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ORIGINAL ARTICLE

# The effects of caffeic acid phenethyl ester and melatonin on age-related vascular remodeling and cardiac damage

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## ABSTRACT

Oxidative stress has been implicated with cardiovascular aging. Most antioxidant intervention studies have involved long-term treatments as a potential means to eliminate age-related oxidative damage in many systems. In the present study, not only light and electron microscopic pictures of the heart and thoracic aorta of young and aged and, caffeic acid phenethyl ester (CAPE) and melatonin and administered aged Sprague Dawley rats, but also antioxidant system status was evaluated. Significantly elevated levels of malondialdehyde (MDA) were observed in the heart and thoracic aorta of aged rats ($P < 0.05$ and $P < 0.001$, respectively). Chronic melatonin and CAPE administration significantly reduced the levels of MDA in the heart ($P = 0.005$ and $P = 0.05$, respectively) and thoracic aorta ($P < 0.001$ and $P < 0.05$, respectively) of aged animals. Additionally, melatonin and CAPE were efficient in stimulating the activities and increasing the levels of the antioxidant enzymes in the heart and aorta. Prominent electron microscopic alterations in cardiac myocytes such as nuclear irregularity, mitochondrial degeneration, myofilament disorganization and disruption, and lipofuscin accumulation were observed in aged rats. The main age-related histologic modifications observed in aorta were irregularity in endothelial cells and their nuclei, divergence of endothelial cells from basement membrane and neighboring cells, and elastic fibril fragmentation and reduction. Melatonin and CAPE obviously reduced these alterations in both heart and aorta of aged rats. Taking the results together, we suggest that supplemental administration of CAPE and melatonin is beneficial in delaying age-related cellular damage in cardiovascular system.

## INTRODUCTION

Aging is a syndrome of changes that are deleterious and progressive, thus far irreversible. Cardiovascular disease is the most frequent cause of death among elderly. Oxidative stress has been implicated with cardiovascular aging. Tissues with few or no cell divisions, such as heart and brain, are theoretically more susceptible to accumulative damage caused by reactive oxygen species (ROS) [1]. There is strong evidence to link the unfavorable accumulation of ROS and the resulting oxidative damages with aging process and human diseases [2,3] Experimental studies have shown that increased oxidative stress and depressed antioxidant status have deleterious effects on cardiac structure and function [4,5]. The myocardium uses enzymatic and nonenzymatic systems to neutralize ROS. However, cardiac muscle may be especially vulnerable to the resulting oxidative damage because of its high rate of oxygen consumption and its low antioxidant defenses relative to other contracting muscle [6]. Most antioxidant intervention studies have involved long-term treatments as a potential means to eliminate age-related oxidative damage in many systems including cardiovascular system.

Melatonin is a highly ubiquitous free radical scavenger and indirectly an antioxidant [7]. Caffeic acid phenethyl ester (CAPE), an active component of propolis, has many biologic and pharmacologic activities including antioxidative capabilities [8,9]. There are a few studies about the beneficial effects of melatonin on cardiac mitochondrial function in aged rats [10,11]. Moreover, studies on age-related ultrastructural changes are relatively scarce. To our knowledge, this is the first experimental study evaluating the effects of melatonin and CAPE on thoracic aorta and of CAPE on the heart in aging rats. Therefore, the aim of this study was designed to evaluate the effect of chronic melatonin and CAPE supplementation on the ultrastructural features, lipid peroxidation levels, and enzymatic and nonenzymatic antioxidants in the heart and aorta of aged rats.

## MATERIALS AND METHODS

### Animals and experimental protocol

Twenty-eight male Sprague-Dawley rats weighing 200–450 g were used. Animals were fed with standard rat chow and tap water ad libitum. They were maintained on a 12-h light/12-h dark cycle at 21°C.

Animals were divided into four groups. First group included rats 4 months of age (young group, $n = 7$) and 2nd, 3rd and 4th groups included rats 18 months of age (old groups, $n = 7$ each). The animals from 3rd group received 5 mg/b.w/day melatonin (Aged + Mel), from 4th group received 15 mg/b.w/day CAPE (Aged + CAPE), both intraperitoneally for 95 days. Melatonin was dissolved in absolute ethanol, and further dilutions were made in saline, with 1% final concentrations of ethanol. CAPE was prepared in the biochemistry laboratory according to standard method described by Grunberg et al. [12]. Animals from 1st and 2nd groups were injected equivalent doses of saline. At the end of the experiment, animals were killed by decapitation.

