of each lysate

was subjected to 12% SDS-PAGE. The protein bands were electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (0.45  $\mu$ m, Immobilon-P, Millipore Eschborn, Germany) at 80 V for 2 hours at 4  $^{\circ}$ C,<sup>17</sup> followed by incubation in blocking buffer 5% skim milk in phosphate buffered saline (SM-PBS) containing 0.1% Tween-20 at 4  $^{\circ}$ C for overnight and washing with PBST (PBS containing 0.1% Tween-20). Membranes were probed with antibodies to Bcl-2 (N-19, 1:500), BAX (N-20, 1:500), cyt c (C-20, 1:250), caspase-3 (N-19, 1:250), and  $\beta$ -actin (AC-15, 1:3000, taken as internal control) for 2 hours at 37  $^{\circ}$ C. After extensive washing, the blots were incubated with the respective HRP-conjugated secondary antibody (1:1500) for 1 hour at 37  $^{\circ}$ C. After washing, the membranes were developed with 3,3'-diaminobenzidine (DAB) tetrahydrochloride/hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), except for cyt c. Cyt c was detected with enhanced chemiluminescence (ECL) plus Western blotting detection system (Amersham, UK). Antibodies were procured from Santa Cruz Biotechnology (CA, USA).

**Assessment of apoptosis in differentiated monocytes:** Total PBMCs of each blood sample were cultured with PMA with percentage of monocytes to be 0.7%-0.8% of total PBMCs. The extent of apoptosis in the monocytes/macrophages obtained from a $\rightarrow$ d groups of patients and control subjects was assessed by Cell Death Detection ELISA (CDD ELISA).

**Expression of BAX and Bcl-2 in differentiated monocytes:** To assess the expression of BAX and Bcl-2 in the monocytes/macrophages obtained from a $\rightarrow$ d groups of patients and control subjects, PBMCs from each group were harvested separately with PBS-ethylenediaminetetraacetic acid (EDTA), washed and fixed in 1% paraformaldehyde (20 minutes, 4  $^{\circ}$ C). After washing, cells were incubated in permeabilization buffer for 10 minutes and centrifuged (1200 rpm, 5 minutes). Subsequently, cells were treated with primary antibody against BAX (1:20)/Bcl-2 (1:50) for 1 hour, washed, and incubated with fluorochrome-conjugated respective secondary antibody (1:1000) for 30 minutes. After washing, cells resuspended in PBS and monocytes/macrophages were analyzed by FC using CellQuest software program. Result was expressed in view of mean fluorescence intensity (MFI) of the FITC-positive cells in each group. In each set, a negative control was used in order to rule out non-specificity.

**Assessment of lectin-like oxidized LDL receptor-1 (LOX-1) expression on differentiated monocytes:** To assess the expression of LOX-1 on differentiated THP-1 cells (group a $\rightarrow$ d), membrane fraction of

the cells from each group was prepared and subjected to Western blotting.<sup>17,18</sup>

Expression of LOX-1 on differentiated monocytes obtained from a→d groups of patients and control subjects was assessed by FC. Total PBMCs isolated from each blood sample were cultured separately in presence of PMA. Cells were harvested and incubated with FITC-conjugated ox-LDL for 1 hour at room temperature. After washing, cells were analyzed by FC. The result was expressed in view of the MFI of the FITC-positive cells in each group. In each set, a negative control was used to rule out irrelevant specificity.

Statistical analysis for normally-distributed parametric tests was performed by paired t-test/independent t-test as per requirement, and further, in patient samples, correlation and regression analysis was done.

## Results

nHDL (d = 1.125-1.210 g/ml) and nLDL (d = 1.019-1.250 g/ml) were isolated and purified, purification of apolipoprotein A-I, nHDL was delipidified and the protein part of the delipidified HDL was resolubilised followed by gel filtration chromatography on Sephacryl S-200 column (1.7 × 82 cm).

**Purification and characterization of apo A-I:** Four different types of lipoproteins (nLDL, ox-LDL, nHDL, and rHDL) were used in the present study. The apo A-I was purified from HDL by sequential gel filtration chromatography on Sephacryl S-200 column and Sephacryl S-300 HR16/60 column. The  $M_r$  of the purified apo A-I was confirmed in SDS-PAGE, in which it migrated as a single band of 29 kDa. The ELISA titer of anti-apo A-1 was 1:10000 with 15.6 ng of the purified apo A-I. The rHDL was prepared using the purified apo A-I along with PC.

