

Exposure to oxLDL impairs TGF- β activity in human tendon cells

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Abstract

Background: Hypercholesterolemia is a known risk factor for the development of acute or chronic tendon injuries, but the mechanisms which link cholesterol and tendon pathology are not completely known. Previous studies have shown that patients with hypercholesterolemia experience elevated levels of oxidized LDL (oxLDL), a molecule which triggers inflammation and collagenase activity. In this study we discovered a potential mechanism that may contribute to tendon damage by oxLDL.

Methods: Isolated human tendon cells were incubated in the presence or absence of oxLDL. The influence of oxLDL on the expression level of key mRNA and proteins was examined using real time quantitative PCR, ELISA and Western blots. The activities of enzymes relevant to collagen synthesis and breakdown (lysyl oxidase and matrix metalloproteinases) were quantified using fluorometry. Finally, the isolated human tendon cells in a 3D construct were exposed to combinations of oxLDL and TGF- β to examine their interacting effects on collagen matrix remodeling.

Results: The expression of key mRNAs including *TGFB*, *COL1A1*, *DCN*, and *LOX* were reduced in human tendon cells by oxLDL while *MMPs* were increased. The oxLDL reduced the activity of LOX, whereas conversely MMP activities were induced. COL1A1 synthesis and TGF- β secretion were also inhibited. Adding recombinant TGF- β reversed the effects of oxLDL on the expression of collagens and *LOX*. OxLDL also impaired collagen matrix remodeling, and adding TGF- β restored the native phenotype.

Conclusions: Exposure oxLDL in patients with hypercholesterolemia may adversely affect the mechanical and structural properties of tendon tissue through a direct action of oxLDL on tendon cells, including impairment of TGF- β expression. This impairment leads to disturbed matrix remodeling and synthesis, thereby potentially leading to increased risk of acute or chronic tendon injury.

Background

Hypercholesterolemia is characterized by high blood cholesterol levels and is influenced by a variety of genetic and environmental factors. A major cause of acquired hypercholesterolemia is a diet high in saturated fat; according to the World Health Organization, the global prevalence of raised total cholesterol among adults in 2008 was 39% and has changed little since 1980 [1]. As well as being a major risk factor for developing cardiovascular disease due to its role in atherosclerosis, hypercholesterolemia is associated with tendon pathologies [2–4], tendon injury prevalence [5–7] and impaired tendon healing [8]. However, there has been little research into the mechanisms by which cholesterol causes tendons to weaken.

The mechanisms of lipid deposition in tendon have been speculated [9]; it is thought that low-density lipoproteins (LDLs) derived from plasma become trapped in the tendon matrix. Subsequent oxidative modification of LDL to oxLDL activates resident tissue macrophages which internalize oxLDL and transform them into foam cells. Accumulation of oxLDL in patients with hypercholesterolemia triggers inflammation and expression of metalloproteinases (MMPs) in atherosclerosis [10, 11].

We have previously found that mice fed a high-fat diet for 30 weeks demonstrated oxLDL deposition in tendon ECM, and that these tendons had reduced biomechanical strength compared to those on a normal diet [7]. These findings suggest a direct link between hypercholesterolemia, intratendinous oxLDL accumulation and tendon health. At the cellular level, human tenocytes cultured with oxLDL-supplemented media demonstrated increased MMP2 gene expression and decreased collagen (COL1A1, COL3A1) gene expression compared to controls [12]. Inflammation, changes in MMP activity and collagen turnover are therefore proposed as relevant pathogenic mechanisms of hypercholesterolemia on tendon pathology [13].

In this study we sought to investigate the role of oxLDL in tendon pathophysiology more completely by further investigating regulatory pathways that influence matrix remodeling and synthesis in human tendon cells. Specifically, we examined the expression, activity and synthesis of genes, enzymes and proteins relating to matrix synthesis and remodeling after exposure to oxLDL.

Methods

Cell culture

Human tendon cells were isolated from healthy human hamstring (semitendinosus) tendons (excess anterior cruciate ligament autograft material) as previously described [14]. Male (N = 2; age 28 ± 1.4) and female (N = 2; age 37 ± 5.7) donors provided written informed consent and the protocol was approved by University of British Columbia Clinical Research Ethics Board (certificate number H10-00220). Equal number of females and males were used in each experiment to exclude the effect of gender as a factor. To expand the tendon cell culture, the isolated tendon cells were grown in high glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum, 2 mm L-glutamine, and 1x Antibiotic/Antimycotic solution in a humidified incubator containing 5% CO₂ at 37°C. Then, 10% calf serum was replaced with 5% Lipoprotein Deficient Serum from fetal calf (Sigma, USA, #S5394) before and during incubation with oxLDL. Recombinant TGF- β protein (PeproTech, USA, #100 – 21) at concentration of 5ng/ml were added during the incubation with oxLDL.