Animal experiments were performed in accordance with the guidelines for animal research from the National Institute of Health and were approved by the Committee of Animal Research at Inonu University, Malatya, Turkey.

### Microscopic examination

The hearts and upper third of thoracic aorta were rapidly removed and divided to three portions. The first part of the samples were placed in 10% buffered formalin and prepared for routine paraffin embedding. Sections of tissues were cut at 5 μm, mounted on slides, stained with hematoxylin–eosin (H–E), Masson's trichrome, and Verhoeff-light green. Sections were examined by a Leica DFC280 Light microscope and Leica Q Win and Image Analysis system (Leica Micros Imaging Solutions Ltd, Cambridge, UK).

The second part of the tissues was processed for electron microscopic examination. For that purpose, samples were fixed in 2.5% glutaraldehyde buffered with 0.2 M $NaH_2PO_4$ + $NaHPO_4$ (pH = 7.2–7.3) and post-fixed in 1% $OsO_4$. After dehydration in acetone, they were embedded in Araldite CY 212. Ultrathin sections were stained with uranyl acetate and lead citrate and were examined in a Carl Zeiss Libra 120 Electron microscope (Carl Zeiss SMT AG Company, Oberkochen, Germany).

### Biochemical determination

The other part of tissue samples was stored at −80 °C for the determination of malondialdehyde (MDA), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GSH-Px), and total glutathione (GSH).

Homogenization: Tissues were homogenized (PCV Kinematica Status Homogenizator) in ice-cold phosphate buffered saline (pH 7.4). The homogenate was sonified with an ultrasonifier (Bronson sonifier 450) by three cycles (20-s sonications and 40-s pause on ice). The homogenate was centrifuged (15 000 ***g***, 10 min, 4 °C), and cell-free supernatant was subjected to enzyme assay immediately.

*Determination of enzyme activities*

*CAT assay.* Catalase activity was measured at 37 °C by following the rate of disappearance of hydrogen peroxide ($H_2O_2$) at 240 nm ($\epsilon_{240} = 40$ per M/cm) [13]. One unit of catalase activity is defined as the amount of enzyme catalyzing the degradation of 1 μmol of $H_2O_2$ per min at 37 °C and specific activity corresponding to transformation of substrate (in μmol) ($H_2O_2$) per min per mg protein.

*SOD assay.* Superoxide dismutase (Cu, Zn-SOD) activity in the supernatant fraction was measured using xanthine oxidase/cytochrome c method [14], where 1 unit (U) of activity is the amount of enzyme needed to cause half-maximal inhibition of cytochrome c reduction. The amount of SOD in the extract was determined as U of enzyme per mg protein, utilizing a commercial SOD as the standard.

*GSH-Px assay.* Glutathione peroxidase activity was determined in a coupled assay with glutathione reductase by measuring the rate of NADPH oxidation at 340 nm using $H_2O_2$ as the substrate [15]. The enzyme reaction contained 30 mM potassium phosphate, pH 7.0, 1 mM EDTA, 0.2 mM NADPH, 2 mM GSH, 1 mM sodium azide, 1 U glutathione reductase, and 0.1 mM $H_2O_2$. One unit (U) of GSH-Px is defined as the amount of NADPH (μmol) disappeared per min per mg protein.

*GSH assay.* The total glutathione level was performed using the method of Theodorus et al. with some modifications [16]. The reaction mixture contained 50 mM sodium phosphate, 1 mM EDTA, 0.5 mM DTNB, 0.2 mM NADPH, and 0.5 U/mL of glutathione reductase. Homogenate (10 μL) was added to initiate the reaction but was omitted for control. The formation of 5-thio-2-nitrobenzoate is followed spectrophotometrically at 412 nm. The amount of GSH in the extract was determined as nmol/mg protein utilizing a commercial GSH as the standard.