**Evaluation of ox-LDL-induced apoptosis in differentiated THP-1 cells:** Differentiation of THP-1 cells in the presence of different doses (5-100 nM) of PMA was evaluated in view of DNA fragmentation analysis. A ladder-like appearance was visualized in the DNA isolated from THP-1 cells triggered with 100 nM PMA for 24 hours. In another set of experiment, when THP-1 cells were cultured with 100 nM PMA for different time intervals (6-24 hours), no ladder was detected in DNA isolated from the cells cultured with PMA till 18 hours. Thus, in the subsequent experiments, THP-1 cells were differentiated with 100 nM PMA for 18 hours.

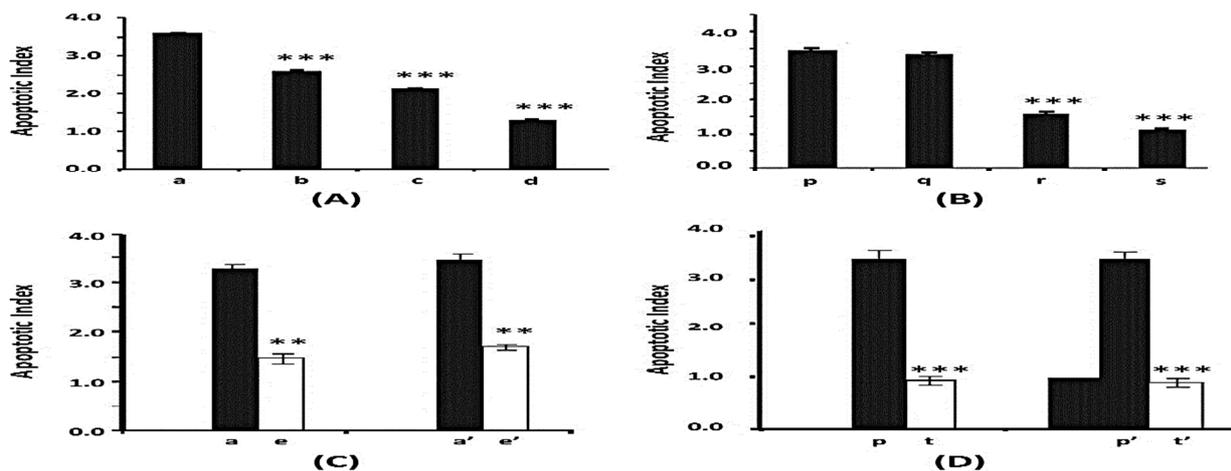
Apoptotic cells were found to be increased significantly ( $P \leq 0.001$ ) with 25 µg/ml, 50 µg/ml, and 100 µg/ml ox-LDL, respectively, at 24 hours as

compared to the untreated cells and they were maximum with 100 µg/ml ox-LDL.

A time-dependent significant ( $P \leq 0.001$ ) increase in % apoptotic cells was observed at 12 hours (10%), 18 hours (25%), and 24 hours (29%) as compared to that of respective control cells (5%). Since no significant increase in the number of apoptotic cells was noticed at 24 hours as compared to that at 18 hours, in the subsequent experiments, differentiated THP-1 cells were cultured with 100 µg/ml ox-LDL for 18 hours.

**Effect of nHDL and rHDL on ox-LDL-induced apoptosis in THP-1 cells:** Effect of various doses (25-100 µg/ml) of nHDL and rHDL on ox-LDL (100 µg/ml, 18 hours)-induced apoptosis in differentiated THP-1 cells was assessed separately by CDD ELISA. A significant ( $P < 0.001$ ) reduction in the extent of apoptosis was noted in presence of nHDL and rHDL (Figure 1A and 1B,  $P < 0.001$ ), which was maximum at 100 µg/ml dose for both molecules. Moreover, ox-LDL-induced apoptosis in the differentiated THP-1 cells was also reduced significantly (\*\* $P < 0.050$ , \*\*\* $P < 0.001$ ) on 12 hours preincubation with nHDL and rHDL (100 µg/ml) separately (Figure 1C and 1D, \*\* $P < 0.050$ , \*\*\* $P < 0.001$ ). Since no significant change in the extent of apoptosis was noted at 12 hours of preincubation with nHDL/rHDL as compared to that at 6 hours, in the subsequent experiments, PMA-differentiated cells were preincubated for 6 hours with 100 µg/ml of nHDL/rHDL and then cultured with ox-LDL (100 µg/ml, 18 hours).

