Research Article

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Homocysteine induces iNOS and oxLDL accumulation in murine immune cells

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Abstract

Objectives: Increased levels of homocysteine (Hcy) in the blood are considered to be a risk factor for atherosclerosis. Endothelial damage induced by Hcy can lead to deterioration of coronary, cerebral, and peripheral vascular beds, contributing to cardiovascular morbidity and mortality. However, the mechanisms underlying Hcy-induced damage have not been fully elucidated. This study aimed to investigate the effect of Hcy on low-density lipoprotein (LDL) oxidation and inducible nitric oxide synthase (iNOS) expression, both of which are thought to play a role in the pathogenesis of atherosclerotic plaque formation and regulation of inflammation.

Methods: L-929 fibroblast-like cells and RAW 264.7 macrophages were treated with varying concentrations of Hcy (50, 100 and 200 µg/mL for L-929 cells, 10, 25, 100, 200 µg/mL for RAW 264.7 cells) to assess LDL oxidation and iNOS expression using immunocytochemical and immunofluorescence methods. Images obtained from cell culture experiments were quantitatively analyzed using color and brightness histograms, followed by statistical evaluation.

Results: Hcy significantly and dose-dependently increased LDL oxidation and intracellular oxidized LDL (ox-LDL) accumulation in both cell groups. Hcy also significantly increased iNOS expression in both cell groups in a dose-dependent manner.

Conclusions: These findings suggest that Hcy may play a role in the pathogenesis of atherosclerosis and inflammatory processes. The increased levels of ox-LDL and the elevated iNOS expression, particularly in macrophages, may contribute to the exacerbation of atherosclerosis and related diseases.

Keywords: atherosclerosis; homocysteine; inflammation; iNOS; oxidative stress; oxidized LDL

Introduction

The endothelium is essential for maintaining vascular homeostasis by modulating vascular tone, the growth of vascular smooth muscle cells (VSMCs), immune cell adhesion, and vascular inflammation through the release of bioactive molecules. Endothelial dysfunction refers to impaired vasodilation or disrupted protective roles of the endothelium [1]. It is widely accepted that there is a significant connection between endothelial dysfunction and inflammation. Endothelial cells regulate vascular contraction, smooth muscle cell functions, inflammation, and homeostasis [2]. Therefore, endothelial dysfunction is considered a systemic pathology and plays a significant role in the formation of atherosclerosis [3]. This condition not only leads to a loss of vascular functionality but also contributes to the development of proinflammatory and prothrombotic processes.

Monocytes, macrophages, B and T lymphocytes play significant roles in the early stages of atherosclerotic lesions. Circulating monocytes and macrophages are primary immune system cells involved in the atherosclerotic process, contributing to plaque formation, progression, and rupture, ultimately resulting in luminal thrombosis and occlusion [4].

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Macrophage internalization of ox-LDL leads to foam cell formation, and this development exacerbates the inflammatory response through the production of various proinflammatory mediators. The soluble inflammatory mediators secreted by these cells further promote lesion progression through the continuous activation of inflammatory cascades [5].

Elevated total homocysteine (tHcy) levels in plasma are recognized as a significant risk factor for vascular diseases, including atherosclerosis and thrombosis [6–8]. Although the precise mechanisms underlying Hcy toxicity remain unclear, several pathways have been proposed to explain its contribution to atherosclerosis development. Current evidence suggests that Hcy promotes the progression of cardiovascular diseases through mechanisms such as oxidative stress and endothelial injury. Specifically, Hcy generates reactive oxygen species (ROS), which exert cytotoxic effects on endothelial and VSMCs [9–12]. ROS also play a central role in atherosclerosis by altering lipid metabolism, leading to the formation of ox-LDL particles, which are more atherogenic than native LDL. These modified LDL particles can initiate and sustain chronic inflammatory responses associated with atherosclerosis [13, 14].

Hcy, which induces oxidative stress by affecting cellular respiration, has been shown to promote LDL oxidation and modify other components of atherosclerotic plaques [15]. Consistent with this, Babu et al. demonstrated that exposure of human THP-1 macrophages and adult human retinal pigment epithelial cells (ARPE-19) to Hcy and homocysteine thiolactone (HTL) led to increased protein expression of the ox-LDL receptor CD36 and enhanced ox-LDL uptake in both cell types [16]. Similarly, a study by Zhang et al. reported that Hcy induced oxidative stress and an inflammatory response in murine macrophages, and this effect could be attenuated by 17β-estradiol [17]. Furthermore, macrophages have been shown to differentiate towards the M1 phenotype, contributing to atherosclerosis progression when exposed to a combination of Hcy and lipopolysaccharide [18]. In addition, Xu et al. reported that fatty acid binding protein 4 (FABP4) plays a key role in Hcy-induced macrophage inflammation and lipid metabolism. FABP4 has been found to activate the Janus kinase 2/signal transducer and activator of transcription 2 (JAK2/STAT2) pathway, thereby amplifying macrophage inflammation [19].