*Lipid peroxidation assay (MDA).* The analysis of lipid peroxidation was carried out as described by Buege et al. [17] with a minor modification. The reaction mixture was prepared by adding 250 μL homogenate into 2 mL reaction solution (15% trichloroacetic acid: 0.375% thiobarbituric acid: 0.25 N HCl, 1 : 1 : 1, w/v) and heated at 100 °C for 15 min. The mixture was cooled to room temperature, centrifuged (10 000 ***g*** for 10 min), and the absorbance of the supernatant was recorded at 532 nm. 1,1,3,3-Tetramethoxypropane was used as MDA standard. MDA results were expressed as nmol per mg protein in the homogenate.

*Determination of protein.* Protein levels of the tissue samples were measured by the Bradford method [18]. The absorbance measurement was taken at 595 nm using a UV–VIS spectrophotometer. Bovine serum albumin was used as protein standard.

### Statistical evaluation

Statistical analysis was carried out using the SPSS 10.0 statistical program (SPSS Inc., Chicago, IL, USA). All data are expressed as arithmetic mean ± SE. The differences between mean values of cell counts, zone thicknesses, and tissue enzyme levels for each group were analyzed by using one-way analysis of variance (ANOVA) and post hoc Duncan tests. Values of $P < 0.05$ were regarded as significant.

## RESULTS

Significantly elevated levels of MDA, an end product of lipid peroxidation, were observed in the heart and thoracic aorta of aged rats ($P < 0.05$ and $P < 0.001$, respectively). Chronic melatonin and CAPE administration significantly reduced the levels of MDA in the heart ($P = 0.005$ and $P = 0.05$, respectively) and thoracic aorta ($P < 0.001$ and $P < 0.05$, respectively) of aged animals. Melatonin was more efficient in reducing MDA levels than CAPE ($P < 0.05$) in aorta. Quantitative analysis of the activities of the antioxidant enzymes CAT, SOD, and GSH-Px in both the heart and thoracic aorta did not reveal any significant difference from those of young rats; however, the level of GSH was significantly higher in the heart of aged rats. Melatonin and CAPE were efficient in stimulating the activities and increasing the levels of the antioxidant enzymes in the heart and thoracic aorta. However, melatonin failed to increase the activity of SOD and CAPE of CAT in the heart. All of the values of cellular antioxidant enzymes were higher in melatonin- and CAPE-administered aged rats, when compared to the values of young rats (*Tables I* and *II*).

Our Masson's trichrome staining results revealed no difference in collagen content of the aged rat heart. The media of thoracic aorta was characterized by numerous elastic membranes, which were arranged concentrically (*Figure 1a*). In the media of aorta of aged rats, the most prominent change was elastin fiber decrease. The Verhoeff-stained elastin fibers were also disorganized and disrupted (*Figure 1b*). Aortic elastin fiber network was dense in melatonin-administered aged rats (*Figure 1c*). However, elastin fiber loss and disorganization was clear in thoracic aorta sections of CAPE-administered aged rats (*Figure 1d*).

The age-related ultrastructural modifications observed in the aorta were irregularity in endothelial cells and their nuclei, divergence of endothelial cells from basement membrane and neighboring cells, elastic fibril fragmentation and reduction. Endothelium was replaced by irregular cells with irregular nuclei and pseudopodia adhering to the vestigial elastic lamina (*Figure 2a*). A prominent change was also found in the subendothelium, which increased in thickness more than fivefold. Subepithelial and luminal lymphocytes were occasionally observed (*Figure 2b*). Basement membrane was generally absent. Smooth muscle cells with irregular nuclei were irregular in shape. Peripheral heterochromatin condensation and mitochondrial degeneration were observed.

**Table I** Mean levels of tissue MDA and GSH, mean activity of tissue SOD, CAT, and GSH-$P_x$ in the heart of the animals from all groups.

| Groups | MDA (nmol/mg prot) | SOD (U/mg prot) | CAT (U/mg prot) | GSH-$P_X$ (U/mg prot) | GSH (nmol/mg prot) |
|---|---|---|---|---|---|
| Group 1 (Young) | 0.27 ± 0.02 | 9.04 ± 0.87 | 18.04 ± 1.11 | 6.55 ± 0.81 | 36.80 ± 1.04 |
| Group 2 (Aged) | 0.35 ± 0.02[a] | 9.57 ± 0.67 | 19.86 ± 1.61 | 5.64 ± 0.5 | 43.36 ± 1.68[a] |
| Group 3 (Aged + Mel) | 0.23 ± 0.03[b] | 9.17 ± 0.42 | 22.49 ± 1.78[a] | 9.78 ± 0.68[d,e] | 46.1 ± 2.46[g] |
| Group 4 (Aged + CAPE) | 0.28 ± 0.012[c] | 10.24 ± 0.64[b] | 19.83 ± 0.56 | 7.33 ± 0.41[f] | 44.60 ± 1.11[a] |

