Original Article

Oxidized-LDL and Fe^{3+}/Ascorbic Acid-Induced Oxidative Modifications and Phosphatidylserine Exposure in Human Platelets are Reduced by Melatonin

(melatonin / ox-LDL / oxidative modifications / iron / ascorbic acid / phosphatidylserine / platelet)

A. SENER[1], D. OZSAVCI[1], O. BINGOL-OZAKPINAR[1], O. CEVIK[1], G. YANIKKAYA-DEMIREL[2], T. YARDIMCI[1]

[1]Department of Biochemistry, Faculty of Pharmacy, Marmara University, Istanbul, Turkey
[2]Centro Laboratory, Istanbul, Turkey

Abstract. Low-density lipoprotein (LDL) modifications and platelet activation are major risk factors for cardiovascular diseases. When platelets are exposed to oxidative stress, they become activated. Oxidized LDL (ox-LDL) and metal-catalysed oxidation systems such as Fe^{3+}/ascorbic acid increase free radical production. We wanted to verify whether melatonin has a protective effect against oxidative modifications and phosphatidylserine externalization in platelets induced by ox-LDL and Fe^{3+}/ascorbic acid. For *in vitro* effects of melatonin on platelets, ADP-activated platelets were incubated with ox-LDL or Fe^{3+}/ascorbic acid for 1 h at 37 ºC with or without melatonin. Then platelet malondialdehyde, protein carbonyl and glutathione levels were measured. Platelet phosphatidylserine exposure was measured with annexin-V using flow cytometry. Malondialdehyde, protein carbonyl and phosphatidylserine levels of platelets treated with Fe^{3+}/ascorbic acid significantly increased compared to the control group. Glutathione contents of Fe^{3+}/ascorbic acid-treated platelets significantly decreased. Melatonin pre-treatment of Fe^{3+}/ascorbic acid-treated platelets caused a marked reduction in malondialdehyde and phosphatidylserine levels and a marked increase in glutathione levels. Melatonin also caused non-significant reduction in protein carbonyl contents of Fe^{3+}/ascorbic acid-treated platelets. Malondialdehyde, protein carbonyl and phosphatidylserine levels of platelets treated with ox-LDL also significantly increased compared to the control group. Platelet glutathione levels non-significantly decreased with ox-LDL. With addition of melatonin, malondialdehyde, protein carbonyl and phosphatidylserine levels of platelets treated with ox-LDL significantly decreased. These data suggest that melatonin may protect platelets from iron overload-induced and ox-LDL-induced oxidative modifications and also from the triggering signals of apoptosis activation, possibly due to its scavenger effect on toxic free radicals.

Introduction

Accumulating evidence indicates that free radical-induced oxidative damage contributes to the development of a range of disorders such as cancer, neurodegenerative and vascular diseases. Increased reactive oxygen species (ROS) lead to the alteration of intracellular signals, thus modifying cellular responses in several cells including platelets (Madamanchi et al., 2005). When platelets are exposed to oxidative stress, important changes occur in their structures. Platelet proteins may be initial targets of ROS/reactive nitrogen species (RNS) (Olas and Wachowicz, 2007).

We have chosen Fe^{3+}/ascorbic acid and oxidized low-density lipoprotein (ox-LDL) as an inducer for oxidative modifications. Iron is an essential element for the growth of almost all living organisms and is required for many metabolic functions (Prakash, 2008). At the same time, free iron may be a strong promoter that triggers oxidative stress in iron overload and increases ROS (Pratico et al., 1999). Iron is transported and stored binding to proteins such as transferrin and ferritin in normal

Received June 24, 2008. Accepted January 28, 2009.

Corresponding author: Azize Sener, Department of Biochemistry, Faculty of Pharmacy, Marmara University, Tibbiye Cad., No: 49, 34668, Haydarpasa-Istanbul, Turkey. Phone: +90 216 414 29 62; e-mail: azizesener@hotmail.com

Abbreviations: apoB100 – apolipoprotein B100, apoER2 – apolipoprotein E receptor 2, FITC – fluorescein isothiocyanate, GpIIb/IIIa – glycoprotein IIb/IIIa, GSH – glutathione, GSH-Px – glutathione peroxidase, GSSGRd – glutathione reductase, LOX-1 – lectin-like oxidized low-density lipoprotein receptor 1, LPA – lysophosphatidic acid, MDA – malondialdehyde, ox-LDL – oxidized low-density lipoprotein, PBS – phosphate-buffered saline, PCO – protein carbonyl, PRP – platelet-rich plasma, PS – phosphatidylserine, RNS – reactive nitrogen species, ROS – reactive oxygen species, SOD – superoxide dismutase.

conditions. However, oxidative stress can itself provide the iron that is necessary for Fenton chemistry by mobilizing iron from ferritin and transferrin or by degrading haem proteins to release iron (Halliwell and Gutteridge, 1986; Prakash, 2008). Especially, in the presence of reducing agents such as ascorbic acid (vitamin C) and thiols, Fe^{3+} converts to Fe^{2+}, and Fe^{2+} initiates Fenton reaction (Halliwell and Gutteridge, 1990). Ascorbic acid is an important element of the antioxidant defence system, but under specific conditions it may also have some prooxidant effects. The combination of Fe^{3+} plus ascorbic acid generates H_2O_2 and a hydroxyl radical with Fenton reaction and may lead to lipid peroxidation and protein oxidation (Macho et al., 1997). The accumulation of ROS promotes oxidative modifications and thus may initiate cell injury and death (Higuchi, 2004).