Increased iNOS expression enhances NO production, which interacts with superoxide anions to yield peroxynitrite, a potent oxidant promoting atherosclerosis and inflammation [20, 21]. The study by Woo et al. showed that Hcy stimulates iNOS-mediated NO production at pathophysiological concentrations [22]. Similarly, Mayo et al. demonstrated that Hcy impairs microvascular endothelial outgrowth by disrupting cell locomotion via an iNOS-dependent mechanism [23]. Moreover, Hcy has been reported to upregulate the expression of iNOS and the nuclear factor kappa B (NF-κB) p65 subunit in macrophages, further contributing to inflammatory processes [24].

Analyzing LDL oxidation and iNOS expression in fibroblasts, key connective tissue cells involved in immunological processes, and macrophages, which are central to inflammation, is crucial, as ox-LDL promotes oxidative stress and plaque formation, while iNOS mediates immune responses contributing to vascular damage. Additionally, evaluating Hcy in a dose-dependent manner provides insight into how varying concentrations of Hcy affect the mechanisms underlying atherosclerosis and inflammation. Therefore, the main aim of this study was to examine the effects of Hcy on two different cell lines simultaneously within the same study and to investigate the dose-dependent changes induced by Hcy.

Materials and methods

Cell culture studies

L-929 murine fibroblast-like cells and RAW 264.7 murine macrophages were obtained from ATCC (Washington DC, USA). The cells were seeded into eight-well chamber slides (Sigma, Germany) with 0.5 mL of culture medium, resulting in a density of 2×10^5 cells per well. Both cell lines were incubated overnight in RPMI-1640 medium (Sigma, Germany) supplemented with 10 % fetal bovine serum (FBS, Gibco, USA) and penicillin/streptomycin (100 IU and 100 µg/mL, respectively), in a humidified environment at 37 °C with 5 % CO_2. The cell culture procedures for each experiment were performed in triplicate under both control and treated conditions to ensure the reliability and consistency of the results. The selection of L-929 fibroblast-like cells was based on the fact that fibroblasts, as connective tissue cells, play a role in both influencing and being influenced by immunological processes (Supplemental Figure A). Furthermore, RAW 264.7 murine macrophages were chosen for the study due to their central role as key cell types in inflammatory processes (Supplemental Figure B).

Selection of homocysteine concentrations

In our preliminary study, cell death was observed following the administration of 400 µg/mL of Hcy, and thus, 200 µg/mL was determined as the maximum Hcy dose, as it was the highest concentration at which cells were able to survive. Additionally, in the preliminary study, various Hcy concentrations were evaluated for their effects on LDL oxidation

and iNOS expression in each cell type. Based on these results, concentrations that exhibited clearer dose-dependent responses were selected to more accurately characterize Hcy's dose-dependent effects.

Importantly, in humans, normal plasma Hcy concentrations range from 5 to 15 µmol/L. The concentrations used in our experimental model are supra-physiological and are intended to mimic pathological conditions. As this is an *in vitro* cell culture study, higher concentrations were selected based on our preliminary findings to ensure the manifestation of detectable effects, which may not be as prominent under physiological conditions. Therefore, our dosing strategy was designed to meet the experimental requirements of an *in vitro* system rather than replicate exact *in vivo* levels.

LDL oxidation in L-929 and RAW 264.7 cells with immunocytochemical method

Confluent L-929 cells were pre-treated with 50 µg/mL LDL, followed by incubation with Hcy at concentrations of 50, 100, and 200 µg/mL for 24 h. The accumulation of intracellular ox-LDL was assessed using an immunocytochemical method. For RAW 264.7 macrophages, cells were pre-treated with 100 µg/mL LDL and then exposed to Hcy at 25, 100, and 200 µg/mL for 24 h, with ox-LDL detection conducted via immunocytochemistry.

