Original Article

Native High-Density Lipoprotein and Melatonin Improve Platelet Response Induced by Glycated Lipoproteins

(high-density lipoproteins / *in vitro* glycation / low-density lipoproteins / melatonin / platelets)

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Abstract. Activated platelets and glycated lipoproteins are responsible for atherothrombosis in diabetics. Melatonin and native high-density lipoproteins are crucial in the preservation of pro/oxidant-antioxidant balance. The aim of the present study was to investigate the *in vitro* effects of native high-density lipoproteins and melatonin on altering the platelet response induced by glycated lipoproteins. Low-density lipoproteins and high-density lipoproteins were purified from plasma by ultracentrifugation and were glycated with glucose for three weeks. After incubation with or without melatonin/or native high-density lipoproteins, low-density lipoproteins, glycated low-density lipoproteins/glycated high-density lipoproteins were added to ADP-induced platelets. Oxidative parameters, caspase-3/9 and nitric oxide levels were measured spectrophotometrically; CD62-P/annexin-V expression was determined by flow cytometry. In glycated low-density lipoprotein/glycated high-density lipoprotein-treated groups, platelet malondialdehyde/protein carbonyl, P-selectin, annexin-V, caspase-3/9 levels were increased (ranging from P < 0.001 to P < 0.01); glutathione and nitric oxide levels were reduced (ranging from P < 0.001 to P < 0.01). In glycated low-density lipoprotein/glycated high-density lipoprotein-treated groups, melatonin treatment reduced malondialdehyde, protein carbonyl, CD62-P, annexin-V and caspase-3/9 (P < 0.001, P < 0.01) levels and elevated nitric oxide (only glycated low-density lipoproteins). In glycated low-density lipoprotein/glycated high-density lipoprotein-treated groups, native high-density lipoprotein treatment reduced malondialdehyde, protein carbonyl, annexin-V, caspase-3/9 levels (P < 0.001, P < 0.01) and increased glutathione; nitric oxide levels (only with gly-HDL). Both melatonin and high-density lipoproteins should be regarded as novel promising mechanism-based potential therapeutic targets to prevent atherothrombosis in diabetics.

Introduction

Low-density lipoprotein (LDL), especially modified LDL, is regarded as a risk factor for atherothrombosis. Contrary to LDL, high-density lipoprotein (HDL) has anti-atherogenic features by preventing LDL oxidation and inhibiting thrombosis (Alsheikh-Ali et al., 2005; Ouweneel and Van Eck, 2016). Modifications in HDL and LDL such as glycosylation could cause abnormal lipoprotein function and contribute to atherogenesis.

Especially glycation may be cytotoxic and immunogenic for many cells; it may cause direct damage to the vessel wall. It has been shown that for several reasons, glycation of lipoproteins stimulates cell signalling pathways (which are important in cell proliferation and differentiation) and oxidative stress in several cells, thus contributing to atherosclerosis (in hyperlipidaemia, renal disease and diabetes) (Veiraiah, 2005; Kong et al., 2015; Toma et al., 2016). At the same time, glycation may generate free radicals, thereby increasing oxidative damage in the lipids and protein structure (apolipoproteins) of lipoproteins. Moreover, lipids and proteins of glycated LDL and HDL have been shown to be more susceptible to oxidative modification compared to na-

Abbreviations: AGE – advanced glycation end products, BHT – butylated hydroxyl toluene, DNPH – 2,4-dinitrophenylhydrazine, FITC – fluorescein isothiocyanate, gly- – glycated, GSH – glutathione, HDL – high-density lipoprotein, LDL – low-density lipoprotein, MDA – malondialdehyde, n- – native, NBT – nitroblue tetrazolium, NO – nitric oxide, PCO – protein carbonyl content, PBS – phosphate-buffered saline, PFA – paraformaldehyde, pNA – p-nitroanilide, PON – paraoxonase, PRP – platelet-rich plasma, PS – phosphatidylserine.

tive LDL/HDL. Although non-enzymatic glycosylation of both LDL and HDL naturally occurs in the plasma, toxic roles of these lipoproteins in atherosclerosis are related to their prolonged presence in circulation and impaired cellular uptake (Zhao et al., 2013; Du et al., 2017; Kashyap et al., 2018). Advanced glycation end product (AGE) proteins also bind to LDL, causing further delay in the plasma clearance of LDL. Recognition of glycated LDL via the classical LDL receptor gets impaired, and this process may contribute to hyperlipidaemia and foam-cell formation, respectively. Meanwhile, glycation of HDL impairs its recognition by cells and reduces its effectiveness in reverse cholesterol transport (Imachi et al., 2003; Veiraiah, 2005).