Modified LDL, especially ox-LDL, contributes to the atherothrombotic process by increasing free radical production, lipid peroxidation and platelet activation (Di Massimo et al., 2001). It is known that ox-LDL reduces platelet life-span and enhances sensitivity to aggregating and oxidative agents (Avriam, 1995; Volf et al., 2000). Blood platelets are susceptible to oxidative damage and apoptosis (Sener et al., 2005). Apoptosis of nucleated cells and anucleated cells is regulated by caspases, a group of cysteine proteases, and is characterized by phosphatidylserine (PS) externalization on the outer surface of the plasma membrane (Jacobson et al., 1994). PS becomes exposed to outer cell membranes during the early stages of apoptosis. PS exposure is reported to be a predictor of platelet activation as well as being an apoptotic marker (Tonon et al., 2002).

Recently, numerous studies have focused on using exogenous antioxidant molecules which prevent the deleterious effects of oxidative agents involving Fe^{2+} and ox-LDL. Antioxidants form the first line of defence against free radicals. Melatonin (N-acetyl-5-methoxytryptamine) is known to be a hormone secreted by the pineal gland and modulates sleepiness, reproduction, circadian rhythm and immunity (Hardeland et al., 2006). Additionally, melatonin is a potent free scavenger for especially hydroxyl and peroxyl radicals (Walters-Laporte et al., 1998), inhibits apoptosis in normal cells (Sainz et al., 2003), influences activities and cellular mRNA levels of antioxidant enzymes including superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and glutathione reductase (GSSGRd). Stimulation and regulation of gene transcription of these enzymes is probably via melatonin receptors (Tomás-Zapico and Coto-Montes, 2005). Specific melatonin receptors have been described in several cell types (Mayo et al., 2002). Platelets also possess melatonin receptors (Vacas et al., 1992). Although there are several studies about the relationship between melatonin and platelet aggregation (Del Zar et al., 1990; Kornblihtt et al., 1993), we couldn't find any reports about its other effects concerning especially oxidative modifications and activation-apoptosis in platelets.

The aim of our study was to investigate the effects of Fe^{3+}/ascorbic acid and ox-LDL-induced oxidative changes in platelets. The main goal of this study was to detect whether melatonin protects platelets from free radical attacks and prevents triggering signals of apoptosis and activation in *in vitro* conditions.

Material and Methods

Materials

Annexin V-FITC and the binding buffer were from Immunotech (Coulter, Miami, FL) Phosphate-buffered saline (PBS), reduced glutathione (GSH), Triton X-100, 5-5-dithiobis-2-nitrobenzoic acid (DTNB), ADP (adenosine diphosphate), paraformaldehyde (PFA), commercial LDL, apyrase and melatonin were from Sigma (Sigma-Aldrich, St Louis, MO). $FeCl_3$, ascorbic acid, $CuSO_4$, guanidine hydrochloride, thiobarbituric acid (TBA), EDTA, tetraethoxypropane (TEP) and dinitrophenylhydrazine (DNPH) were of reagent grade from Merck (Merck, Darmstadt, Germany).

Subjects

The subjects included seven healthy donors. Exclusion criteria for the subjects included smoking, alcohol consumption, pregnancy, haemochromatosis, iron overload, medical history of cardiovascular diseases, diabetes mellitus and use of antiplatelet, anticoagulant, vitamin and mineral supplements, antihistamines, and anti-inflammatory drugs. Inclusion criteria: normolipidaemic, ageing between 30–40 years, no medical history of platelet function disorders.

Preparation of platelet samples

Venous blood of the subjects (N = 7) was centrifuged at 200 *g* for 8 min to obtain platelet-rich plasma (PRP). PRP was centrifuged at 8000 *g* for 10 min at 4 °C (Hettich, Universal 32R, DLB Labcare, Newport Pagnell, England). The platelet pellet was washed once with Tris-NaCl buffer (0.03 M Tris, 0.12 M NaCl, pH 7.4) containing 5 mM EDTA and 1 U/ml apyrase.

Oxidation of LDL

The commercial LDL was dialysed against PBS (10 mM NaH_2PO_4, 120 mM NaCl, 2.7 mM KCl, pH 7.4) to remove EDTA. Oxidation of LDL (1 mg/ml) was carried out with 10 μM $CuSO_4$ in PBS for 24 h at 37 °C. Oxidation was terminated by refrigeration. Ox-LDL was dialysed for 24 h in PBS buffer at +4 °C. Oxidation of LDL was confirmed by the presence of malondialdehyde (MDA) (an end-product of lipid peroxidation) after dialysis. Ox-LDL was freshly used.

Incubation of platelet samples with agents

Melatonin was dissolved in a minimum amount of ethanol (95%) diluted with distilled water to obtain 100 mM stock solution. Platelets were suspended with Tris-NaCl buffer in plastic tubes. Before adding Fe^{3+}/

ascorbic acid or ox-LDL, platelets of all groups were activated with ADP (5 μM) and pre-incubated with or without melatonin for 15 min at 37 °C.

Then Fe^{3+}/ascorbic acid or ox-LDL was added to tubes and incubated for 1 h at 37 °C. Platelet suspensions (1 ml) were incubated at final concentrations as follows:

1. Platelets (Control)
2. Platelets + Melatonin (10 mM)
3. Platelets + Fe^{3+} (150 μM)/ascorbic acid (250 μM)
4. Platelets + Melatonin (10 mM) + Fe^{3+} (150 μM)/ ascorbic acid (250 μM)
5. Platelets + ox-LDL (100 μg /ml)
6. Platelets + Melatonin (10 mM) + ox-LDL (100 μg/ ml)

After the incubation process above, media were removed by centrifugation and washed. The platelets were re-suspended in distilled water. Then platelet suspensions were frozen and thawed four times. After centrifugation for 10 min at 8000 *g*, the supernatant was used for GSH determination and the precipitate for MDA and protein carbonyl (PCO) determination.

Measurement of GSH

GSH levels were assayed according to the method of Mergel and Anderman (1979) using DTNB. GSH contents of platelets were determined with GSH (2–30 μg/ ml) as the standard. The results were expressed as μg per 10^9 platelets.