Ox-LDL staining was performed with biotinylated anti-ox-LDL antibodies (IMMCO Diagnostics, New York, USA). Streptavidin conjugated with horseradish peroxidase (HRP) and diaminobenzidine (DAB) were purchased from DAKO-Cytomation (Denmark). Ethanol fixed L-929 and RAW 264.7 cells were treated with biotinylated anti-ox-LDL antibodies for 30 min, washed three times with PBS, incubated for an additional 30 min with streptavidin-HRP and washed three times with PBS. Then, 0.03 % 3,3-diamino benzidine tetrahydrochloride plus 0.01 % hydrogen peroxide in 50 mmol/L Tris-HCl buffer (pH 7.4) were applied for 10 min. All incubations were performed at room temperature. The slides were examined under a light microscope at ×200 magnification (LEICA DMRX, Wetzlar, Germany).

iNOS expression in L-929 and RAW 264.7 cells with immunocytochemical and immunofluorescence methods

L-929 cells were incubated with Hcy at doses of 50 and 100 µg/mL for 24 h, and iNOS expression was measured using an immunocytochemical method. In L-929 cells, iNOS staining was performed with biotinylated anti-iNOS antibodies (Neomarkers, Fremont, CA 94539 USA). Ethanol fixed L-929 cells were treated with biotinylated anti-iNOS antibodies for 30 min, washed three times with PBS, incubated for an additional 30 min with streptavidin-HRP and washed three times with PBS. Then, 0.03 % 3,3-diamino benzidine tetrahydrochloride plus 0.01 % hydrogen peroxide in 50 mmol/L Tris-HCl buffer (pH 7.4) were applied for 10 min. All incubations were performed at room temperature. The slides were examined under a light microscope at ×200 magnification (LEICA DMRX, Wetzlar, Germany).

RAW 264.7 macrophages were treated with Hcy at concentrations of 10, 25, and 200 µg/mL for 36 h, with iNOS detection performed using an immunofluorescence method. In RAW 264.7 macrophages, 30 µL of anti-iNOS antibody (mouse origin) (Neomarkers, Fremont, CA 94539 USA) were added as the primary antibody on test slides, while the control slides were treated with the same amount of phosphate buffered saline solution (PBS). After 30 min of incubation in a humid chamber at room temperature, both the control and test slides were washed with PBS, and 30 µl FITC (fluorescent isothiocyanate)-labeled anti-mouse IgG was administered as a conjugate substance.

For another 30 min, slides were kept and incubated at room temperature and then, washed again with the standard PBS solution. After air drying, the slides were examined under a fluorescence microscope at ×200 magnification (LEICA DMRX, Wetzlar, Germany).

Quantitative assessment of LDL oxidation and iNOS expression intensity

For the quantitative evaluation of immunocytochemical analysis, images were assessed using an image analysis program (Adobe Photoshop 7.0). Different regions of the images were examined, and cells stained brown were selected. The mean luminosity values were determined through histogram analysis. A low luminosity value was interpreted as an indicator of high ox-LDL levels in L-929 fibroblast-like cells and RAW 264.7 macrophages or elevated iNOS expression in L-929 fibroblast-like cells.

For the quantitative assessment of immunofluorescence analysis, the same program was used to examine different regions of the images. Green-stained cells were selected, and the mean values were determined using the green channel in histogram analysis. An increase in green intensity was

interpreted as indicating high iNOS expression in RAW 264.7 macrophages. Both luminosity and green intensity histogram values were quantified on a scale of 0–255. The quantitative data were then subjected to statistical analysis.

Statistical analyses of results

Statistical analyses were performed using Jamovi 2.3.21, with data presented as median (min.-max.). The normality of the data distribution was assessed using the Shapiro-Wilk test. For variables that did not exhibit a normal distribution, the Kruskal-Wallis test was applied, followed by the Mann-Whitney U test for subgroup comparisons with Bonferroni correction. A p-value of <0.05 was considered statistically significant.

Results

Effect of Hcy on LDL oxidation

Hcy induced a significant, dose-dependent increase in the intracellular accumulation of ox-LDL in both L-929 fibroblast-like cells and RAW 264.7 macrophages, as assessed by immunocytochemical analysis (Figure 1). The increase in LDL oxidation was statistically significant when the Hcy dose was increased from 50 µg/mL to 200 µg/mL in L-929 fibroblast-like cells and from 25 µg/mL to 200 µg/mL in RAW 264.7 macrophages ($p<0.05$). A low luminosity value was interpreted as an indicator of high ox-LDL presence. The experimental observations were shown in Figure 2A–D for the L-929 fibroblast-like cells and in Figure 2E–H for the RAW 264.7 macrophages.