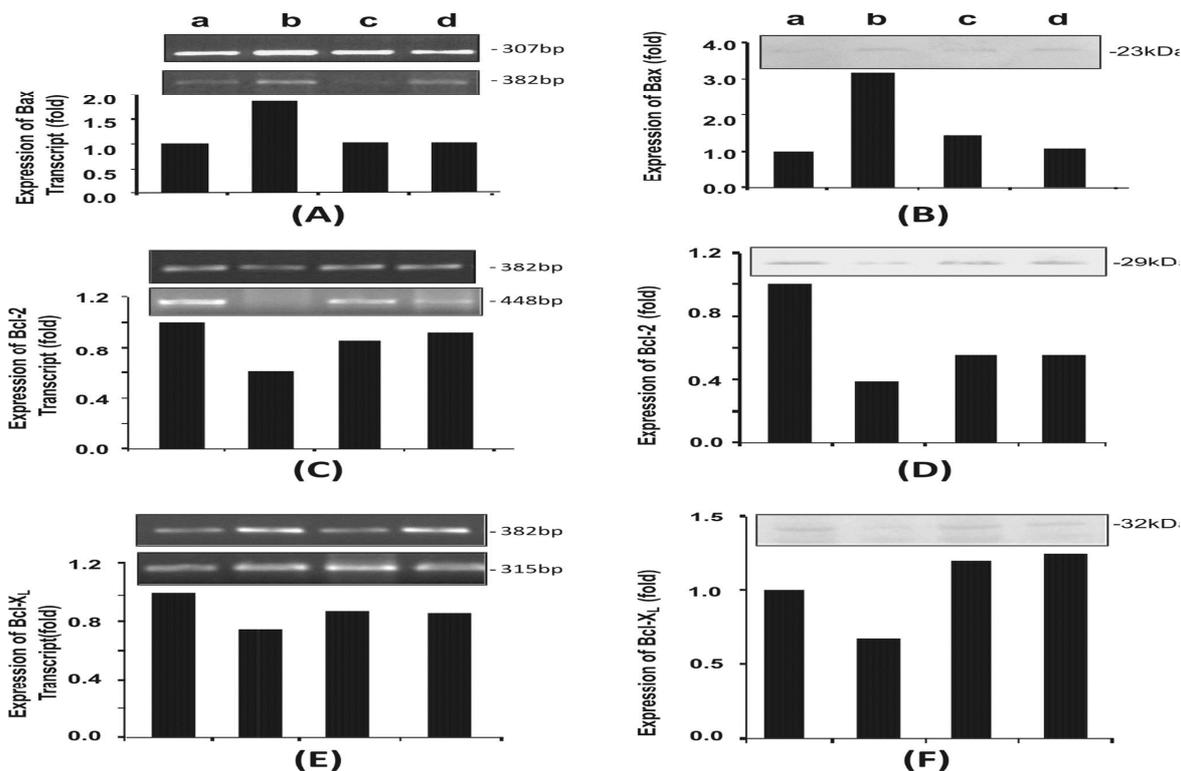
**HDL suppresses apoptosis in differentiated THP-1 cells:** Impact of nHDL/rHDL on ox-LDL-induced apoptotic signal transduction pathways was assessed in differentiated THP-1 cells with respect to the expression level of different regulatory and effector proteins of apoptosis, at both transcript and protein levels of BAX (Figure 2A and 2B), Bcl-2 (Figure 2C and 2D), and Bcl-xL (Figure 2E and 2F). An enhanced expression of BAX was noted in group b as compared to group a, while it reduced appreciably in group c and d when compared to group b. The level of expression of Bcl-2 and Bcl-xL decreased in group b as compared to group a, whereas it was upregulated in group c and d. We examined the effect of nHDL/rHDL on ox-LDL-induced release of cyt c and activation of caspase-3 in differentiated THP-1 cells as shown in figure 3. Cyt c expression was downregulated in group c and d as compared to group b. The level of expression of caspase-3 transcript was upregulated in group b as compared to group a, whereas an appreciable decrease in its level was noted in group c and d as compared to group b.