On the other hand, platelets play crucial roles in inflammation, atherosclerosis, diabetes and metastasis (Russo et al., 2017; Olsson and Cedervall, 2018). Platelets cannot synthesize cholesterol; therefore, they use plasma lipoproteins as a cholesterol source. Activated platelets and modified lipoproteins get involved in the atherosclerotic lesion formation together; therefore, correlation between platelets and plasma lipoproteins play a relatively important role in the pathogenesis of several diseases. Blood platelets are continuously exposed to LDL and HDL. We previously reported that HDL has antiplatelet effects (Sener et al., 2011). Moreover, modified, especially oxidized, LDL promotes atherothrombotic events by increasing radical levels, lipid peroxidation and activation in platelets. Recent studies have suggested that although platelets are anucleated cells, they can undergo apoptosis. Platelets undergo apoptosis for several reasons (hyperlipidaemia, diabetes), and this leads to their activation and shortens their lifespan (Thushara et al., 2015; De Silva and Kim, 2018). Antioxidants are crucial in the preservation of pro/oxidant-antioxidant balance in the metabolism and suppression of cell apoptosis.

Melatonin is an antioxidant neurohormone synthesized in mammals. It has been shown that melatonin protects normal cells from apoptosis and at the same time, it promotes apoptosis in several cancer cells. Its antioxidant effects via several mechanisms minimize oxidative stress, inhibit apoptosis and influence activities of several antioxidant enzymes in various conditions (Reiter et al., 2016). Generally, studies focusing on platelet-melatonin interaction are related with the effect of melatonin on platelet aggregation. Little evidence was found about other platelet functions. It was demonstrated in a human study that melatonin triggers platelet generation, powerfully suppresses platelet aggregation and micro-thrombosis formation (Evsyukova, 2011). Despite a few opposing results (pro-apoptotic in high concentration) (Kumari and Dash, 2011), melatonin may be beneficial as a therapeutic agent for cardiovascular diseases or thrombotic status.

Furthermore, native HDL, which has a protective role in cardiovascular events, also has anti-inflammatory, anti-thrombotic, anti-oxidant and pro-fibrinolytic effects. Human HDL is found to be heterogeneous regarding its

density, size, shape, surface charge, and composition. HDL particles can be classified into two major subclasses: 1) small, dense (1.125–1.21 g/ml) HDL_3, and 2) larger, dense (1.063–1.125 g/ml) HDL_2 according to their apolipoprotein composition. The biological activities and atheroprotective function of HDL are related to the physicochemical properties of both lipid and protein moieties, and equally to the particle structure. Total HDL, diverse HDL subclasses, and even reconstituted HDL particles were used in various studies and have shown different effects on cells including platelets. HDL_2 (Lp A-I)'nin is shown to be cardioprotective and HDL_3 (Lp A-I/A-II) atherogenic (Asztalos et al., 2004; Martin et al., 2015). Contrary to LDL/ox-LDL, HDL reverses the effects of LDL on platelet activation (Sener et al., 2011). According to our investigations in the literature, the actions of melatonin and HDL on especially platelet apoptosis and platelet response induced by glycated (gly) LDL and glycated HDL have not been documented.

The aim of our present study was to investigate the *in vitro* effects of glycated LDL and HDL on platelet features such as activation, apoptosis, oxidative status and also to investigate the effects of melatonin and native HDL on altering platelet response with glycated lipoproteins.

Material and Methods

Subjects

After signing a written consent approved by the local ethical committee (Medical Faculty of Marmara University), 12 healthy subjects (aged 25–45) were enrolled in this study. Exclusion criteria were medical history of renal, liver and cardiovascular/thrombotic disease; smoking; use of antiplatelet, anticoagulant, or lipid-lowering drugs for at least 10 days.

Lipoprotein isolation and preparation of glycated lipoproteins

LDL and HDL were isolated from plasma samples of 12 healthy normolipidemic subjects by density gradient ultracentrifugation (density range d = 1.019 to 1.063 g/ml for LDL and d = 1.063 to 1.24 g/ml for HDL) by a previously described modified method (Sclavons et al., 1985). After dialysing with phosphate-buffered saline (0.1 M PBS, 1 mM EDTA, pH 7.4), lipoproteins were sterilized by filtration through 0.45 µm pore membranes and stored at 4 °C until use. Total protein concentration of isolated lipoproteins was determined spectrophotometrically by the Bradford method (Bradford, 1976). Total lipid and apoprotein contents of isolated lipoprotein fractions were measured by using standard methods in a Hithachi 917 Autoanalyzer (Boehringer Mannheim, GmbH., Mannheim, Germany).