Measurement of lipid peroxidation

For the measurement of lipid peroxidation and protein oxidation, the precipitate was solubilized for 5 h with Tris-NaCl buffer containing 1% Triton X-100 at 4 °C and then centrifuged. Lipid peroxidation was evaluated with TBA according to the method of Buege and Aust (Buege and Aust, 1978). After solubilization, platelet crude membrane MDA levels were assayed in supernatants. The results were expressed as nmol/mg protein.

Measurement of PCO

The contents of PCO were detected by the method of Cardoso et al. (2004). Supernatants were incubated with 0.5 ml of 10 mM DNPH in 2 N HCl for 1 h at room temperature. Protein hydrazone derivatives were precipitated with 0.5 ml 20% TCA and then the precipitates were washed three times with 1 ml ethanol : ethyl-acetate (1 : 1). The final pellet was re-suspended in 6 M guanidine hydrochloride and incubated for 15 min at 37 °C. The absorbance was measured at 360 nm, using a molar extinction coefficient of 2.2×10^4 M^{-1} cm^{-1}. The results were expressed as nmol/mg protein.

Flow cytometric analysis of phosphatidylserine (PS) externalization

For the measurement of PS exposure, annexin-V-FITC (fluorescein isothiocyanate) was used. Annexin-V-FITC (25 μg/ml) was added to the samples that were incubated with agents described above (under the heading of "Incubation of platelet samples with agents"). Sample tubes were kept on ice until analysis for 10 min in the dark. After addition of an equal volume of cold binding buffer, flow cytometric analysis was made in the FACS Calibur flow cytometry system (Becton-Dickinson, Franklin Lakes, NJ) (Vermes et al., 1995).

For the estimation of non-specific and background fluorescence, inactivated samples were stained with an isotype FITC-conjugated immunoglobulin G (IgG) control. The analysis of all the samples was carried out in a flow cytometer. The system was equipped with 488 nm argon ion laser. CaliBrite beads (BD Biosciences, San Jose, CA) were used for daily quality control. 50,000 cells were counted in each tube and the results represent the mean value of the duplicate samples. Platelets were identified by staining with FITC-conjugated CD41a and by gating from logarithmic scaled forward scatter/side scatter scattergram. The results were expressed as the percentage of positive cells. The negative control cursor was set to 2 % of cells on histograms.

Determination of protein

Protein was determined according to Bradford method (Bradford, 1976) using bovine serum albumin as the standard.

Statistical analysis

All results were expressed as mean ± SD. Data were analysed with ANOVA followed by a Tukey post-hoc test for multiple comparisons. P values lower than 0.05 were regarded as significant.

Results

The effects of melatonin on Fe^{3+}/ascorbic acid-mediated oxidative modifications, PS exposure and GSH contents

In our study, in healthy subjects (N = 7) the values of platelet MDA, which is widely used as an indicator of lipid peroxidation, increased significantly with the addition of Fe^{3+}/ascorbic acid ($P < 0.001$). After Fe^{3+}/ascorbic acid was added to platelets that had been pre-incubated with melatonin, the levels of MDA decreased significantly compared to the group treated with only Fe^{3+}/ascorbic acid ($P < 0.001$) (Fig. 1A).

As shown in Fig. 1B, the values of platelet PCO increased significantly with Fe^{3+}/ascorbic acid ($P < 0.01$). On the other hand, following the incubation of Fe^{3+}/ ascorbic acid with platelets that had been treated with melatonin, the values of PCO decreased by 18 %, but this was not statistically significant ($P > 0.05$).

The platelet GSH contents decreased significantly with the addition of Fe^{3+}/ascorbic acid ($P < 0.001$). When Fe^{3+}/ascorbic acid was added to platelets that had been pre-incubated with melatonin, GSH contents increased significantly ($P < 0.001$) (Fig. 1C).

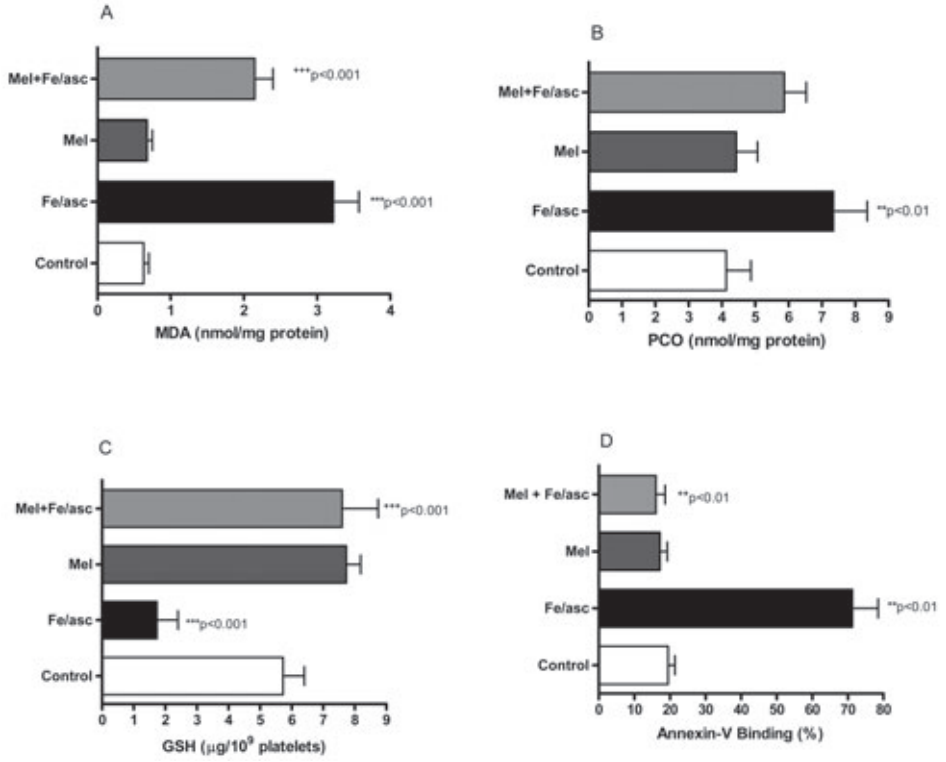

Fig 1. The effects of melatonin (Mel) and/or Fe/asc (Fe3+/ascorbic acid) on MDA levels (A), PCO contents (B), GSH contents (C) and PS externalization (D) in human platelets
(***,** significantly different from the control group; $^{+++}$,$^{++}$ significantly different from the Fe/asc group)