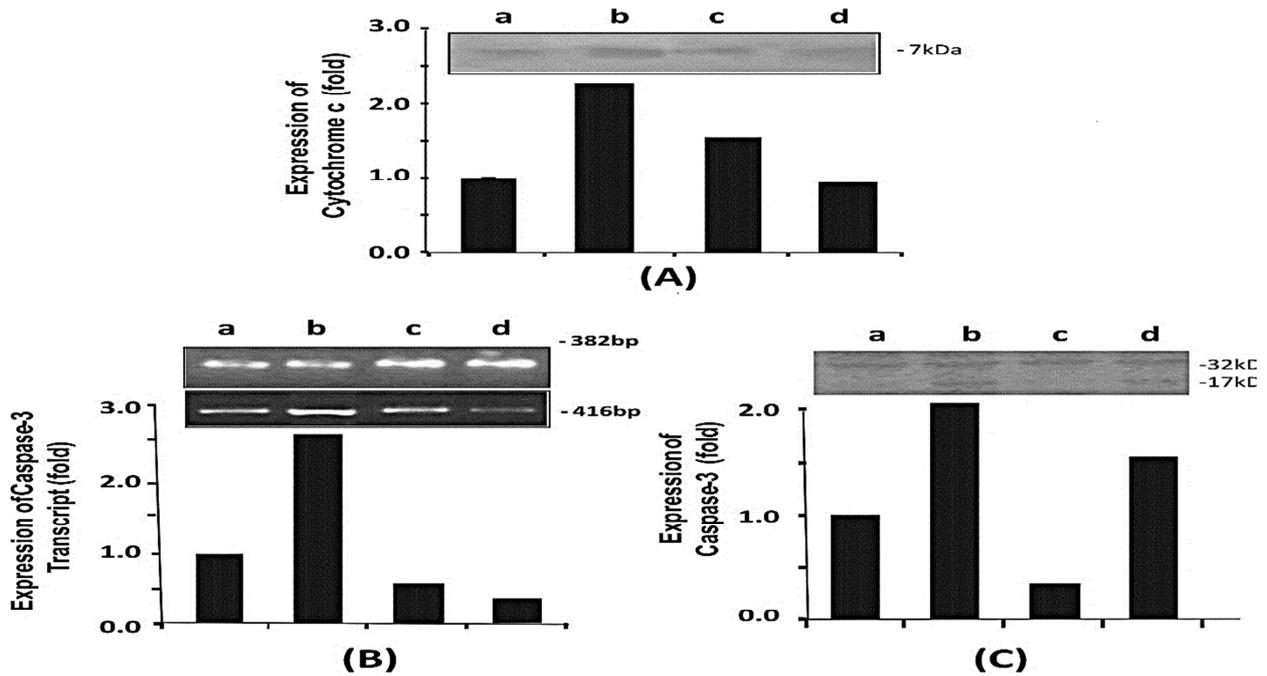
**Figure 1.** A and B) The bar graph showing the apoptosis in phorbol-12 myristate-13-acetate (PMA)-differentiated THP-1 cells cultured in presence of oxidized low-density lipoprotein (ox-LDL) preincubated without (a and p) and with different doses of 25 µg/ml (b and q), 50 µg/ml (c and r), and 100 µg/ml (d and s) of normal high-density lipoprotein (nHDL) and reconstituted HDL (rHDL), respectively; C and D) The assessment of apoptosis in PMA-differentiated THP-1 cells cultured in presence of ox-LDL without (a and e) and with 100 µg/ml of nHDL (a' and e') for 6 hours and without (p and t) and with (p' and t') 100 µg/ml rHDL, respectively, for 12 hours. Each bar represents the mean ± standard deviation (SD) of three independent experiments performed in duplicates.

Further, in Western immune blot, a prominent band (17 kDa) of activated caspase-3 was found in

group b, which was not detectable in group c and reduced in group d.