In vitro glycation of lipoproteins was carried out in the absence of EDTA at 37 °C for 21 days (3 weeks) under sterilized conditions. Gly-LDL and gly-HDL were

prepared by incubating isolated native HDL (n-HDL) / native LDL (n-LDL) with 200 mM/l glucose solution containing 25 μmol/l butylated hydroxyl toluene (BHT) in PBS. Native lipoproteins were similarly incubated in PBS without glucose and used as a control. After dialysis against PBS samples, the gly-lipoproteins were stored at –20 °C prior to analysis.

Determination of glycation and paraoxonase (PON) levels

The degree of early glycation products in modified lipoproteins was assessed by the colorimetric method using a commercial kit (Fructosamine Test, Hoffman-La Roche, Basel, Switzerland). The kit is based on reduced nitroblue tetrazolium (NBT) (pH 10.3). The difference in NBT was monitored at 530 nm in a spectrophotometer and formation of fructosamine was calculated according to the manufacturer's protocol. Fructosamine levels of samples were expressed as mmol/l.

The activities of PON of n-HDL and gly-HDL were measured using paraoxon (1 mmol/l) (Sigma-Aldrich, St. Louis, MO). n-HDL and gly-HDL samples (100 μg) were re-suspended in 5 mmol/LTris-HCl, at pH 7.4. After adding substrate paraoxon, the increase in the absorbance at 412 nm was monitored by a spectrophotometer. n/gly-HDL-PON activity was expressed as U/mg of HDL proteins (Gan et al., 1991).

Preparation of platelet samples

Venous blood samples (N = 12) were obtained with a 21-gauge butterfly needle from healthy volunteers. Whole blood was collected into tubes containing 3.2% sodium citrate and 1 U/ml apyrase used to prevent platelet activation during the isolation. The blood samples were centrifuged at 200 g for 8 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 2,500 g for 15 min to obtain a platelet pellet. The platelets were washed with Tris-NaCl buffer containing EDTA and apyrase and used as previously described (Sener et al., 2011).

Interaction of melatonin and lipoproteins with platelets

Platelet pellets were suspended in HEPES-Tyrode's buffer and incubated with either native-HDL/native-LDL (100 μg/ml) or gly-HDL/LDL (100 μg/ml) or melatonin (10 μM) (Sigma-Aldrich) at 37 °C for 3 h. Then ADP (10 μM) stimulated platelets were centrifuged and re-suspended in HEPES-Tyrode's buffer. After the centrifugation (Hettich GmbH & Co. KG, Universal 32R, Tuttlingen, Germany) at 2,500 g for 15 min, the supernatant and pellet were used for several analyses.

Measurement of lipid peroxidation and protein carbonyl content (PCO)

Lipid peroxidation was evaluated by quantitation of malondialdehyde (MDA) according to the method of Buege and Aust (1978). The results were expressed as nmol/mg protein. PCO levels in platelet pellets were measured spectrophotometrically (Levine et al., 1990). PCO groups react with 2,4-dinitrophenylhydrazine (DNPH) to generate chromophoric dinitrophenyl hydrazones. Supernatants were incubated with 0.5 ml of 10 mM DNPH in 2N HCl for 1 h at room temperature. After the DNPH reaction, proteins were precipitated with an equal volume of 20% (w/v) trichloroacetic acid and washed three times with 1 ml of an ethanol/ethylacetate mixture (1 : 1) and then centrifuged at 6,000 g for 5 min. Finally, the precipitates were dissolved in 6M guanidine-HCl solution and the absorbance was measured at 360 nm, using a molar extinction coefficient of $2.2 \times 10^4 M^{-1} cm^{-1}$. The results were expressed as nmol/mg protein.

Determination of glutathione (GSH) and nitric oxide (NO) levels

The GSH levels in supernatants were determined spectrophotometrically (Mergel et.al., 1979) using DTNB. The results were expressed as μmol/mg protein. The NO levels in the supernatant were determined using a colorimetric NO assay kit (Abcam, Cambridge, MA) according to the manufacturer's protocol. In this colorimetric assay, nitrate is first converted to nitrite by NADH-dependent nitrate reductase, followed by spectrophotometric measurement of the nitrite levels using Griess reagent (Sigma-Aldrich). Results were expressed as μmol.

Caspase-3 and caspase-9 activity assay

Caspase-3 and caspase-9 activities were determined using caspase colorimetric assay kits (EMD Millipore, Caspase-3/9 Colorimetric Cellular Activity Assay Kit, Ottawa, Canada) according to the manufacturer's instructions. Briefly, cells were sonicated in ice-cold lysis buffer supplied by the kits. Samples were centrifuged for 10 min at 10,000 g at 4 °C. Then, the supernatants were collected, samples containing 200 μg protein were incubated for 2 h at 37 °C in a buffer mixture containing p-nitroanilide (pNA) substrates (Ac-DEVD-pNA for caspase-3 and Ac-LEHD-pNA for caspase-9). pNA cleavage was followed spectrophotometrically. The Ac-DEVD-pNA cleavage activity was calculated as picomoles per minute. Caspase-9 levels were expressed as percentage of the activity measured vs controls.