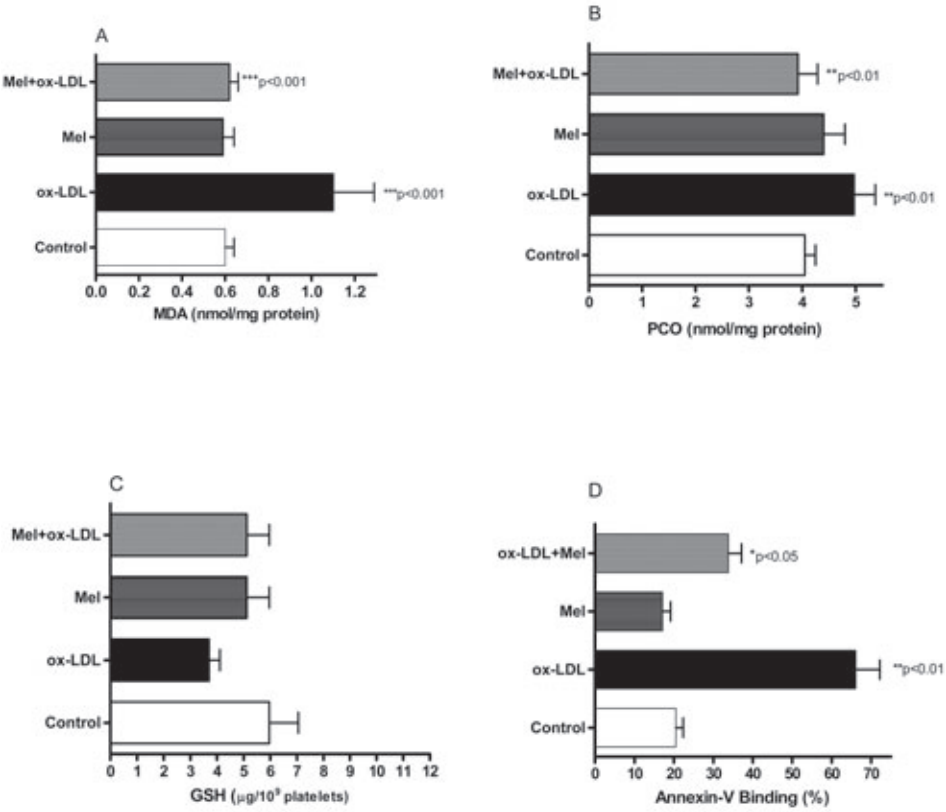

Fig 2. The effects of melatonin (Mel) and/or ox-LDL on MDA levels (A), PCO contents (B), GSH contents (C) and PS externalization (D) in human platelets
(***,** significantly different from the control group; $^{+++}$,$^{++}$,$^{+}$ significantly different from the ox-LDL group)

The response of platelet PS increased significantly with Fe^{3+}/ascorbic acid ($P < 0.01$). With the addition of melatonin, the levels of PS decreased significantly compared to the group treated with only Fe^{3+}/ascorbic acid ($P < 0.01$) (Fig. 1D).

The addition of only melatonin to platelets did not change MDA, PCO, GSH and PS response compared to the control group ($P > 0.05$).

The effects of melatonin on ox-LDL-mediated oxidative modifications, PS exposure and GSH contents

MDA values obtained from commercial LDL oxidation (37.1 ± 12.4 nmol/mg protein) were found significantly higher than non-ox-LDL (2.3 ± 0.85 nmol/mg protein) ($P < 0.001$).

After incubation with ox-LDL, the levels of platelet MDA increased significantly compared to the control group ($P < 0.001$). After the incubation of melatonin with platelets and addition of ox-LDL, the levels of MDA significantly decreased compared to the group incubated with only ox-LDL ($P < 0.001$) (Fig. 2A).

When platelets were incubated with ox-LDL, the levels of PCO increased significantly, similarly as lipid peroxidation ($P < 0.01$). However, after the incubation of melatonin with platelets and the addition of ox-LDL, the levels of PCO significantly decreased compared to the ox-LDL group ($P < 0.01$) (Fig. 2B).

On the other hand, as shown in Fig. 2C, ox-LDL decreased the platelet GSH levels (20 %), but this was not statistically significant ($P > 0.05$). The slightly decreased platelet GSH levels with ox-LDL approached the control levels with the incubation of melatonin.

Ox-LDL significantly increased the platelet PS expression compared to the control ($P < 0.01$). Pre-incubation of platelets with melatonin before the addition of ox-LDL significantly decreased the PS expression compared to platelets treated with only ox-LDL ($P < 0.05$) (Fig. 2D).

When only melatonin was added to platelets, there was no change in the levels of MDA and PCO, GSH contents and PS response ($P > 0.05$).

Discussion

Oxidative stress resulting from ROS leads to oxidative modification of proteins, lipid peroxidation, apoptosis and DNA damage in most peripheral cells including platelets, which are sensitive to oxidative agents (Di Massimo et al., 2001; Luliano et al., 2007). On the other hand, living organisms have several defence systems in order to remove ROS from intracellular environment and protect cells from oxidative damage caused by free radicals. These radicals are removed through enzymatic and non-enzymatic antioxidant systems during metabolism. The antioxidant system represents the first line of defence against these toxic radicals by metabolizing them to non-toxic products (Di Massimo et al., 2001).