Effect of Hcy on iNOS expression

Hcy significantly increased iNOS expression in L-929 fibroblast-like cells in a dose-dependent manner, as indicated by the immunocytochemical results presented in Figure 3A. The iNOS expression significantly increased when the Hcy dose was increased from 50 µg/mL to 100 µg/mL ($p<0.05$). A low luminosity value was interpreted as an indicator of high iNOS expression. The experimental observations were shown in Figure 4A–C. In RAW 264.7 macrophages, Hcy also significantly increased iNOS expression in a dose-dependent manner, as shown by the immunofluorescence analysis in Figure 3B. The increase in iNOS expression was statistically significant when the Hcy dose was increased from 10 µg/mL to 25 µg/mL and 200 µg/mL ($p<0.05$). An increase in green intensity was interpreted as an indicator of high iNOS expression in RAW 264.7 macrophages. This effect was shown in Figure 4D–G through immunofluorescence analysis.

Discussion

The present study demonstrates that Hcy may contribute to the pathogenesis of atherosclerosis by increasing LDL oxidation and iNOS expression in fibroblast-like L-929 cells and RAW 264.7 macrophages. These findings suggest that Hcy-mediated LDL oxidation and increased iNOS

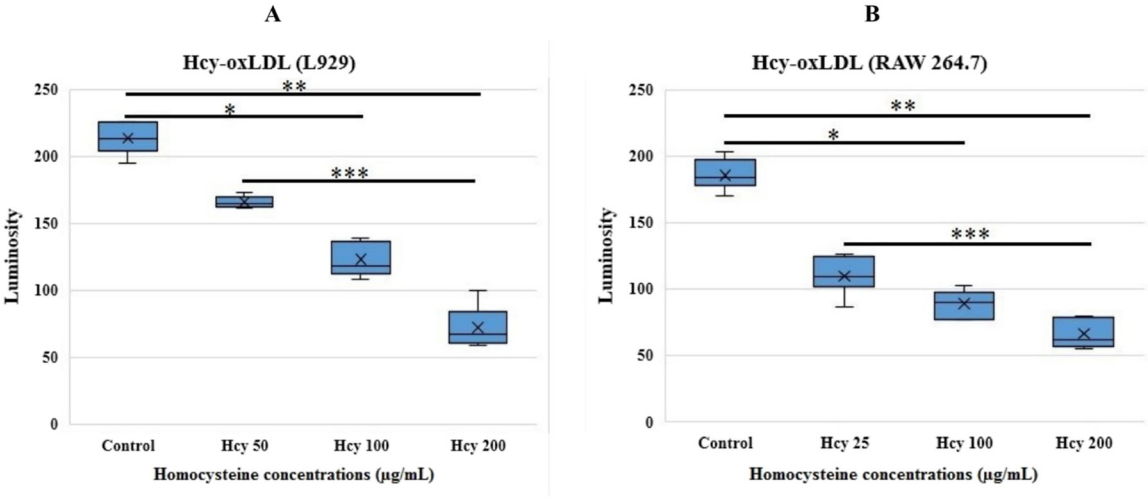

Figure 1: The effect of Hcy on LDL oxidation in L-929 murine fibroblast-like cells (A) and RAW 264.7 murine macrophages (B). Data are shown as median (min.-max.). *p, **p, ***p<0.05.