Bioartificial tendon (BAT)

BATs were prepared as previously described [15] with some modifications. Human tendon cells were seeded in the mixture of Purecol EZ Gel (Sigma-Aldrich, USA, # 5074), 5x DMEM, and 5% Lipoprotein Deficient Serum from fetal calf (Sigma, USA, #S5394) with or without 50 μ g/ml oxLDL and 5 ng/ml TGF- β . The mixture was pipetted between the anchor stems of untreated Tissue Train plate (Flexcell International Corp., USA, #TT-5001U) in each well. After setting the BATs in the incubator, 3ml 1x DMEM media with or without oxLDL and TGF- β was added to each well and the plate incubated for 72 hours. The BATs were then scanned using an image scanner and the images analyzed in ImageJ to measure the diameter of the BATs.

oxLDL preparation

Isolated Human Low Density Lipoprotein (LDL) (Lee Biosolutions, USA, #360 – 10) was oxidized as previously described [12]. LDL was diluted in DPBS containing 5 μ M copper sulfate and incubated at 37°C for 24 hours. The oxidation was stopped by adding 1 mM EDTA. The oxLDL was washed and concentrated using Amicon Ultra centrifugal filter unit with the pore size of 10 kDa MWCO (Millipore, USA, #UFC901024). The concentration of oxLDL was measured by a bicinchoninic acid protein assay kit. The concentrated oxLDL was aliquoted and stored in -80°C.

Gene expression analysis

The changes in mRNA expression was analyzed using Real-Time Quantitative Reverse Transcription PCR (RT-qPCR) as previously described [16]. Δ Ct values were calculated as $Ct_{\text{Target}} - Ct_{\text{GAPDH}}$ and used for graphs and statistical analysis. The oligo primers that were used are listed in Table 1.

Table 1
The sequences of oligo DNA used as primer for qRT-PCR

Gene	Forward primer	Reverse primer
COL1A1	CACCAATCACCTGCGGTACAGAA	CAGATCACGTCATCGCACAAAC
COL1A2	AGAGTGGAGCAGTGGTTACTA	GATACAGGTTTCGCCAGTAGAG
COL3A1	AATCAGGTAGACCCGGACGA	TTCGTCCATCGAAGCCTCTG
DCN	GTCACAGAGCAGCACCTACC	TTGTCCAGACCCAAATCAGAACA
ILK	CCCACGACATGCACTCAATA	GACCAGGACATTGGAAAGAGAA
ITGB1	TGATCCTGTGTCCCATTGTAAG	TGACCTCGTTGTTCCCATTC
LOX	TACCCAGCCGACCAAGATA	TGGCATCAAGCAGGTCATAG
MMP1	CAGAAAGAGACAGGAGACATGAG	GAAGAGTTATCCCTTGCCTATCC
MMP2	AGAGAACCTCAGGGAGAGTAAG	CCTCGAACAGATGCCACAATA
MMP3	GTGAGGACACCAGCATGAA	GACCACTGTCCTTTCTCCTAAC
MMP8	GGGTGGGCTCTAAATCCATTAT	CCAATCTCTGCCTCTGTCTTC
MMP9	GAACCTTGACAGCGACAAGAAG	CGGCACTGAGGAATGATCTAA
MMP10	GGCCCTCTCTTCCATCATATTT	CCTGCTTGTACCTCATTTCT
MMP13	AGCATCTGGAGTAACCGTATTG	CCCGCACTTCTGGAAGTATT
MMP14	GCCGACTAAGCAGAAGAAAGA	TGTCGGCTTGGAGTTAAAGG
TGFB1	CCTGCCTGTCTGCACTATTC	TGCCCAAGGTGCTCAATAAA
GAPDH	TCTTTTGCGTCGCCAGCCGAG	TGACCAGGCGCCCAATACGAC

Enzymatic assays

To evaluate the activity of LOX and MMPs in protein lysate of human tendon cells, fluorometric assay kits were used. According to the MMP activity assay kit (Abcam, USA, #ab112146), protein lysates were mixed with 4-aminophenylmercuric acetate (APMA) and incubated at 37°C for 3 hours to activate MMPs. Then, MMP green substrate solution was added and incubated for 30 minutes. The fluorescence intensity was measured at 490/525 nm (Ex/Em) using a microplate reader (Infinite F500, Tecan, Austria). Amplite™ Fluorimetric Lysyl Oxidase Assay kit (AAT Bioquest, USA, #15255) was used according to the manual to measure LOX activity in protein lysate. The recorded relative fluorescence units from the MMP and LOX assay kits were normalized to the total protein concentrations measured by BCA assay.