It was shown that in the presence of ascorbic acid, iron leads to the oxidation of fats and triggers DNA damage (Friel et al., 2007). On the contrary, Yang et al. (1999) indicated that iron and ascorbic acid did not have a pro-oxidative effect. As it is known, ascorbic acid converts ferric iron to ferrous iron. Ferrous iron moves more easily in the body and contributes to oxidative changes by Fenton reaction, which generates highly reactive hydroxyl radicals. Hydroxyl radical, a strong electrophilic compound, can easily react with double-bound unsaturated lipids and propagate the chain reaction of lipid auto-oxidation (Choe and Min, 2006). It can also cause oxidation of proteins in biological systems (Stadtman and Oliver, 1991).

In our study using oxidant systems containing ferric iron and ascorbic acid, we investigated the effects of ferrous iron on oxidative modifications and PS exposure in platelets. We found that with the addition of ferrous iron, lipid peroxidation and protein oxidation product contents increased, but the levels of GSH, which is one of potent endogenous antioxidants, decreased in platelets. It has been shown that oxidative stress and deficiency of antioxidant capacity are common mediators of apoptosis (Buttke and Sandstrom, 1994; Chandra et al., 2000). Similarly, we observed in our experimental conditions that ferrous iron-mediated excessive oxidative stress and depletion of GSH triggered PS expression, which is a pro-apoptotic signal and an activation marker of platelets.

On the other hand, in the second part of the study, ox-LDL was used as an oxidative agent and its effects on platelet parameters were examined as mentioned above. It is obvious that ox-LDL has a critical role in the development of oxidative damage associated with cardiovascular diseases (Liu et al., 1998). Ox-LDL often has toxic effects on cells. Oxidation causes differences in ox-LDL's electrophoretic mobility, density and fragmentation of apolipoprotein B100 (apoB100) (Korporaal et al., 2007). Ox-LDL contributes to the atherothrombotic process by increasing monocyte adhesion to endothelium, proliferating smooth muscle cells, inducing prothrombinase complex activity on platelets, injuring cells (apoptotic and necrotic pathways) and promoting procoagulant properties of vascular cells (Chisolm and Steinberg, 2000). When LDL is oxidized, biologically active lipids generate within the ox-LDL complex such as lysophosphatidic acid (LPA), lysophosphatidylcholine, F_2-isoprostanes, 4-hydroxy-2,3-trans-nonenal (Siess et al., 1999), MDA and cholesterol hydroperoxides (Young and Mc Enemy, 2001). It is not clear whether the biological effect of ox-LDL on the cells is created via lysophosphatidic acid or lipid peroxides existing in its structure. However, once formed, oxidatively modified lipids are able to react with other cellular components. These lipids may enhance oxidative changes in the cell membrane and short chain aldehydes in its structure may also cause cross-linking of membrane components, loss of enzymatic activities and deformation of ion transport (Esterbauer et al., 1991; Nishio et al., 1996;

Keller and Mattson, 1998). In addition, ox-LDL may cause an increase in platelet response (aggregation, activation, granule secretion) by stimulating the platelet signal pathways via some receptors (GpIIb/IIIa, LOX-1, CD36, apoER2, etc.) with the lipid radicals in its structure (Ozsavcı et al., 2006; Korporaal et al., 2007). In these pathways induced by ox-LDL, mitogen-activated protein kinase phosphorylation, cytosolic phospholipase A_2 activation, increase of arachidonic acid synthesis, cyclooxygenase activation, thromboxane A_2 formation and calcium signals take place.

Thus, ox-LDL may increase ROS production within the cell through signal transduction. Even ROS produced by platelets may lead to additional platelet response in the platelets. Additionally, ROS released from activated platelets also enhance generation of ox-LDL from native LDL (Avriam, 1995). Furthermore, it has been demonstrated that ox-LDL increases free radical production and platelet activation via reducing NO synthase expression in platelets (Liu et al., 1998). Our results indicate that although ox-LDL is a promoter of platelet lipid peroxidation and protein oxidation status, it slightly decreases GSH levels (non-significant). These data show that ox-LDL is a weaker inducer of oxidative stress in our experimental system. Therefore, its effect on GSH consumption is modest. In addition, ox-LDL induces PS externalization in platelets and may initiate the apoptotic pathway. Artwohl et al. (2003) also showed that modified LDL triggers apoptosis in endothelial cells. In our previous study we also pointed out that increasing oxidative stress triggers platelet apoptosis and activation in hyperlipidaemia (Sener et al., 2005). This situation may result from either direct effects of ox-LDL or increased MDA response. It is difficult to distinguish these from each other because both of them may be effective on apoptosis following common ways. One of these common ways may be activation of the caspase cascade. It has been shown that there are various caspases and apoptosis in platelets (Wachowicz et al., 2008). Moreover, when platelets are activated, molecules such as superoxide radical and H_2O_2 that they themselves produce may cause reactivation, aggregation and apoptosis in platelets (Forde and Fitzgerald, 1997). Our results provide evidence that ox-LDL-dependent platelet response may pave the way for thrombosis and cardiac disorders in the future.

In the body there are enough antioxidant reserves to protect living cells against the accumulation of free radicals. The equilibrium in the prooxidant-antioxidant system is controlled via intracellular mechanisms. Antioxidant defence systems may be generally classified as indirect enzymatic antioxidant enzymes and small molecular weight molecules that directly scavenge free radicals. Melatonin has a direct free radical scavenging activity and a regulating effect on gene transcription for antioxidant enzymes (Rodriguez et al., 2004). The antioxidant properties of melatonin have been extensively studied in different cell types. Melatonin inhibits apoptosis in normal cells, but it increases the rate of apoptosis in various cancer cells (Sainz et al., 2003). It has been observed that melatonin can inhibit ox-LDL-induced apoptosis of endothelial progenitor cells (Li et al., 2007). According to the study of Sainz et al. (1995), preincubation with melatonin for 3 h decreases the DNA fragmentation in rat thymocytes. On the other hand, melatonin treatment does not reduce the rate of total body irradiation-induced apoptosis in cultured rat lymphocytes. Investigators have concluded that the lack of melatonin effect on the apoptosis rate in rat lymphocytes may be due to the dose-dependent effect of melatonin (Yurtcu et al., 2007) .