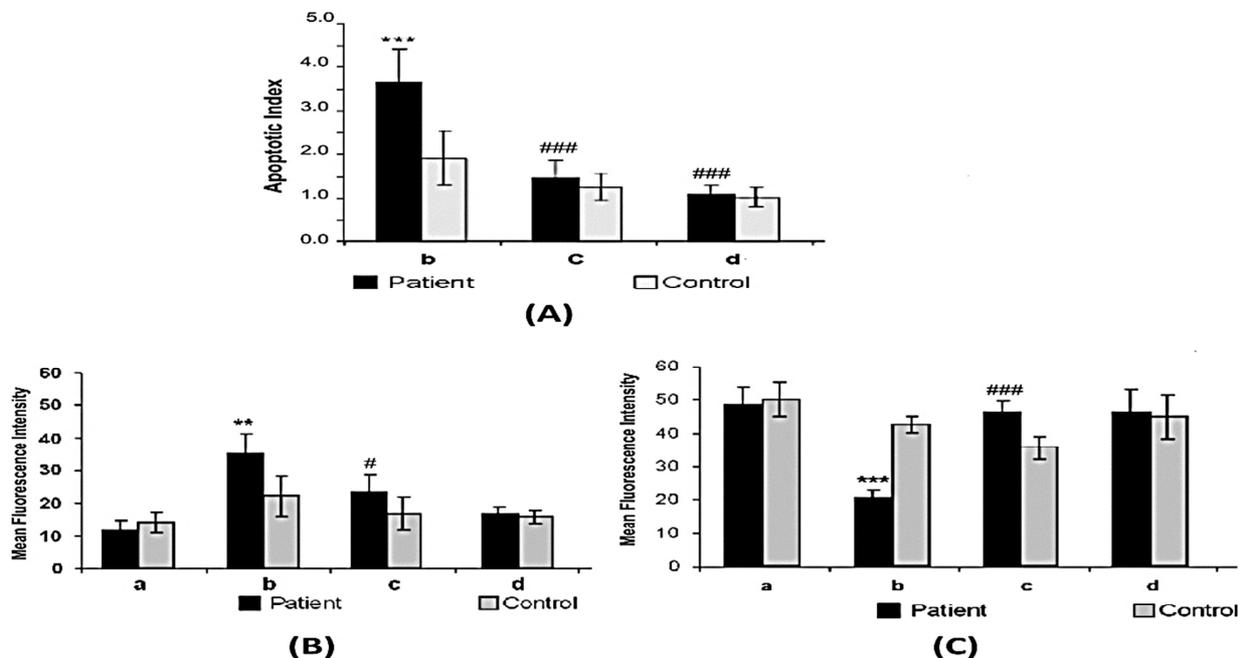
**Figure 2.** Effect of normal high-density lipoprotein (nHDL) and reconstituted HDL (rHDL) on oxidized low-density lipoprotein (ox-LDL)-induced alteration in expression of B-cell lymphoma-2 (Bcl-2)-associated X-protein (BAX) (A), Bcl-2 (C), and Bcl-extra large (Bcl-xL) (E) in differentiated THP-1 cells detected by reverse transcription polymerase chain reaction (RT-PCR) and Western immunoblotting BAX (B), Bcl-2 (D), Bcl-xL (F), respectively. Bar graphs represent the level of expression of BAX, Bcl-2, and Bcl-xL molecules under different conditions both at messenger ribonucleic acid (mRNA) and protein levels.



**Figure 3.** Effect of normal high-density lipoprotein (nHDL) and reconstituted HDL (rHDL) on oxidized low-density lipoprotein (ox-LDL)-induced release of cytochrome c (cyt c) (A) and activation of caspase-3 in differentiated THP-1 cells was determined both at messenger ribonucleic acid (mRNA) (B) and protein level (C). Bar graph represents the level of expression of caspase-3 under different conditions with respect to that of control.

*Effect of nHDL/rHDL on ox-LDL-induced apoptosis in differentiated monocytes:* We further substantiated our

studies on monocytes obtained from patients and control subjects (Figure 4A).