Protein quantification: The secretion of TGF-β1 was quantified in the conditioned media of human tendon cells using an ELISA kit (R&D System, USA, #DY246) according to the manual. The level of COL1A1 protein in cell lysate was measured using western blot as previously described [16]. The western blots of Col1A1 and Vinculin were detected by IRDye 680 and HRP-conjugated antibodies, then visualized using the G:BOX Chemi XT4 Gel Documentation System (Syngene, UK) and Odyssey CLx Imaging System (LI-COR, USA) with default settings, respectively.

Statistics

All data were analyzed by either paired t-test or one-way ANOVA followed by Dunnett's multiple comparison test. GraphPad prism version 7.03 was used to analyze data and generate graphs. The number of biological replicates is reported in the figure captions. *P* values of less than 0.05 were regarded as statistically significant.

Results

oxLDL changes the expression of collagen and the factors that mediate matrix remodeling

In order to determine the effect of oxLDL on mRNA expression of other matrix proteins and factors that regulate tendon matrix synthesis and remodeling, we used qRT-PCR. Our data indicate a significant reduction of extracellular matrix mRNA (*COL1A1*, *COL1A2*, *COL3A1* and *DCN*) while the expression of *MMPs* (*MMP1*, *MMP3* and *MMP14*) was increased. *LOX* mRNA also was reduced by oxLDL. The qRT-PCR results showed that oxLDL reduced the expression of *ILK*, *ITGB1* and *TGF-β*, which mediate collagen synthesis and mechanotransduction [16, 17] (Fig. 1).

oxLDL changes the activity of MMPs and LOX

MMPs are degradative enzymes that regulate extracellular matrix remodeling in tendon tissue. Increased activity of MMPs is associated with tendon injuries [18]. The family of LOX enzymes regulate collagen fibrillogenesis by enzymatic cross-linking of collagen matrix, a process which is critical for the proper development of tendon tissue [19, 20]. To measure the activity of LOX and MMPs, we used fluorometric assays that utilize specific synthetic substrates as probes for detection of oxidization and peptide

degradation by LOX and MMPs, respectively. Our data indicate oxLDL decreased the activity of LOX in protein lysates of human tendon cells while the MMP activity was increased (Fig. 2a, b).

oxLDL reduces collagen synthesis by human tendon cells

Collagen type I, as a major component of the tendon matrix, has a pivotal role in the mechanical strength of tendon tissue [21]. We used Western immunoblot to measure the rate of collagen synthesis in incubated human tendon cells with different concentrations of oxLDL. The scans of immunoblots indicate diminished synthesis of COL1A1 protein, the major subunit of collagen type I, in protein lysates of treated human tendon cells with oxLDL (Fig. 2c). The densitometry data show a significant, dose-responsive reduction of COL1A1 protein in the presence of oxLDL (Fig. 2d).

Reduced expression of TGF- β by oxLDL is attributed to changes in collagen and LOX expression but not MMPs

We examined the possible upstream role of TGF- β in regulating the response of tenocytes to oxLDL, hypothesizing that TGF- β may be responsible for some of the observed changes. The ELISA and qRT-PCR results showed that oxLDL decreases TGF- β expression at the level of mRNA (Fig. 3a) and protein (Fig. 3b). Adding recombinant TGF- β to human tendon cells treated with oxLDL rescued the expression of collagen and *LOX* genes but not *MMPs* (Fig. 3c, d).

oxLDL disturbs matrix remodeling

Remodeling of the collagen matrix is prominent during tissue development and wound healing [22, 23]. We used a modified BAT composed of human tendon cells in 3d collagen matrix to examine the impact of oxLDL on tendon remodeling. oxLDL disrupted the remodeling of BAT compared to the control while adding recombinant TGF- β rescued the ability of tendon cells for matrix remodeling (Fig. 4a). Measuring the thickness of BATs showed that the effect of oxLDL was significant (Fig. 4b).