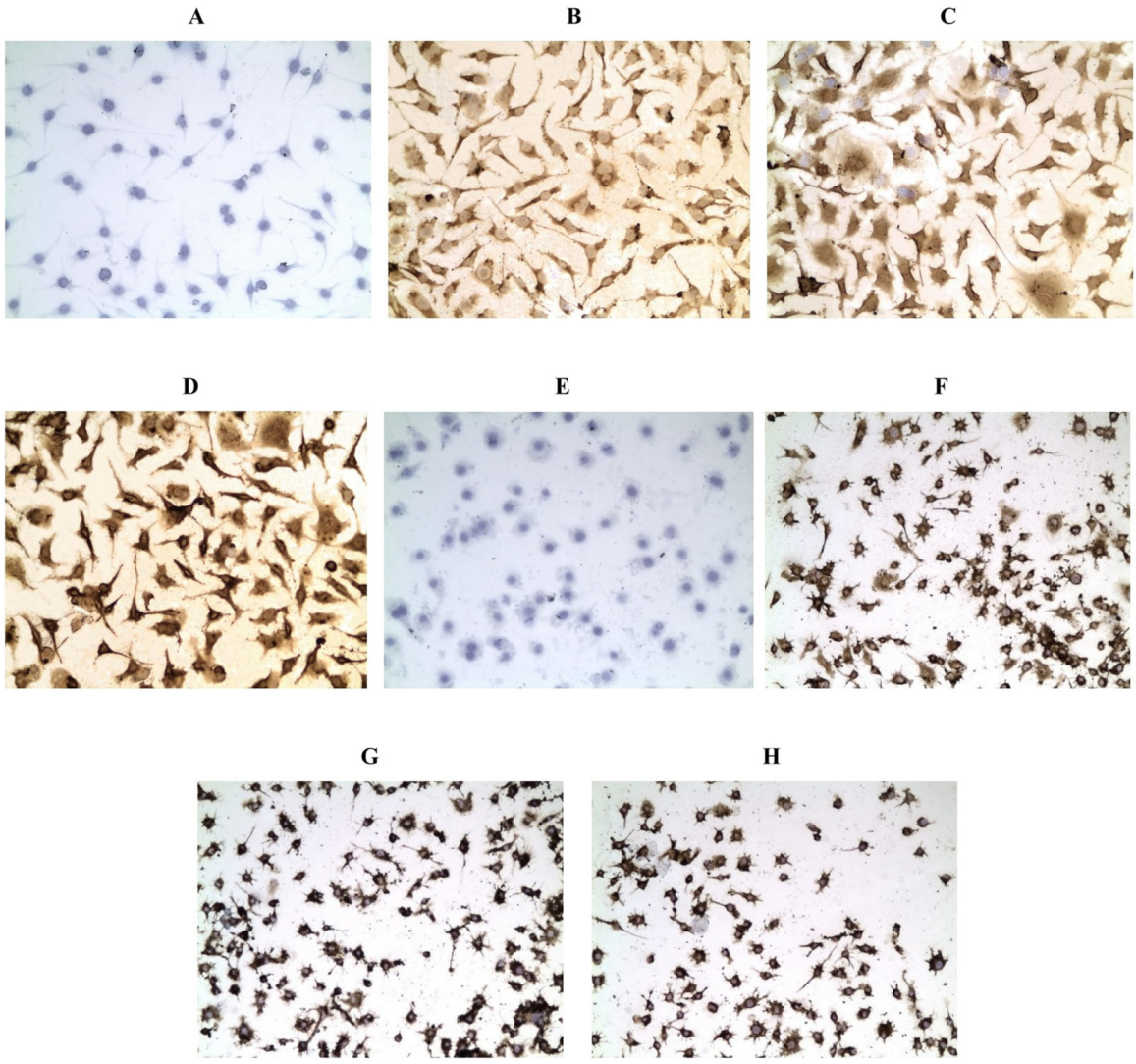

Figure 2: Figure represents the effect of Hcy **(A:Control, B:50 µg/mL, C:100 µg/mL, D:200 µg/mL, E:Control, F:25 µg/mL, G:100 µg/mL and H:200 µg/mL Hcy)** on LDL oxidation in L-929 murine fibroblast-like cells and RAW 264.7 murine macrophages indicated by immunocytochemical method (×200).

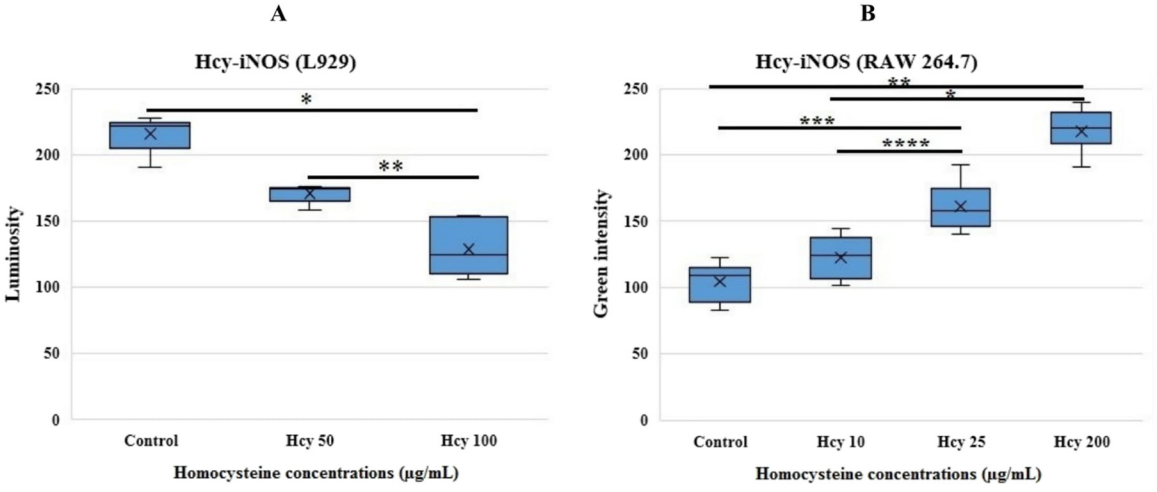

Figure 3: The effect of Hcy on iNOS expression in L-929 murine fibroblast-like cells (A) and RAW 264.7 murine macrophages (B). Data are shown as median (min.-max.). *p, **p, ***p, ****p<0.05.