**Figure 4.** Bar graphs showing the effect of normal high-density lipoprotein (nHDL) and reconstituted HDL (rHDL) on oxidized low-density lipoprotein (ox-LDL)-induced apoptosis (A) and expression of B-cell lymphoma-2 (Bcl-2)-associated X-protein (BAX) (B) and Bcl-2 (C) proteins in differentiated monocytes obtained from patients and control subjects

Apoptotic index and the extent of apoptosis was increased by 4-fold and 1.2-fold in group b in patients and control subjects, respectively. A significant ( $P < 0.001$ ) decrease in the extent of apoptosis was noted in group c and d as compared to that in group b of patients' samples.

Our findings were authenticated by the study on the level of expression of BAX and Bcl-2 in the monocytes obtained from a→d groups of patients and control subjects. In case of patients' samples, BAX was significantly increased in group b ( $P < 0.050$ ) as compared to group a, while its expression was found to be decreased in group c ( $P < 0.010$ ) and d as compared to group b (Figure 4B). Further, levels of Bcl-2 was significantly reduced in group b ( $P < 0.001$ ) as compared to group a, whereas it was increased in group c ( $P < 0.001$ ) as compared to group b (Figure 4C) in patient sample.

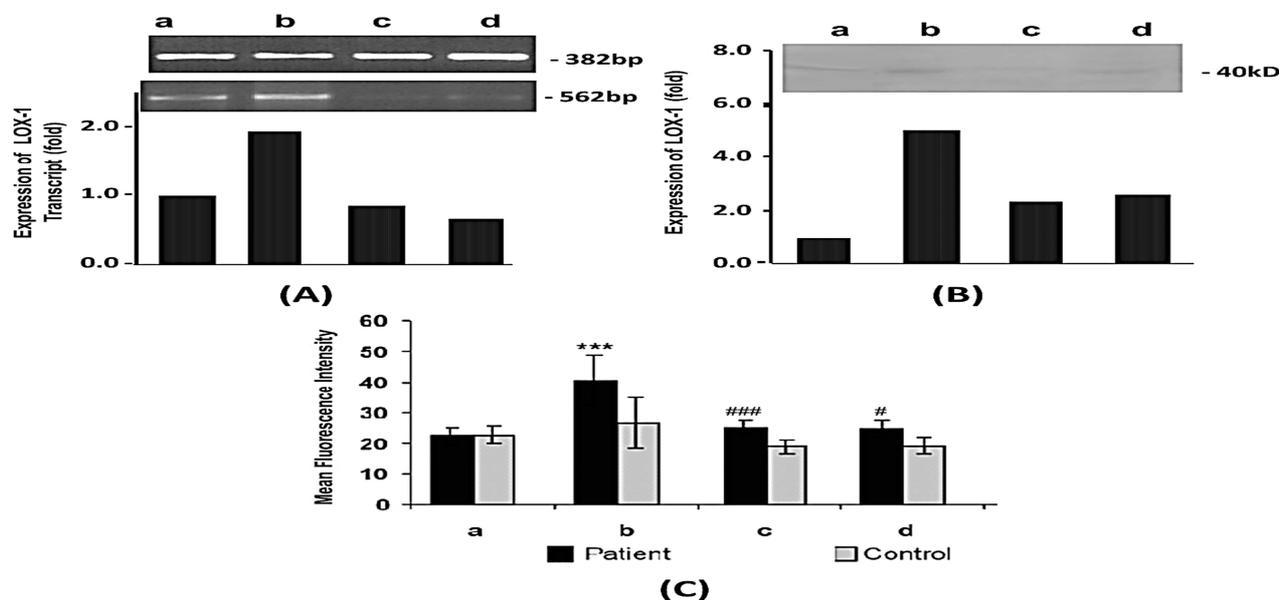
**Assessment of LOX-1 expression:** The level of LOX-1 was variable in different groups (a→d) of THP-1 cells. It was found to be increased in group b as compared to group a, while its expression was decreased in group c and d in comparison to group b (Figure 5A and 5B). Our result was further substantiated by studying the LOX-1 expression in a→d groups of monocytes obtained from hyper-LDL and normal-LDL subjects (Figure 5C). In case of hyper-LDL subjects, the level of LOX-1 was increased significantly in group b ( $P < 0.001$ )

compared to group a. However, a significant decrease in its level of expression was noted in group c ( $P < 0.001$ ) and d ( $P < 0.010$ ) as compared to group b. In case of control subjects, no significant alteration in the level of LOX-1 expression was noted between the groups.