Melatonin metabolites have a direct radical scavenger effect and anti-apoptotic activity (Seeger et al., 1997). In another report, it was demonstrated that melatonin administration can prevent oxidative toxic effects induced by iron-dependent free radical damage in rabbit erythrocytes (Koyu et al., 2000). In addition, melatonin also protects cells from NADPH, Fenton reaction and H_2O_2-induced lipid peroxidation (Sewerynek et al., 1995; Milczarek et al., 2000; Karbownik and Lewinski, 2003).

However, we could not find any literature associated with the effects of melatonin on the platelet oxidative modifications and especially PS response. Although there is much evidence about the relationship between other antioxidants and platelet functions, studies with melatonin have focused on platelet aggregation only. It is well established that vitamin E inhibits platelet aggregation *in vitro* (Steiner, 1978), platelet adhesion *in vivo* (Jandak et al., 1989) and also the platelet release reaction (Steiner and Anastasi, 1976). In another study, Kornblihtt et al. (1993) stressed that melatonin has a marked inhibitory effect on collagen, ADP, and epinephrine-induced platelet aggregation. It has been observed that high-affinity melatonin receptors are present in platelets (Vacas et al., 1992). However, the radical scavenging effects of melatonin are receptor-independent because the experimental evidence confirmed that melatonin is a direct ROS scavenger (Rodriguez et al., 2004). In our findings, we detected beneficial effects of melatonin on iron and ox-LDL-induced oxidative stress in platelets. Especially, melatonin has a critical role in the modulation of platelet lipid peroxidation and PS exposure. Thus, melatonin protects platelets from free radical attacks and prevents the triggering signals of apoptosis and activation.

When we compared ferrous iron-mediated oxidative stress and ox-LDL-mediated oxidative stress, we found that ferrous iron was more severe than ox-LDL on platelets in our experimental conditions. Our Fe^{3+}/ascorbic acid-related results confirm the studies carried out by Almaas et al. (1997) and Friel et al. (2007). They found that hydroxyl radical production (oxidative stress) induced by the *in vitro* addition of ascorbic acid to iron was greater than ascorbic acid supplement alone.

In conclusion, there is a close relationship between oxidative stress and platelet functions and the regulation of this interaction with antioxidants should be a critical

background to the progression of atherothrombotic diseases. The reduction in melatonin with age may be responsible for increased oxidative damage and apoptosis of cells in the elderly (Reiter et al., 1996). Our data showed that melatonin blocks either Fe^{3+}/ascorbic acid oxidant system or ox-LDL (as an oxidant inducer or as a trigger of the signal mechanism)-mediated oxidative stress in activated platelets. From this aspect, the antioxidant effects of melatonin on platelets may be important for several diseases related with oxidative stress including ageing and ageing-related chronic diseases such as atherosclerosis, diabetes mellitus, ischaemia reperfusion injury and cancer.

Acknowledgement

This study was performed in accordance with the Declaration of Helsinki.

References

Almaas, R., Rootwelt, T., Oyaseter, S., Saugtad, O. D. (1997) Ascorbic acid enhances hydroxyl radical formation in iron-fortified infant cereals and infant formulas. *Eur. J. Pediatr.* **156,** 488-492.

Artwohl, M., Graier, W. F., Rodem, M., Bischof, M., Freudenthaler, A. (2003) Diabetic LDL triggers apoptosis in vascular endothelial cells. *Diabetes* **52**, 501-510.

Avriam, M. (1995) LDL-platelet interaction under oxidative stress induces macrophage foam cell formation. *Thromb. Haemost.* **74**, 560-564.

Bradford, M. M. (1976) A rapid and sensitive method for quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Anal. Biochem.* **72**, 248-254.

Buege, J. A., Aust, S. D. (1978) Microsomal lipid peroxidation. *Methods Enzymol.* **52**, 302-310.

Buttke, T. M., Sandstrom P. A. (1994) Oxidative stress as a mediator of apoptosis. *Immunol. Today* **15**, 7-10.

Cardoso, S. M., Santana, I., Sweardlow, R. H., Oliveria, C. R. (2004) Mitochondria dysfunction of Alzhemier's disease cybrids enhances Abeta toxicity. *J. Neurochem.* **89**, 1417-1426.

Chandra, J., Samali, A., Orrenius, S. (2000) Triggering and modulation of apoptosis by oxidative stress. *Free Radic. Biol. Med.* **29**, 323-33.

Chisolm, G. M., Steinberg, D. (2000) The oxidative modification hypothesis of atherogenesis. *Free Radic. Biol. Med.* **28**, 1815-1826.

Choe, E., Min, D. B. (2006) Chemistry and reactions of reactive oxygen species in foods. *Crit. Rev. Food Sci. Nutr.* **46,** 1-22.

Del Zar, M. M., Martinuzzo, M., Falcon, C., Cardinali, D. P., Carreas, L. O., Vacas, M. I. (1990) Inhibition of human platelet aggregation and thromboxane-B_2 production by melatonin: Evidence for a diurnal variation. *J. Clin. Endocrinol. Metab.* **70**, 246-251.

Di Massimo, C., Penco, M., Seri, F., Tozzi-Ciancarelli, M. G. (2001) Possible involment of increased susceptibility of LDL to oxidation in age-related platelet activation. *Clin. Hemorheol. Microcirc.* **25**, 13-20.