Data are expressed as mean ± SE.
[a]$P < 0.05$ vs. Group 1.
[b]$P = 0.005$ vs. Group 2.
[c]$P = 0.05$ vs. Group 2.
[d]$P = 0.001$ vs. Group 2.
[e]$P < 0.001$ vs. Group 1.
[f]$P < 0.05$ vs. Group 3.
[g]$P < 0.005$ vs. Group 1.

**Table II** Mean levels of tissue MDA and GSH, mean activity of tissue SOD, CAT, and GSH-$P_x$ in thoracic aorta of the animals from all groups.

| Groups | MDA (nmol/mg prot) | SOD (U/mg prot) | CAT (U/mg prot) | GSH-$P_X$ (U/mg prot) | GSH (nmol/mg prot) |
|---|---|---|---|---|---|
| Group 1 (Young) | 0.42 ± 0.02 | 8.39 ± 0.98 | 42.05 ± 1.91 | 25.9 ± 1.44 | 22.08 ± 1.34 |
| Group 2 (Aged) | 0.59 ± 0.02[a] | 8.08 ± 1.15 | 41.95 ± 3.2 | 27.87 ± 3.67 | 21.32 ± 1.15 |
| Group 3 (Aged + Mel) | 0.39 ± 0.02[b] | 10.85 ± 0.86 | 45.99 ± 2.03 | 53.94 ± 3.63[b,g] | 24.17 ± 1.62 |
| Group 4 (Aged + CAPE) | 0.49 ± 0.03[c,d] | 13.44 ± 1.47[e,f] | 44.43 ± 1.92 | 40.94 ± 2.77[c,h,i] | 22.30 ± 1.03 |

Data are expressed as mean ± SE.
[a]$P < 0.001$ vs. Group 1.
[b]$P < 0.001$ vs. Group 2.
[c]$P < 0.05$ vs. Group 2.
[d]$P < 0.05$ vs. Group 3.
[e]$P = 0.005$ vs. Group 1.
[f]$P < 0.005$ vs. Group 2.
[g]$P < 0.001$ vs. Group 1.
[h]$P < 0.005$ vs. Group 1.
[i]$P < 0.05$ vs. Group 3.

In cardiac myocytes of young rats, myofibrils were separated by sarcoplasm containing many healthy mitochondria arranged as rows, with consequent obvious longitudinal striations (*Figure 3a*). Prominent electron microscopic alterations in cardiac myocytes such as nuclear irregularity (*Figure 3b,c*), mitochondrial degeneration (*Figure 3c,d*), myofilament disorganization and disruption (*Figure 3b,e*), and lipofuscin accumulation were observed in aged rats. Abnormalities in the mitochondrial structure such as mitochondrial swelling, cristae loss, myelinic figure and vacuole formation, and a mottled matrix were particular. Other cellular ultrastructural changes were interstitial edema and the appearance of intracellular vacuoles.

Melatonin and CAPE obviously reduced these alterations in both the heart and aorta of aged rats. Ultrastructural picture of the heart and thoracic aorta of CAPE-administered (*Figure 4a*) and melatonin-administered (*Figure 4b*) rats were generally normal. Occasional lipofuscin granules were observed in cardiac myocytes. Rarely, endothelial alterations, irregularity, and subendothelial thickening, were also seen in melatonin- and CAPE-administered rats.