## Discussion

The major finding of this study is that the increased expression of pro-apoptotic members (BAX, caspase-3, cyt c) was found to be decreased in the presence of nHDL and rHDL in differentiated monocytes. Ox-LDL was used to induce apoptosis in PMA-differentiated human monocytes. It has been reported that PMA could trigger differentiation or apoptosis, depending on the cell type.<sup>19</sup> This dual effect of PMA may be due to its ability to activate different isoforms of protein kinase C (PKC), the activation of which can either lead to the disordered growth, cell transformation, and inhibition of apoptosis or it can result in cell growth inhibition and induction of apoptosis.<sup>20</sup>

In the present study, it was observed that pre-incubation of differentiated cells with 100  $\mu\text{g/ml}$  of nHDL/rHDL for 6 hours followed by co-incubation with ox-LDL for 18 hours could inhibit apoptosis significantly. Nofer et al.<sup>21</sup> reported that apoptosis of endothelial cells could also be suppressed by HDL and associated lysosphingolipids.



**Figure 5.** A and B) The expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) at messenger ribonucleic acid (mRNA) and protein level in differentiated monocytes triggered in presence of oxidized low-density lipoprotein (ox-LDL) with and without normal high-density lipoprotein (nHDL) and reconstituted HDL (rHDL), respectively; C) Bar graphs showing the effect of nHDL and rHDL on ox-LDL-induced LOX-1 expression in differentiated monocytes obtained from patients and control subjects in different experimental groups (a→d)

A significantly higher extent of apoptosis and an increased expression of BAX was found in group b of hyper-LDL subjects as compared to that of normal-LDL subjects which was consistent with the reports of Rosa et al., who demonstrated the effect of mitochondrial apoptotic pathway in macrophages during atherosclerosis.<sup>22</sup> It has also been reported that macrophages could express BAX in atherosclerotic plaques and cholesterol withdrawal could reduce the expression of BAX and apoptotic cells in these plaques,<sup>23</sup> which supported our observation in group c and d in patients' samples, in which, decrease in the extent of apoptosis and BAX level was observed.

Mehta et al.<sup>24</sup> reported that LOX-1 mediated ox-LDL-induced apoptosis in vascular endothelial cells by stimulating p38 mitogen-activated protein kinase (MAPK) which was in good agreement with our observations. It is possible that uptake of ox-LDL through LOX-1 could activate mitochondrial apoptotic signal transduction pathways.

It can be suggested that nHDL/rHDL could either prevent the binding of ox-LDL to LOX-1 by preventing the accumulation of oxidized lipids in the ox-LDL through lecithin-cholesterol acyltransferase (LCAT) enzyme, thereby inhibiting ox-LDL-mediated signaling through this receptor or it might degrade the ox-LDL in the system because several apolipoproteins, including apo A-I, have intrinsic antioxidant property.<sup>1</sup>

### Conclusion

Apo A-I plays a prominent role and should be measured as a component for assessing cardiovascular risk in humans. Role of LOX-1 in macrophage-cholesterol homeostasis might provide some interesting targets for atheroprotection. The present study showed the protective role of HDL in the disease process as substantiated by inhibition of ox-LDL-mediated apoptosis in differentiated monocytes obtained from patients. Despite a wealth of information on this subject, further research is required to substantiate the findings in more numbers of patient samples, so that identification of appropriate drug targets can be done which will prevent the progression of atherosclerosis.

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### Conflict of Interests

Authors have no conflict of interests.