Discussion

This study showed that oxLDL disturbed a number of important but divergent processes in human tendon cells, including the remodeling, synthesis and cross-linking of key tendon extracellular matrix components. The expression and activity of degrading enzymes such as MMP1, MMP3 and MMP14 were increased while LOX expression and activity was reduced. Also, the expression of genes with key functions in the adaptive response to mechanical loading - *ILK*, *ITGB1* and *TGF- β* - were diminished in human tendon cells by oxLDL. Our results indicate that downregulation of *TGF- β* by oxLDL promotes reduced expression of collagen and *LOX* which may contribute to changes in BAT matrix remodeling, but further unidentified mechanisms must be responsible for the increased MMP activity.

Surprisingly, this appears to be the first study that has examined the effect of oxLDL on LOX in tendon cells. LOX activity is critical for crosslinking of collagen fibrils and proper assembly of the tendon matrix to confer tensile strength to the tendon tissue [24]. Dysregulation of LOX has been reported as the

etiology of various disorders, which introduces LOX as a potential therapeutic target [25]. The inhibition of LOX prevents collagen fibril formation by human tendon cells [19], and the treatment of tendon with recombinant LOX improves the mechanical properties of tendon without any changes in collagen synthesis [26]. LOX also promotes cell-mediated collagen contraction due to collagen crosslinking and subsequent increased ECM stiffness [27, 28]. The reduced activity and expression of LOX in human tendon cells by oxLDL may impair collagen fiber formation and remodeling in tendon.

Our study demonstrated a significant effect of oxLDL on the regulation of MMP activity in human tendon cells. This effect, in conjunction with the diminished LOX activity discussed above, may explain the inferior mechanical properties of tendon tissues in animals with high cholesterol [8, 12, 29]. A balance of LOX and MMP is essential for tendon homeostasis, which involves both crosslinking and degradation of the ECM, respectively [30, 31]. The impaired regulation of MMP activity has been suggested to have a major role in the development of tendon injuries [24, 32]. Our gene expression analysis showed increased expression of *MMP-1*, *MMP-3* and *MMP-14* in human tendon cells by oxLDL but no significant changes in other MMPs (*MMP-2*, *8*, *9*, *13*). MMP1, MMP13 and MMP14 catalyze the degradation of collagen type 1 and 3 [33]. MMP14, known as membrane type 1 MMP (MT1-MMP), also activates pro-MMP2 by its proteolytic cleavage to the mature MMP2 which has collagenolytic activity [34, 35]. The imbalanced activity of MMP can lead to tendon pathology or injuries [36]. The overall pattern of oxLDL increasing specific MMPs has been observed in other cell types as well: oxLDL induces the expression of *MMP9* in human monocyte-derived macrophages while its inhibitor *TIMP1* was reduced [37] whereas in endothelial cells, oxLDL increases *MMP1* expression. Increased activity and expression of MMPs by oxLDL in tendon cells potentially contribute to destruction of tendon matrix by degradation of collagen fibers.

The uptake of LDL particles into cells is regulated by the LDL receptor [38]. However, oxidation of LDL prevents its binding to the receptors [39]. The scavenger receptors, including Lectin-like oxidized LDL receptor-1 (LOX-1); CD36; and scavenger receptor A, can bind to oxLDL to promote its internalization in certain cell types [39]. However, the process of binding or uptake of oxLDL by tendon cells which exerts its effect on tendon tissue has not yet been determined. Our results indicate that oxLDL reduced collagen and *LOX* expression via downregulation of *TGF-β*, which was reversed by adding recombinant TGF-β to the human tendon cell culture.

The remodeling of the collagen matrix, which was disturbed by oxLDL in BATs, is mainly controlled by TGF-β [40, 41]. TGF-β is a key factor in various biological processes that regulate healing, development and mechanical properties of tendon [42–44] including tendon cell migration and collagen synthesis [45]. In tendons, tissue adaptation is heavily influenced by physical activity, which is experienced by cells as mechanical forces transmitted from the extracellular matrix to the cells in a continuous feedback process [46]. Integrins and TGF-β are thought to play an important role in this ongoing physiological process [16, 43]. The observed downregulation of integrins, integrin-related proteins (ITGB1 and ILK) and TGF-β in the presence of oxLDL may impair the normal adaptive response to exercise in individuals with hypercholesterolemia.

Conclusions

Overall, this study describes potentially detrimental effects of oxLDL on the homeostasis of tendons, which occurs through direct action of oxLDL on tendon fibroblasts. These effects may be driven by changes in ECM degradation, crosslinking, and synthesis. TGF- β appears to restore collagen crosslinking and synthesis. However, the effects of oxLDL on ECM degrading enzymes is affected by different mechanisms. Further mechanistic studies may unravel novel therapeutic targets for managing tendon injuries in patients with high lipid conditions.