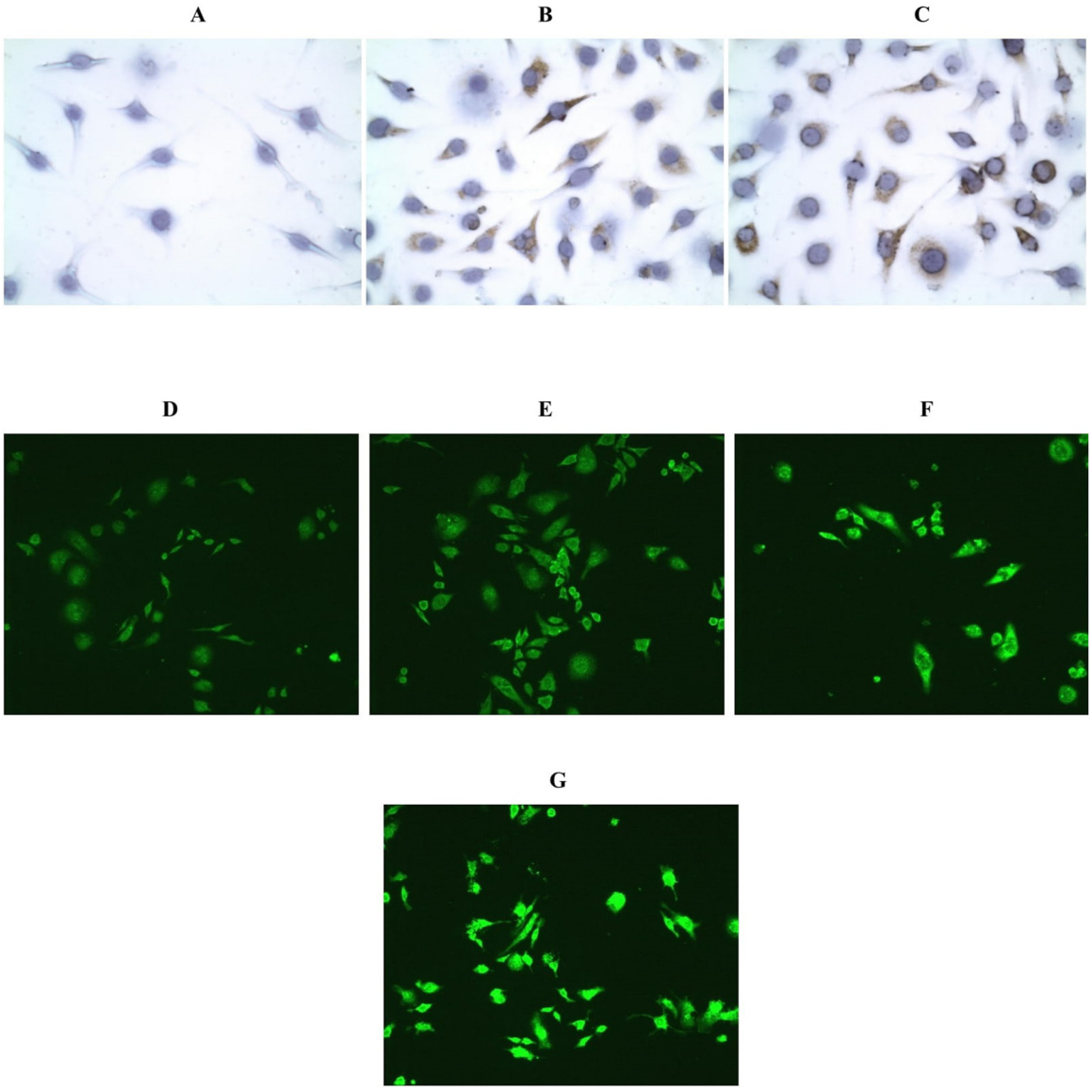

Figure 4: Figure represents the effect of Hcy **(A:Control, B:50 µg/mL, C:100 µg/mL, D:Control, E:10 µg/mL, F:25 µg/mL and G:200 µg/mL Hcy)** on iNOS expression in L-929 murine fibroblast-like cells and RAW 264.7 murine macrophages indicated by immunocytochemical and immunofluorescence methods respectively (×200).

expression confirm its pro-atherogenic and pro-inflammatory properties, further supporting a close link between Hcy, endothelial dysfunction, and inflammation. These results align with our previous studies, which revealed elevated Hcy levels as a significant risk factor for coronary artery disease and restenosis. We previously showed that high plasma Hcy levels in Turkish subjects are associated with coronary artery disease [6] and that Hcy is an independent risk factor for restenosis following percutaneous transluminal coronary angioplasty (PTCA) and coronary stenting [9].