Esterbauer, H., Zollner, H., Lang, J. (1991) Chemistry and biochemistry of 4-hydroxynonenal, malondialdhyde and related aldehydes. *Free Radic. Biol. Med.* **13**, 341-390.

Friel, K. J., Diehl-Jones, L. W., Suh, M., Tsopmo, A., Shirwadkar, P. V. (2007) Impact of iron and vitamin C-containing supplements on preterm human milk: In vitro. *Free Radic. Biol. Med.* **42**, 1591-1598.

Forde, R. C., Fitzgerald, D. J. (1997) Reactive oxygen species and platelet activation in repefusion injury. *Circulation* **95,** 787-789.

Halliwel, B., Gutteridge, J. M. (1986) Iron and free radical reactions: two aspects of antioxidant protection. *TIBS* **11**, 372–375.

Halliwell, B., Gutteridge, J. M. (1990) The antioxidant of human extracellular fluids. *Arch. Biochem. Biophys.* **280**, 1-8.

Hardeland, R., Perumal-Pandi, S. R., Cardinali, P. D. (2006) Melatonin. *Int. J. Biochem. Cell Biol.* **38,** 313-316.

Higuchi, Y. (2004) Glutathione depletion-induced chromosomal DNA fragmentation associated with apoptosis and necrosis. J. Cell Mol. Med. 8, 455-464.

Jacobson, M. D., Burne, J. F., Raff, M. C. (1994) Programmed cell death and Bcl-2 protection in the absence of a nucleus. *EMBO J.* **13**, 1899–1910.

Jandak, J., Steiner, M., Richardson, P. D. (1989) α-tocopherol, an effective inhibitor of platelet adhesion. *Blood* **73**, 141-149.

Keller, J. N., Mattson, M. P. (1998) Roles of lipid peroxidation in modulation of cellular signaling pathways, cell dysfunction, and death in nervous system. *Rev. Neurosci.* **9**, 105-116.

Karbownik, M., Lewinski, A. (2003) Melatonin reduces fenton reaction-induced lipid peroxidation in porcine thyroid tissue. *J. Biol. Chem.* **90**(4), 806-811.

Kornblihtt, L. I., Finocchiaro, L., Molinas, F. C. (1993) Inhibitory effect of melatonin on platelet activation induced by collagen and arachidonic acid. *J. Pineal Res.* **14**,184-191.

Korporaal, S. J. A., Van Eck, M., Adelmejjer, J., Ijsseldijk, M., Out, R., Lisman, T., Lenting P. J., Van Berkel, T. J. C., Akkerman, J. W. N. (2007) Platelet activation by oxidized low density lipoprotein is mediated by CD 36 and scavenger receptor-A. *Arterioscler. Thromb. Vasc. Biol.* **22**, 2476-2483.

Koyu, A., Özgüner, M. F., Çalışkan, S., Karaca, H., Köylü, H. (2000) Effect of melatonin on iron treatment-induced oxidative damage in rabbits. *T. Clin. J. Med*. **20**,143-148.

Li, X. L., Xie, X. M., Chen, X. B., He, J., Fang, Y. Q. (2007) Effect of melatonin on the proliferation, apoptosis, and expression of bcl-2 in oxidized low-density lipoprotein-induced endothelial progenitor cells. *Zhong Nan Da Xue Xue Bao Yi Xue Ban* **32**, 862-867.

Liu, S., Chen, Y., Zhou, M., Wan, J. (1998) Oxidized cholesterol in low density lipoprotein may be responsible for the inhibition of LPS-induced nitric oxide production in macrophages. *Atherosclerosis* **136**, 43-49.

Luliano, L., Colavita, R. A., Leo, R., Pratico, D., Violi, F. (2007) Oxygen free radicals and platelet activation. *Free Radic. Biol. Med.* **22**, 999-1006.

Macho, A., Hirsch, T., Marzo, I. (1997) Glutatione depletion is an early and calcium elevation is a late event of thymocyte apoptosis. *J. Immunol.* **158**, 4612-4619.

Madamanchi, R. N., Hakim, Z. S., Runge, M. S. (2005) Oxidative stress in atherogenesis and arterial thrombosis: The disconnect between cellular studies and clinical outcomes. *J. Thromb. Haemost.* **3**, 254-267.

Mayo, J. C., Sainz, R. M., Antoli, I., Herrera, F., Martin, V., Rodriguez, C. (2002) Melatonin regulation of antioxidant enzyme gene expression. *Cell. Mol. Life Sci.* **59**, 1706-1713.

Mergel, D. C., Anderman, G. (1979) Simultaneous determination of oxidized and reduced glutathione in human rabbit red cells. *Methods Find. Exp. Clin. Pharmacol.* **1**, 277-283.

Milczarek. R., Klimek, J., Zelewski, L. (2000) Melatonin inhibits NADPH-dependent lipid peroxidation in human placental mitochondria. *Horm. Metab. Res.* **32**, 84-85.

Nishio, E., Arimura, S., Watanabe, Y. (1996) Oxidized LDL induces apoptosis in cultured smooth muscle cells: A possible role for 7-ketocholesterol. *Biochem. Biophys. Res. Commun.* **223**, 413-418.

Olas, B., Wachowicz, B. (2007) Role of reactive nitrogen species in blood platelet function. Platelets **23**, 1-11.

Ozsavcı D., Avcı, H., Şener, A., Enç, E., Vanizor-Kural, B., Yanıkkaya-Demirel, G., Yardımcı, T. (2006) Isolated native, oxidized LDL and HDL influence platelet binding characteristics of fibrinogen and glycoprotein IIb/IIIa. *FEBS J.* **273**, Suppl 1, 99**.**

Prakash, M. (2008) Role of non-transferrin-bound iron in chronic renal failure and other disease conditions. *Ind. J. Nephrol*. **12**, 188-193.