Flow cytometric analysis of platelet activation (CD62-P) and phosphatidylserine (PS) externalization

For the measurement of platelet activation, CD62-P FITC (fluorescein isothiocyanate), and for PS exposure (early apoptotic marker), annexin-V-FITC was used. Flow cytometric analysis was made in the FACS Calibur flow cytometry system (Becton-Dickinson, Franklin Lakes, NJ) (Vermes et al., 1995; Sener et al., 2011). For flow cytometric analysis, aliquots were taken from each

tube and diluted in HEPES buffer (pH = 7.4). Appropriate amounts of related antibodies (CD62-P-FITC and annexin-V-FITC) were added to the tubes. The tubes were incubated for 15 min in the dark. An equal volume of 2% paraformaldehyde (PFA) was added to each tube for fixation. Before analysis, the samples were re-suspended in HEPES buffer for CD62-P measurement and/or incubated with cold binding buffer for annexin-V-FITC. To estimate the unspecific and background fluorescence, an inactivated sample was stained with an isotype FITC-conjugated IgG control. All samples were investigated in the FACS Calibur flow cytometry system (Becton-Dickinson). A total of 50,000 cells were counted in each tube. Platelets were identified by staining the platelets with FITC-conjugated CD41a; platelet discrimination was made according to scatter properties of cells at FS/SS (Forward Scatter/Side Scatter) scattergram with logarithmic scale. The results were expressed as the percentage of positive cells.

Statistical analysis

Results were expressed as mean ± SD of 12 independent experiments. Statistical significance among groups was determined by one-way analysis of variance (ANOVA) followed by Tukey's test using Graph Pad Prism software. $P < 0.05$ was considered statistically significant.

Results

Analysis of isolated native lipoproteins, glycation and PON levels

The composition of pure lipoproteins was determined by examining Apo A-I, Apo B, triglyceride and cholesterol contents (data not shown).Total protein ratios of n-LDL and n-HDL fractions were 1.830 ± 0.532 and 17.520 ± 3.321 mg/ml, respectively.

After glycation, glycation levels (for LDL and HDL) were assayed as fructosamine levels. The early glycation levels (fructosamine formation) of LDL and HDL were found 0.5 mmol/l and 3.5 mmol/l, respectively, while glycation values were previously measured as 0.1 mmol/l, 0.46 mmol/l, respectively (about 4-fold increase for LDL, 8-fold increase for HDL). Additionally, PON levels of n-HDL and gly-HDL were measured as 38.43 U/mg and 5.23 U/mg, respectively.

Determination of oxidative stress in platelets. MDA, GSH, PCO, NO levels

Here, in the gly-LDL and gly-HDL-treated groups, MDA levels were found to be significantly higher than those of the control group (P < 0.001, P < 0.001 respectively). It has been observed that rather than native lipoproteins (n-LDL, n-HDL), glycated lipoproteins are effective on triggering lipid peroxidation (P < 0.001, P < 0.001, respectively) (Fig. 1a).The increase in carbonyls

with glycation of LDL and HDL was also observed as significant (P < 0.001, P < 0.001 respectively) vs the control group (Fig. 1b). Anti-atherogenic properties of HDL are mainly related to reverse cholesterol transport and protection from oxidation/inflammation via its PON activity. The activity of PON was lower in diabetics because glycation severely impairs HDL/PON-mediated functions (as in our study), Here, it was inevitable to see the increase of oxidative stress markers of gly-HDL with reduced PON activity.

Gly-HDL partly loses its protective role and the cellular antioxidant capacity. This situation was supported by the observation that platelet GSH levels were reduced with LDL, gly-LDL and gly-HDL (P < 0.001, P < 0.001, P < 0.001 respectively). This can be explained by the depletion of platelet GSH after glycation (Fig. 1c).

On the other hand, in diabetics, the impairment of the NO-related signalling pathway may contribute to the platelet dysfunction (decreasing nitric oxide bioavailability). The NO levels in LDL, gly-LDL and gly-HDL-treated platelets were significantly lower as compared to the control group (P < 0.001, P < 0.001, P < 0.01, respectively) (Fig. 1d).

The effects of melatonin and native HDL on the oxidative status in platelets

In order to investigate the functional consequences of melatonin and native HDL (which are strong antioxidants), melatonin (10 μM) and native HDL (100 μg/ml) were treated for 1 h with platelets incubated by gly-lipoproteins. Melatonin acts protectively by reducing oxidative stress and it inhibits the damage to proteins and lipids. To confirm this concept, we observed the platelets incubated with gly-LDL and gly-HDL.