Recent studies have highlighted that ox-LDL actively participates in atherogenesis by inducing oxidative stress and immune cell activation, further contributing to plaque development and instability [25, 26]. In the current study, significant increases in ox-LDL levels were observed in L-929 fibroblast-like cells at Hcy concentrations as low as 50 µg/mL, with the effect becoming more pronounced at 200 µg/mL. This result suggests that even moderate elevations in Hcy levels can promote LDL oxidation, leading to the accumulation of pro-atherogenic lipid species. A similar trend was observed in RAW 264.7 macrophages, with a significant

increase in ox-LDL at concentrations starting at 25 μg/mL. This result indicates that the effect of Hcy in accelerating LDL oxidation in a dose-dependent manner was also confirmed in macrophage cells.

The mechanisms through which Hcy induces LDL oxidation are multifaceted. Hcy has been shown to enhance oxidative stress by increasing ROS production via nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activation [27, 28]. Elevated ROS levels can trigger the oxidation of LDL particles, making them more susceptible to uptake by macrophages, a critical step in foam cell formation [25]. Moreover, Hcy may directly impair antioxidant defense mechanisms, further exacerbating oxidative damage and promoting the modification of LDL [27].

In addition to these effects, Zou et al. demonstrated that Hcy rapidly induces p38 mitogen-activated protein kinase (MAPK) phosphorylation, activating p47phox and NADPH oxidase, which increases ROS production. These ROS phosphorylate Akt, likely contributing to VSMCs proliferation [29]. Similarly, Wang et al. found that hyperhomocysteinemia impairs myocardial response to ischemia-reperfusion by downregulating antioxidants and increasing ROS via p38 MAPK activation, resulting in apoptosis [30]. In support of these findings, it has been reported that in Hcy-injured cells, the protein expression of phosphorylated p38 and extracellular signal-regulated kinase 1/2 (ERK1/2) is significantly increased, with Hcy also activating these phosphorylations in VSMCs, an effect that is reversed by simvastatin [31, 32].

Ji et al. showed that Hcy increases the protein expression of p-p65 and p-IκBα, suggesting the activation of the NF-κB pathway and subsequent pro-inflammatory cytokine release [33]. Hcy has also been found to induce an inflammatory response in VSMCs by enhancing C-reactive protein (CRP) expression through the N-methyl-D-aspartate receptor (NMDAr)-ROS-ERK1/2/p38-NF-κB signaling pathway, which involves oxidative stress [34]. Moreover, Hcy has been suggested to activate the NF-κB pathway in endothelial cells, leading to increased expression of monocyte chemoattractant protein-1 (MCP-1), vascular cell adhesion molecule-1 (VCAM-1), and intercellular adhesion molecule-1 (ICAM-1), triggering an inflammatory response [35]. Gao et al. reported that endothelial dysfunction induced by hyperhomocysteinemia may also be associated with the inositol-requiring enzyme 1α (IRE1α)-c-Jun N-terminal kinase (JNK)-autophagy axis pathway, highlighting the involvement of multiple signaling mechanisms in Hcy-mediated endothelial injury [36]. All these studies demonstrate that homocysteine can influence specific molecular pathways related to oxidative stress and inflammatory responses, thereby underscoring the importance of the current study.

Previous results also indicate a significant relationship between Hcy levels and ox-LDL in patients with coronary artery disease [37–39]. These findings highlight the critical role of oxidative stress in the atherogenic effects of hyperhomocysteinemia. Additionally, elevated Hcy levels have been shown to increase the concentration of HTL, which, through its interaction with proteins, may contribute to functional impairment and further vascular damage [40]. Paraoxonase 1 (PON1), an antioxidant associated with HDL (high-density lipoprotein), helps prevent LDL oxidation [41]. Large-scale studies reveal that high PON1 activity protects against cardiovascular disease, while reduced homocysteine thiolactonase activity of PON1 correlates with worse long-term outcomes [42]. Notably, PON1 activity inversely correlates with new atherosclerotic cardiovascular disease events in diabetic individuals [43]. Conversely, plasma Hcy levels have demonstrated a positive correlation with LDL levels [44]. Hcy has also been found to promote myofibroblast activation and stimulate cardiac fibrosis through modulating the Akt/FoxO3 pathway [45]. Furthermore, Hcy is linked to increased expression of lectin-like oxidized LDL receptor-1 (LOX-1) in hyperhomocysteinemic mice, which may exacerbate fibrosis through enhanced ox-LDL uptake [46]. Considered together, it can be suggested that high levels of Hcy lead to high levels of ox-LDL, and these factors contribute to a heightened risk of atherosclerosis.