### References

1. Ibanez B, Giannarelli C, Cimmino G, Santos-Gallego CG, Alique M, Pinero A, et al. Recombinant HDL(Milano) exerts greater anti-inflammatory and plaque stabilizing properties than HDL(wild-type). *Atherosclerosis* 2012; 220(1): 72-7.
2. Boyle JJ. Macrophage activation in atherosclerosis: Pathogenesis and pharmacology of plaque rupture. *Curr Vasc Pharmacol* 2005; 3(1): 63-8.
3. Tiwari RL, Singh V, Barthwal MK. Macrophages: An elusive yet emerging therapeutic target of atherosclerosis. *Med Res Rev* 2008; 28(4): 483-544.
4. Greig FH, Kennedy S, Spickett CM. Physiological effects of oxidized phospholipids and their cellular signaling mechanisms in inflammation. *Free Radic Biol Med* 2012; 52(2): 266-80.
5. Petraki MP, Mantani PT, Tselepis AD. Recent advances on the antiatherogenic effects of HDL-derived proteins and mimetic peptides. *Curr Pharm Des* 2009; 15(27): 3146-66.
6. Schlenke P, Kluter H, Muller-Steinhardt M, Hammers HJ, Borchert K, Bein G. Evaluation of a novel mononuclear cell isolation procedure for serological HLA typing. *Clin Diagn Lab Immunol* 1998; 5(6): 808-13.
7. Phillips HJ. Dye exclusion test for cell viability. In: Kruse PF, Patterson MK, Editors. *Tissue culture: Methods and applications*. Cambridge, MA: Academic Press; 1973. p. 406-8.
8. Via DP, Pons L, Dennison DK, Fanslow AE, Bernini F. Induction of acetyl-LDL receptor activity by phorbol ester in human monocyte cell line THP-1. *J Lipid Res* 1989; 30(10): 1515-24.
9. Chung BH, Segrest JP, Ray MJ, Brunzell JD, Hokanson JE, Krauss RM, et al. Single vertical spin density gradient ultracentrifugation. *Methods Enzymol* 1986; 128: 181-209.
10. Jonas A, Wald JH, Toohill KL, Krul ES, Kezdy KE. Apolipoprotein A-I structure and lipid properties in homogeneous, reconstituted spherical and discoidal high density lipoproteins. *J Biol Chem* 1990; 265(36): 22123-9.
11. Brewer HB Jr, Ronan R, Meng M, Bishop C. Isolation and characterization of apolipoproteins A-I, A-II, and A-IV. *Methods Enzymol* 1986; 128: 223-46.
12. Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, et al. Measurement of protein using bicinchoninic acid. *Anal Biochem* 1985; 150(1): 76-85.
13. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227(5259): 680-5.
14. Engvall E, Perlmann P. Enzyme-linked

- immunosorbent assay, Elisa. 3. Quantitation of specific antibodies by enzyme-labeled anti-immunoglobulin in antigen-coated tubes. *J Immunol* 1972; 109(1): 129-35.
15. Esterbauer H, Striegl G, Puhl H, Rotheneder M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radic Res Commun* 1989; 6(1): 67-75.
  16. Ohkawa H, Ohishi N, Yagi K. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Anal Biochem* 1979; 95(2): 351-8.
  17. Towbin H, Staehelin T, Gordon J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc Natl Acad Sci U S A* 1979; 76(9): 4350-4.
  18. Hensel U, Wellensiek HJ, Bhakdi S. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis immunoblotting as a serological tool in the diagnosis of syphilitic infections. *J Clin Microbiol* 1985; 21(1): 82-7.
  19. Ghosh S. Macrophage cholesterol homeostasis and metabolic diseases: Critical role of cholesteryl ester mobilization. *Expert Rev Cardiovasc Ther* 2011; 9(3): 329-40.
  20. Camont L, Chapman MJ, Kontush A. Biological activities of HDL subpopulations and their relevance to cardiovascular disease. *Trends Mol Med* 2011; 17(10): 594-603.
  21. Nofer JR, Levkau B, Wolinska I, Junker R, Fobker M, von Eckardstein A., et al. Suppression of endothelial cell apoptosis by high density lipoproteins (HDL) and HDL-associated lysosphingolipids. *J Biol Chem* 2001; 276(37): 34480-5.
  22. Rosa BL, Vicenta LC, Victor GC, Dolores LC, Fernando C, Ricardo GH, et al. Alterations of specific biomarkers of metabolic pathways in vascular tree from patients with Type 2 diabetes. *Cardiovasc Diabetol* 2012; 11: 86.
  23. Kockx MM, De Meyer GR, Buysse N, Knaapen MW, Bult H, Herman AG. Cell composition, replication, and apoptosis in atherosclerotic plaques after 6 months of cholesterol withdrawal. *Circ Res* 1998; 83(4): 378-87.
  24. Mehta JL, Li DY, Chen HJ, Joseph J, Romeo F. Inhibition of LOX-1 by statins may relate to upregulation of eNOS. *Biochem Biophys Res Commun* 2001; 289(4): 857-61.