Melatonin treatment reduced MDA (P < 0.001, P < 0.01, respectively) (Fig. 1a), PCO levels (P < 0.001, P < 0.001, respectively) (Fig. 1b), and elevated NO (P < 0.001; P > 0.05, respectively) (Fig. 1d). Moreover, when we detected effects of melatonin on platelet GSH levels, although melatonin slightly increased the GSH levels, this increase was not statistically significant (P > 0.05, Fig. 1c).

Native HDL regulates the cell properties. Actually, when n-HDL was added to platelets incubated with gly-LDL and gly-HDL, MDA (P < 0.001, P < 0.001, respectively) (Fig. 1a) and PCO levels (P < 0.001, P < 0.01 respectively) decreased (Fig. 1b); GSH (P < 0.001, P < 0.001, respectively) (Fig. 1c) and NO levels (only with gly-HDL, P < 0.05) (Fig. 1d) increased significantly. It seems that here, n-HDL strongly suppressed glycation-mediated oxidative stress, probably due to the high PON levels (3.5 mmol/l).

Determination of platelet activation/apoptosis

Platelet activation was estimated from the increase of P-selectin at the platelet surface, which was determined utilizing specific antibodies (CD62-P). However, under-
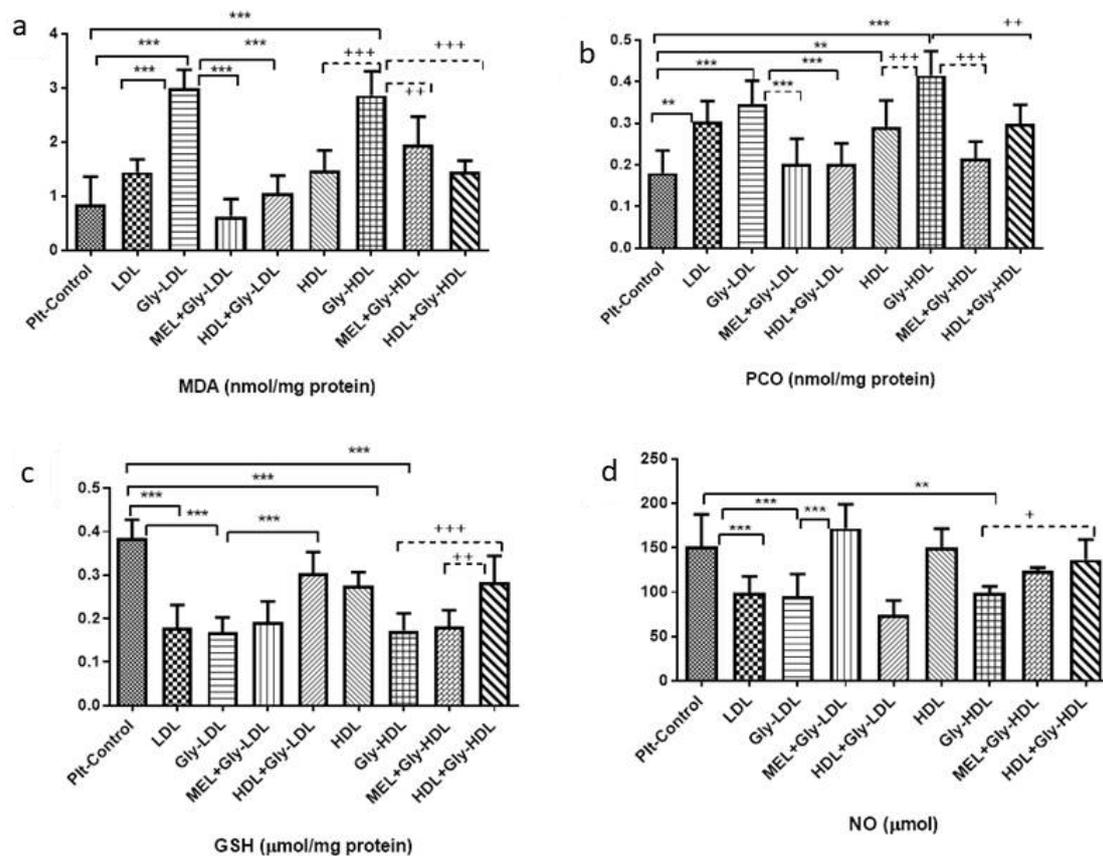
Fig. 1. The effects of n-LDL, n-HDL, gly-LDL, gly-HDL and melatonin on platelet oxidative parameters. Each value is expressed as mean ± SD.
*P < 0.05, **P < 0.01, ***P < 0.001, +P < 0.05, ++P < 0.01, +++P < 0.001

going apoptosis, platelets express PS (annexin-V). As illustrated in Fig. 2a; both gly-LDL /gly-HDL (P < 0.01, P < 0.01, respectively) and n-LDL significantly increased P-selectin abundance (P < 0.05). When platelets were determined by flow cytometry, compared to the control platelets (Fig. 2b), the results revealed that only gly-LDL, gly-HDL, and n-HDL-treated groups had significant annexin-V (early apoptotic cells) positivity (increased with gly-LDL/gly-HDL P < 0.001, P < 0.01 respectively; decreased with n-HDL, P < 0.05), whereas native-LDL did not have any significant effect on the annexin-V positivity.