Abbreviations

BAT	Bio-artificial tendon
ECM	extracellular matrix
ILK	Integrin-linked kinase
ITGB1	Integrin Subunit Beta 1
LOX	Lysyl oxidase
MMP	Matrix metalloproteinase
oxLDL	oxidized Low Density Lipoprotein
TGF- β	Transforming growth factor beta
TIMP	tissue inhibitor of metalloproteinases

Declarations

- **Ethics approval and consent to participate:** Tissue donors provided written informed consent and the protocol was approved by University of British Columbia Clinical Research Ethics Board (certificate number H10-00220). All methods were carried out in accordance with relevant guidelines and regulations.
- **Consent for publication:** Not applicable.
- **Availability of data and materials:** The RT-qPCR dataset is available in the Supplementary Dataset File. The uncropped images of blots used in figure 2c are included as Supplementary Info File. We included individual data points in the graphs wherever was possible.
- **Competing interests:** The authors declare no competing interests.
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- **Authors’ contributions:** R.M. designed and performed the experiments. C.M.W assisted with experiments and editing the manuscript. E.R. assisted with the experiments. R.G.M. provided human

tendon tissues. V.D. and A.S. contributed to designing the experiments. R.M. and A.S. wrote the manuscript.

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Figures

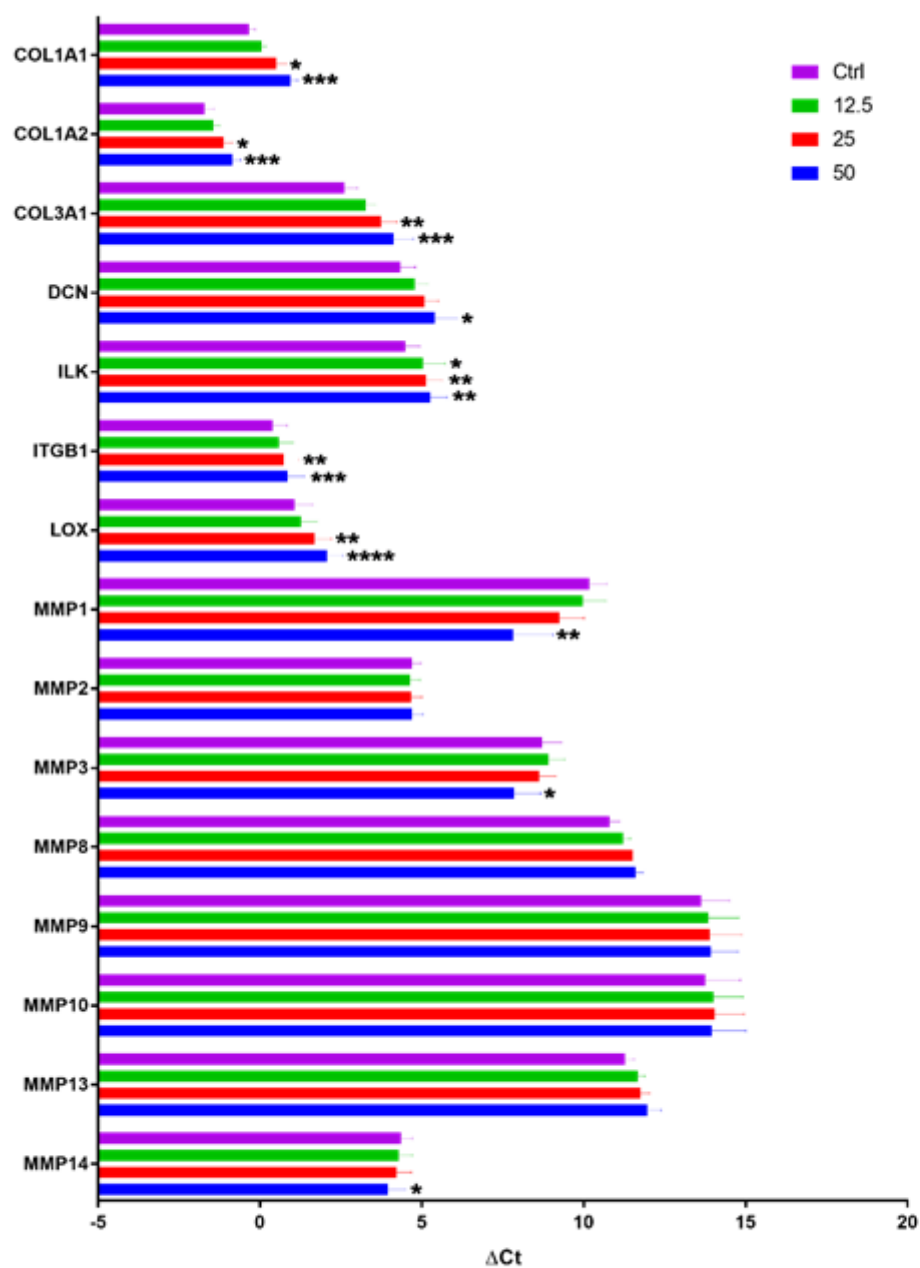


Figure 1

oxLDL changes the expression of collagen and the factors that mediate matrix remodeling.

Exposure of human tendon cells to progressively increasing doses of oxLDL resulted in reduction of key mRNAs involved in anabolic processes such as *COL 1A1*, *COL 1A2*, *COL3A1*, *DCN*, *ILK*, *ITGB1*, and *LOX*.

Conversely, the expression of specific MMPs (*MMP1*, *MMP3*, and *MMP14*) were increased. Mean \pm SE; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; **** $P \leq 0.0001$; n=4 biological replicates.