Pratico, D., Pasin, M., Barry, O. P., Ghiselli, A., Sabatino, G., Iuliano, L., Fitzgerald, G. A., Violi, F. (1999) Iron-dependent human platelet activation and hydroxyl radical formation: involvement of protein kinase C. *Circulation* **99**, 3118-1124.

Reiter, R. J., Pablos, M. I., Agapito, T. T., Guerrero, J. M. (1996) Melatonin in the context of the free radical theory of aging. *Ann. N. Y. Acad. Sci*. **15**, 362-378.

Rodriguez, C., Mayo, J. C., Sainz, R. M., Antolin, I., Herrera, F., Martin, V., Reiter, R. J. (2004) Regulation of antioxidant enzymes: a significant role for melatonin. *J. Pineal Res.* **36**, 1-9.

Sainz, R. M., Mayo, J. C., Uria, H., Kotler, M., Antolin, I., Rodriguez, C., Menendez-Pelaez, A. (1995) The pineal neurohormone melatonin prevents in vivo and in vitro apoptosis in thymocytes. *J. Pineal Res.* **19**, 178-188.

Sainz, R. M., Mayo, J. C., Rodriguez, C., Tan, D. X., Lopez-Burillo, S., Reiter, R. J. (2003) Melatonin and cell death: Differential actions on apoptosis in normal and cancer cells. *Cell Mol. Life Sci.* **60**, 1407-1426.

Seeger, H., Mueck, A. O., Lippert, T. H. **(**1997) Effect of melatonin and metabolites on copper-mediated oxidation of low density lipoprotein. *Br. J. Clin. Pharmacol.* **44**, 283-284.

Sener, A., Ozsavci, D., Oba, R., Demirel, G. Y., Uras, F., Yardimci, T. (2005) Do platelet apoptosis, activation, aggregation, lipid peroxidation and platelet–leukocyte aggregate formation occur simultaneously in hyperlipidemia? *Clin. Biochem.* **38**, 1081-1087.

Sewerynek, E., Poeggeler, B., Melchiorri, D., Melchiorri, D., Reiter, R. J. (1995) H_2O_2-induced lipid peroxidation in rat brain homogenates is greatly reduced by melatonin. *Neurosci. Lett.* **195**, 203-205.

Siess, W., Zang, K. J., Essler, M., Bauer, M., Brandl, R., Corrinth, C., Bittman R., Tigyi, G., Aepfelbacher, M. (1999) Lysophosphatidic acid mediates the rapid activation of platelets and endothelial cells by mildly oxidized low density lipoprotein and accumulates in human atherosclerotic lesions. *Proc. Natl. Acad. Sci. USA* **96**, 6931-6936.

Stadtman, E. R., Oliver, C. N. (1991) Metal-catalyzed oxidation of proteins. *J. Biol. Chem*. **266**, 2005-2008.

Steiner, M., Anastasi, J. (1976) Vitamin E. An inhibitor of the platelet release reaction. *J. Clin. Invest.* **57,** 732-737.

Steiner, M. (1978) Inhibition of platelet aggregation by α-tocopherol. In: *Tocopherol, Oxygen and Biomembranes*, eds. De Duve, C., Hayaishi, O. pp. 142–163, Elsevier, Amsterdam.

Tomás-Zapico, C., Coto-Montes, A. (2005) A proposed mechanism to explain the stimulatory effect of melatonin on antioxidative enzymes. *J. Pineal Res.* **39**, 99-104.

Tonon, G., Luo, X., Greco, J. N., Chen, W., Shi, Y., Jamieson, A. G. (2002) Weak platelet agonists and U46619 induce apoptosis-like events in platelets, in the absence of phosphatidylserine exposure. *Thromb. Res.* **107**, 345-350.

Vacas, M. I., Del Zar, M. M., Martinuzzo, M., Cardinali, D. P. (1992) Binding sites for [^{3}H]-melatonin in human platelets. *J. Pineal Res.* **13**, 60-65.

Vermes, I., Hanen, C., Reutelingsperger, C. P. M. (1995) A novel assay for apoptosis: Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using florescein labelled Annexin V. *J. Immunol. Methods* **180**, 39-52.

Volf, J., Roth, A., Moeslinger, T. (2000) Stimulating effect of biologically modified low density lipoproteins on ADP-induced aggregation of washed platelets persists in absence of specific binding. *Thromb. Res.* **97**, 441-449.

Walters-Laporte, E., Furman, C., Fouquet, S., Martin-Nizard, F., Lestavel, S., Gozzo, A., Lesieur, D., Fruchart, J. C., Duriez, P., Teissier, E. **(**1998) A high concentration of melatonin inhibits in vitro LDL peroxidation but not oxidized LDL toxicity toward cultured endothelial cells. *J. Cardiovasc. Pharmacol.* **32**, 582-592.

Wachowicz, B., Rywaniak J. Z., Nowak, P. (2008) Apoptotic markers in human platelets treated with peroxynitrite. *Platelets* **19**, 624-635.

Yang, M., Collis, C. S., Kelly, M., Diplock, A. T., Evans-Rice, C. (1999) Do iron and vitamin C co-supplementation influence platelet function or LDL oxidizability in healty volunteers? *Eur. J. Clin. Nutr.* **53**, 367-374.

Young, I. S., Mc Enemy, J. (2001) Lipoprotein oxidation and atherosclerosis. *Biochem. Soc. Trans.* **29**, 358-362.

Yurtcu, E., Guney, Y., Ergun, M. A., Guney H. Z., Uluoğlu, C., Hicsonmez, A., Yucel, B., Zengil, H. (2007) Lack of a time-dependent effect of melatonin on radiation-induced apoptosis in cultured rat lymphocytes. *Cell Biol. Int.* **31**, 1144-1149.