To examine the effects of native and glycated lipoproteins on caspase-3/9 activity, ADP-induced platelets were incubated with LDL (100 μg/ml), gly-LDL (100 μg/ml), HDL (100 μg/ml), and gly-HDL(100 μg/ml) for approximately 1 h. As shown in Fig. 2c, d; both gly-LDL and gly-HDL significantly promoted platelet caspase-3 (P < 0.001; P < 0.01 respectively) and caspase-9 (P < 0.001, P < 0.001, respectively) activity; nevertheless, platelet caspase activities did not change after n-LDL and n-HDL treatment (P > 0.05). Furthermore, these data suggest that gly-LDL has stronger effects on caspase-3 activity than gly-HDL (P < 0.001). Thus, elevating platelet caspase activity and oxidative stress together may contribute to the hypercoagulability of diabetics.

The effects of melatonin and native HDL on activation/apoptosis in platelets

To establish whether melatonin prevents glycation-induced activation/early apoptosis process in platelets, gly-LDL/gly-HDL-treated platelets were incubated with melatonin (10 μM). Melatonin reduced CD62-P (P < 0.001, P < 0.05, respectively) and annexin-V levels (P < 0.01, P < 0.001) (Fig. 2a, b). In addition, melatonin treatment significantly reduced both caspases for caspase-3, P < 0.001, P < 0.001, respectively (for caspase-9, P < 0.001, P < 0.001, respectively) (Fig. 2c, d).

When we observed the effect of n-HDL on platelet (incubated with gly-LDL and gly-HDL) activation/apoptosis, n-HL did not change platelet activation, but n-HDL significantly reduced the PS response (P < 0.001, P < 0.001, respectively) (Fig. 2a, b). On the other hand, native-HDL treatment significantly reduced caspase-3 activity (P < 0.001, P < 0.01, respectively). Similarly, native-HDL treatment caused almost two-fold decrease in caspase-9 activity (P < 0.001, P < 0.001, respectively) (Fig. 2c, d).

Discussion

Dysfunction of platelets may cause excessive activation or programmed cell death, and these events may
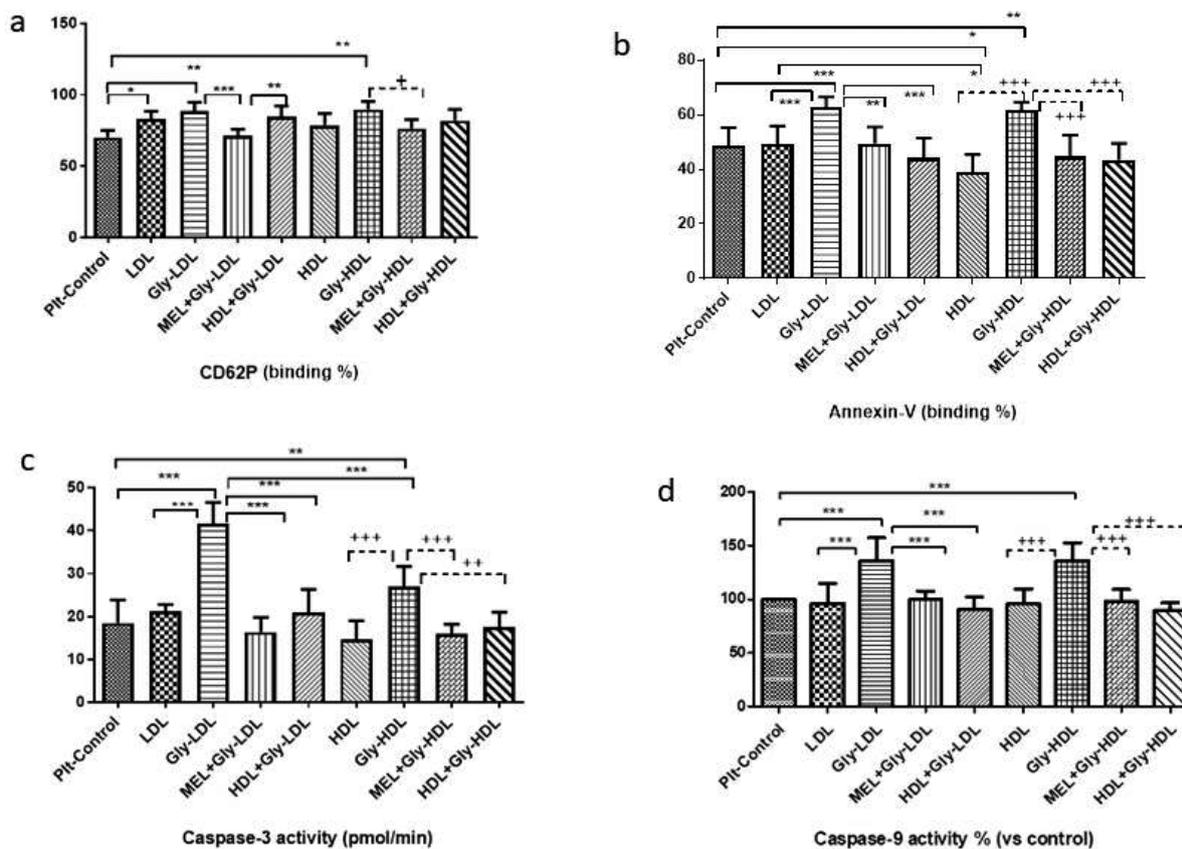
Fig. 2. The effects of n-LDL, n-HDL, gly-LDL, gly-HDL and melatonin on platelet activation/apoptosis. Each value is expressed as mean ± SD.