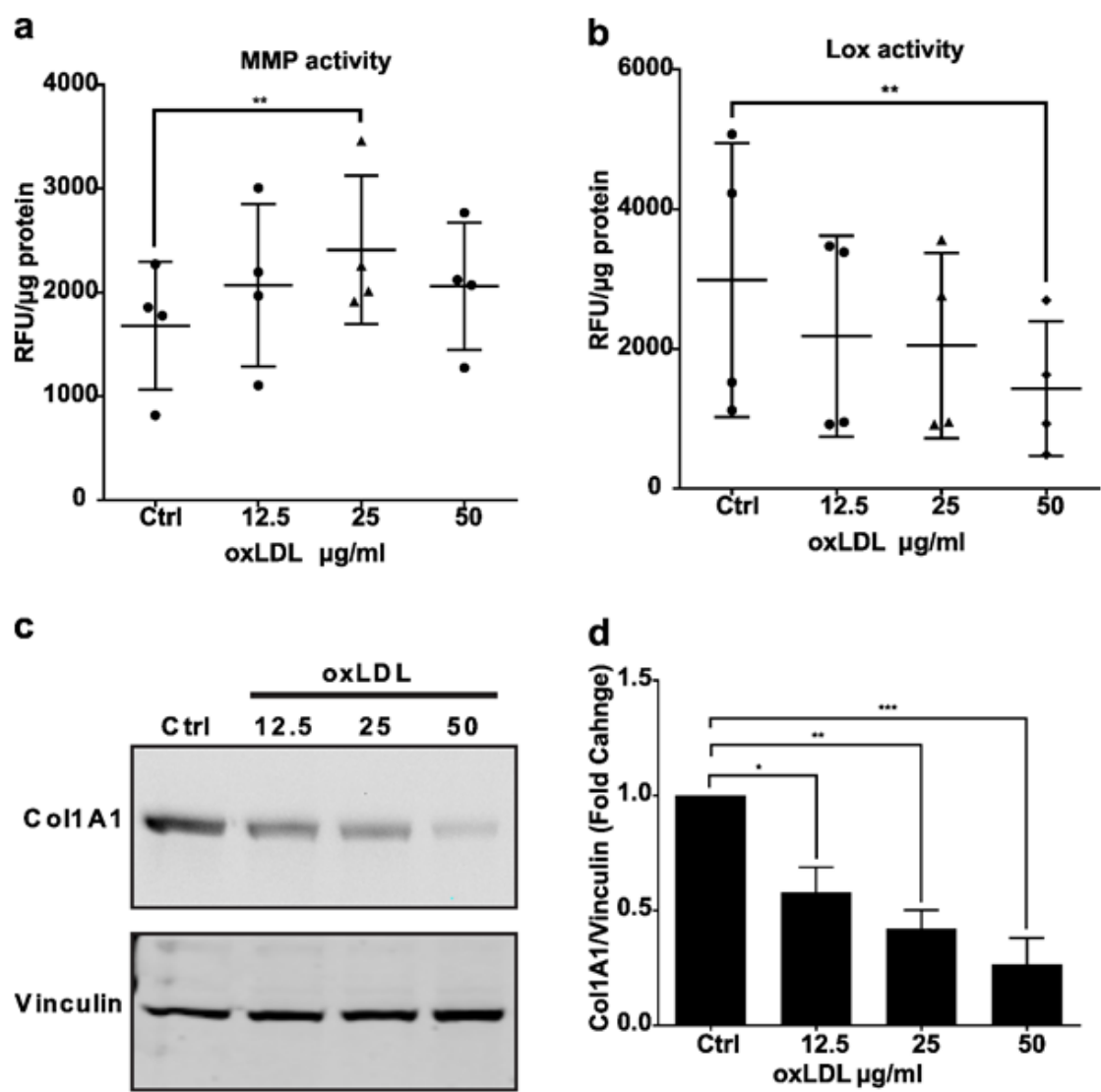


Figure 2

oxLDL changes the activity of enzymes regulating matrix remodeling, and protein expression of collagen.

OxLDL increases MMPs activity (a) and reduces LOX activity (b) in human tendon cells. Western blot shows reductions of COL1A1 protein in human tendon cells after incubation with oxLDL (c). The densitometry analysis of COL1A1/Vinculin ratios shows the effect is statically significant and responsive

to the dose of oxLDL (d). The uncropped images of blots are included in Supplementary information. Mean \pm SE; ns $P > 0.05$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; n=4 biological replicates.