The L-929 cell line is frequently employed in wound healing research, and fibroblasts play a critical role in fibrotic processes. The association between ox-LDL and various fibrotic conditions is well-documented. Additionally, LOX-1 influences cell growth and is involved in inflammation, oxidative stress, and tissue remodeling, suggesting a regulatory role in fibroblast activity and collagen secretion [47–50]. Consistent with existing literature, our study further highlights the crucial role of LDL oxidation in Hcy-related pathology and reveals a significant relationship between Hcy, ox-LDL, and fibroblast cells.

Monocyte infiltration into the damaged arterial wall, followed by differentiation into macrophages, is a hallmark of atherosclerosis. These macrophages can take up lipoproteins and convert into foam cells [51]. Beyond foam cell formation, macrophages can produce factors that exacerbate atherosclerosis, including NO [52]. NO synthesis is mediated by nitric oxide synthase (NOS), which converts L-arginine to NO and citrulline, with various isoforms including iNOS primarily found in macrophages [53]. Given

that macrophages are also present in atherosclerotic lesions and have the capacity to generate significant quantities of NO when activated, it is plausible that Hcy could play a role in the progression of atherosclerosis by promoting iNOS-mediated NO production in these macrophages.

Our study shows that Hcy significantly increased iNOS expression in both L-929 and RAW 264.7 cells. iNOS is a molecule that plays a dual role in vascular function. While NO may have vasoprotective effects, iNOS overexpression may contribute to vascular injury by causing the formation of high levels of peroxynitrite (ONOO-), a potent oxidant that can promote endothelial dysfunction [54]. In L-929 fibroblast-like cells, significant upregulation of iNOS was observed when the Hcy concentration was increased from 50 µg/mL to 100 µg/mL. This finding is consistent with the studies showing that Hcy induces iNOS expression in smooth-muscle cells or macrophages [24, 55]. Similarly, in RAW 264.7 macrophages, iNOS expression was notably elevated at Hcy concentrations as low as 25 µg/mL. These observations highlight the pro-inflammatory effects of Hcy, particularly in macrophages, which play a pivotal role in atherosclerosis development through the secretion of pro-inflammatory cytokines and the uptake of modified LDL particles.

The upregulation of iNOS by Hcy may be mediated through oxidative stress-induced activation of NF-κB, which regulates iNOS expression [24, 55]. In this regard, Hcy-induced iNOS expression contributes to local NO production, which exacerbates inflammation and oxidative stress in the vascular wall, promoting atherosclerotic plaque formation. Excessive NO production in macrophages may also enhance cellular injury and destabilize the plaques [52, 53].

Taken together, the data suggest that Hcy, an important risk factor for atherosclerosis, has a role in increasing ox-LDL levels in both fibroblasts and macrophages. This emphasizes that Hcy may contribute to the pathophysiological mechanisms underlying various diseases. Moreover, Hcy's ability to modulate iNOS expression further highlights its involvement in inflammatory processes. Our results indicate that targeting oxidative stress and inflammation may provide an effective strategy to mitigate the pro-atherogenic effects of hyperhomocysteinemia. Future research should assess the therapeutic potential of these approaches, particularly for individuals with elevated Hcy levels and a predisposition to cardiovascular disease.

Limitations

In our study, oxidative and inflammatory markers such as MDA, catalase, TNF-alpha, IL-6, and IL-8 could not be analyzed due to budgetary constraints. Although cell culture and immunocytochemical analysis provided important data to demonstrate the modulatory effects of Hcy on ox-LDL and iNOS, these findings could not be corroborated by *in vivo* studies due to similar budget limitations.

Conclusions

This study demonstrates that Hcy significantly promotes LDL oxidation and iNOS expression in L-929 fibroblast-like cells and RAW 264.7 macrophages in a dose-dependent manner. These findings suggest that elevated Hcy levels contribute to oxidative stress and inflammation, key processes that drive plaque formation and progression. Further research is needed to investigate the potential therapeutic strategies aimed at alleviating the adverse effects of hyperhomocysteinemia on vascular health.

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