$*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $+P < 0.05$, $++P < 0.01$, $+++P < 0.001$

play a role in the development of atherothrombosis (Russo et al., 2017). Several agents are able to threaten the platelet's life-span both *in vivo* and *in vitro* (Thushara et al., 2015; De Silva and Kim, 2018). It is known that lipoproteins are important players in atherogenesis; they change the features of cells including platelets. Modified, particularly glycated lipoproteins were responsible for atherothrombosis in diabetics (Alsheikh-Ali et al., 2005; Zhao et al., 2013; Ouweneel and Van Eck, 2016; Kashyap et al., 2018). Although there are a limited number of *in vitro* studies related to the relationship between platelets and glycated lipoproteins, this is the first study about the roles of melatonin and native HDL (as natural antioxidants) on glycated lipoprotein-mediated platelet haemostasis. It is well known that oxidative stress plays a relevant role in the pathogenesis of diabetes mellitus (Mohamed et al., 2016). In the first part of this study, we showed that gly-LDL/gly-HDL markedly triggered platelet membrane lipid peroxidation and protein oxidation. The exact mechanism underlying the gly-lipoprotein-induced oxidative stress in platelets may involve the fact that non-enzymatic glycosylation of lipoproteins proceeds through early and advanced stages. Bioreactive glycation products (as in our results, we found them rather high, 4-fold increase for LDL, 8-fold increase for HDL) alter the function of biomolecules, cause depletion of antioxidant GSH in cells and increase

radical-mediated oxidative stress. According to our data, the possible sources of oxidative stress in diabetics might include depletion of platelet GSH with gly-LDL/HDL and hence impaired activities of antioxidant defence enzymes, as shown in the literature (Santilli et al., 2015).

Several studies have reported that hyperglycaemia also has an accelerating effect on platelet apoptosis and activation (Fatmah et al., 2012). Thus, glycated lipoproteins may possibly interact with scavenger receptors on the platelet membrane and alter the membrane properties (Lam et al., 2004; Ashraf and Gupta, 2011; Lê et al., 2015). This status may result in increased expression of receptors (Ghoshal and Bhattacharya, 2014) such as P-selectin. In this study, both n-LDL and gly-lipoproteins stimulated P-selectin release from platelet granules. As shown in a previous study, in fact, there is a significant relationship between platelet activation, apoptosis, and oxidative stress and they all can trigger each other (Sener et al., 2005). Clinical evidence shows that both oxidative stress and apoptosis have a substantial role in the progression of diabetes mellitus (Kowluru, 2005; Santilli et al., 2015; Thushara et al., 2015). We found here that the elevation in P-selectin was parallel to the elevation of PS expression, which is one of the other platelet activation/apoptosis indicators. Previously, we found that oxidized LDL induces PS externalization

in platelets and may initiate the apoptotic pathway (Sener et al., 2009). Additionally, our results also demonstrate that gly-lipoproteins increased the intracellular activity of caspase-3 and 9. It has been hypothesized that apoptotic caspase activation may cause PS flip from the inner to the outer membrane leaflet (Segawa et al., 2015). According to our findings, the loss of membrane phospholipid asymmetry, elevated activation/caspases and increased platelet procoagulant activity may cause prothrombotic risk in diabetics.