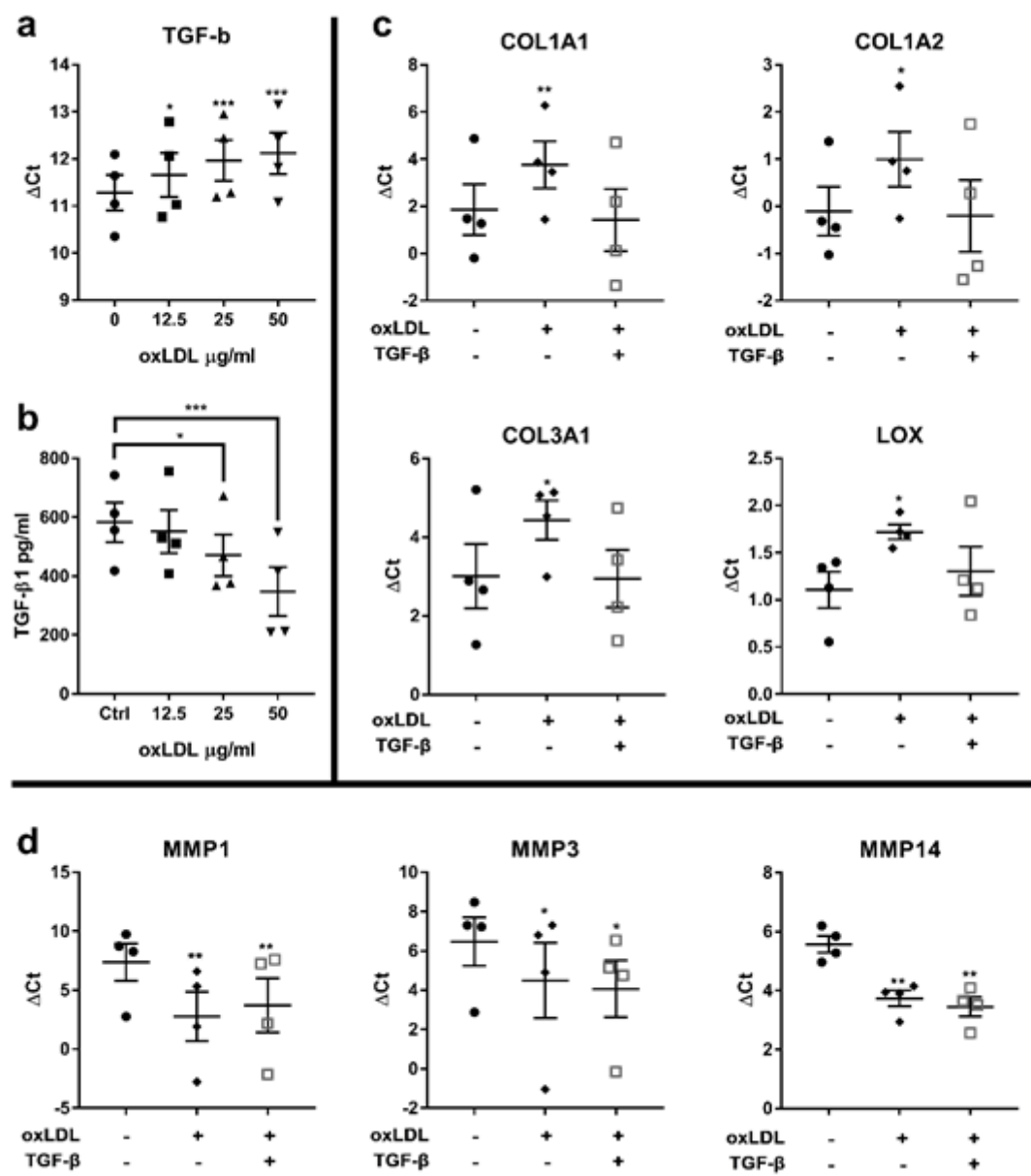


Figure 3

oxLDL changes the expression of collagen and lox via TGF- β .

oxLDL decreases TGF- β expression at the level of mRNA (a) and protein (b). Adding recombinant TGF- β to human tendon cells treated with oxLDL rescued the expression of collagen and *LOX* mRNA (c) but not *MMPs* (d). Mean \pm SE; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; n=4 biological replicates.

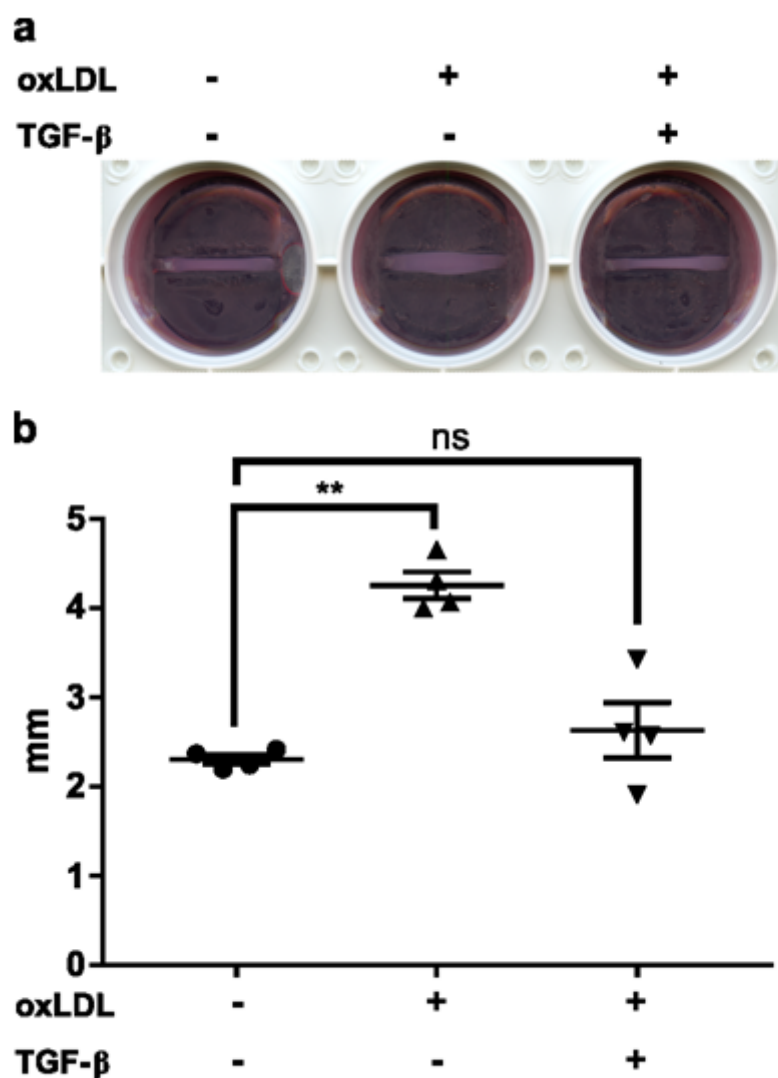


Figure 4

oxLDL disturbed matrix remodeling of BAT via TGF- β .

The scanned micrograph of BATs shows impaired BATs remodeling in presence of oxLDL which was reversed by adding recombinant TGF- β (a). The impact of oxLDL on thickness of BAT was significant while adding TGF- β abrogated this effect (b). Mean \pm SE; ns $P > 0.05$; ** $P \leq 0.01$; $n=4$ biological replicates.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- [SupplementaryDatasetFile.xls](#)
- [SupplementaryInfoFile.pdf](#)