On the other hand, endothelial/platelet-derived NO is an important regulator of platelet activation. Gly-LDL supressed expression of endothelial NO synthase and stimulated apoptosis of endothelial cells (Artwohl et al., 2003; Badimon et al., 2009; Sabbatinelli et al., 2017). In this study, according to our results, gly-lipoproteins had inhibiting effects on the platelet NO status, and thus we hypothesize that a decrease in the bioavailability of platelet NO may contribute to atherothrombosis in diabetics by enhancing the platelet function.

In another part of the study, we compared the impact of melatonin and n-HDL on the gly-lipoprotein-induced platelet response. The pineal secretory product melatonin, an indoleamine, is a natural compound and anti-inflammatory, antioxidant, anti-lipidemic and potent free radical scavenger (Evsyukova, 2011; Erşahin et al., 2012; Galano et al., 2018). In view of the data presented here, it is likely that melatonin has several properties in the platelets. Furthermore, we demonstrated that pre-incubation of gly-lipoprotein-stimulated platelets with melatonin markedly inhibited lipid/protein oxidation, activation (especially with glycated LDL), PS externalization and apoptotic caspases. Moreover, it had an increasing effect on gly-LDL-stimulated platelet NO reduction. Furthermore, melatonin is synthesized not only in the pineal gland, but also in platelets. We have previously shown that melatonin may protect platelets from oxidative modifications with oxidized LDL (Sener et al., 2009). Similarly, in our present study, we observed that the blocking effects of melatonin on elevated oxidation/activation of platelets may be due to its powerful antioxidant properties. In accordance with the presented data, it seems that this molecule can readily cross the platelet membrane, increase platelet membrane micro-fluidity induced by gly-lipoproteins, and thus protect the platelet membranes (García et al., 2014; Galano et al., 2018).

On the other hand, melatonin modulates platelet activation through the interaction of its receptors (Emet et al., 2016) or other mechanisms. As it is known, NO is a considerable regulator in platelet activation, and melatonin has also the ability to regulate the NO pathway. Thus, here we speculated that melatonin may prevent gly-LDL-mediated platelet activation via inhibition of NO down-regulation. There is strong evidence that melatonin protects several cell types against damage-induced apoptosis (Sener et al., 2009; Kumari and Dash, 2011; Galano et al., 2018). Actually, melatonin inhibits the apoptotic process via its antioxidant/scavenger properties; alternatively, melatonin also reduces intracellular signals. It has been shown that at supra-physiological concentrations (about > 10 mM), melatonin may have pro-oxidant activities in some cells (Munik and Ekmek-çioğlu, 2015); these effects were not observed in physiological doses of melatonin (10 μM) like in our study. Therefore, we concluded that at physiological doses, melatonin inhibits the intrinsic and the extrinsic apoptotic signals triggered by gly-lipoproteins in platelets. Our results as presented here are consistent with other different cell studies (Yu et al., 2000; Chetsawang et al., 2006; Gobbo et al., 2015).

At the same time, in the present study we also investigated the effects of n-HDL, which is a powerful antioxidant and atheroprotective agent, on the platelet functions. In fact, HDL has a cardio-protective role via its anti-inflammatory and anti-thrombotic effects (Alsheikh-Ali et al., 2005). HDL-mediated protection in platelets as well as in other cells occurs through several mechanisms: a) HDL collects and transports excess cholesterol from platelets to the liver, thus altering the properties of platelet membranes (Alsheikh-Ali et al., 2005; Ouweneel and Van Eck, 2016). b) As in our previous study, HDL binds to platelets via Apo-A1 and thus shows protective effects (Ozsavci et al., 2001). Consistently, we observed here that as an antioxidant, addition of HDL to platelets caused inhibition of the platelet oxidation status (probably via Apo-A1 receptors) as much as melatonin. Besides, HDL was stronger than melatonin in modulating GSH levels. c) As shown here, n-HDL prevents platelets from glycated lipoprotein-mediated apoptotic signals. d) HDL regulates platelet features via up-regulating NO levels. On the other hand, glycation damages HDL function and PON activity (Du et al., 2017; Kashyap et al., 2018); thus, gly- HDL accelerates type II diabetes due to the reduction in PON levels, as shown in our results (Kashyap et al., 2018).

In conclusion, we are just beginning to learn more about the relationship between platelets and atherogenic lipoproteins and about the role of vascular wall changes. This interaction as an inducer or triggering factor of atherogenesis will be of great interest concerning the mechanisms of diabetes and related events. However, the role of melatonin and native HDL as potential therapeutic targets in atherothrombosis prevention in diabetics remains to be further elucidated.

Disclosure of conflict of interest

The authors declare that they have no conflict of interest.

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