## DISCUSSION

In the last two decades, 'the free-radical theory of aging' has been widely examined and has gained a substantial support from research at the molecular and cellular levels [19–21]. It is suggested that mitochondria are both the most important source and also the major target of free radical attack that leads to human aging. It has been proposed that aging is caused by injury to mitochondrial DNA and lipid peroxidation by free

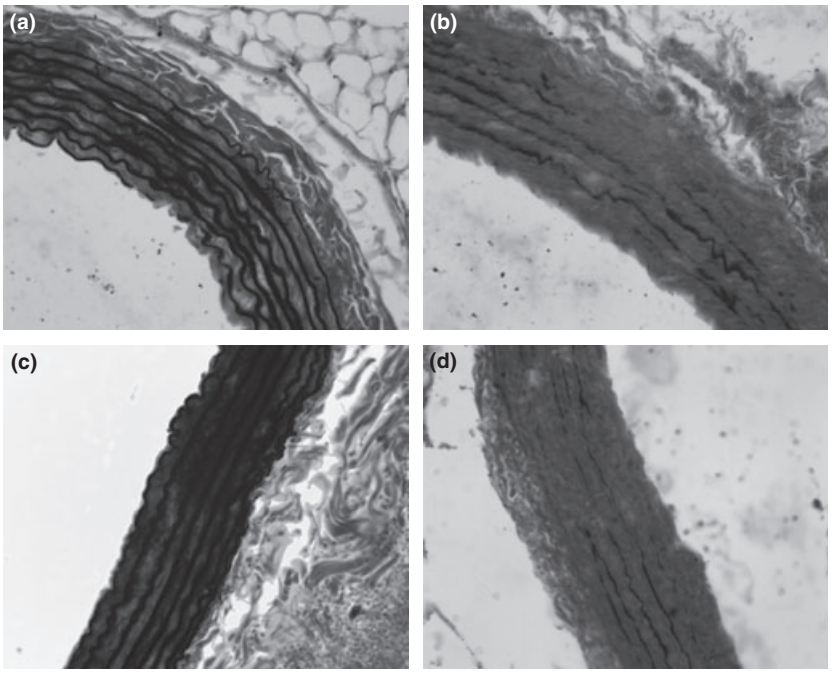


**Figure 1** Examples of thoracic aorta sections stained with Verhoeff-light green method from all groups. (a) Thoracic aorta of a young rat. Concentrically oriented many elastic membranes are observed. (b) Thoracic aorta of an aged rat. Elastic fiber decrease and fragmentation are seen. (c) Thoracic aorta of a melatonin-administered rat. Many elastic thick membranes are present. (d) Thoracic aorta of a caffeic acid phenethyl ester-administered rat. Prominent elastic fiber loss is observed. Verhoeff-light green × 40.

radicals from inner mitochondrial membrane [19,22,23]. Free radicals from mitochondria result in damage to cellular protein, lipids, and DNA throughout the cell. Moreover, the activities of free radical-scavenging enzymes are altered in the aging process [23,24]. In the aging process, the oxidative damage is mostly found in parallel with the declined capacities of antioxidant system [24]. Enzymic SOD, GSH-Px, and nonenzymic GSH play important roles during the process by scavenging of ROS or preventing their formation [25,26]. Quantitative analysis of the activities of the antioxidant enzymes CAT, SOD, and GSH-Px revealed significantly lower values in the liver, kidneys, heart, and brain of aged rats [27]. Several studies focus on the changes of antioxidative enzyme activity in different organs and species in the process of aging, although there have been no conclusive results so far. In fact, it is not clear how aging affects the antioxidative enzyme activity. SOD activity decreased in relation to age in the hearts of rats and mice [28,29]. However, the lack of age-related changes in SOD antioxidant enzymes was reported [1,30]. Higher levels of GSH-Px activity were found in the heart of 19- month Sprague-Dawley rats [31] and 8-month Fisher 344 rats [29] than those of aged rats. CAT activity has been shown to increase significantly in the heart of old Fisher rats [29]. However, we found no significant changes in the activities of SOD, GSH-Px, and CAT enzymes in both the heart and aorta during aging in agreement with results reported by Wu et al. [32].

Glutathione is strongly associated with other age-related pathologies [33]. As individuals age, there is a gradual lowering of GSH levels and of general antioxidant defenses, as well as a decline in the ability of these systems to be induced by exogenous stimuli [34–37], and this decline is associated with a higher incidence of age-related chronic illnesses [38]. On the contrary, we report here a significant increase in cardiac GSH levels in aged Sprague-Dawley rats. Wu et al. [32] have found an increase in the level of cardiac GSH of aged Sprague-Dawley rats too. Moreover, there was no significant decrease in the activities of SOD, CAT, and GSH-Px and in the levels of GSH in aorta of aged rats. Previous studies have demonstrated a low antioxidant capacity in the rat heart [39,40], but an effect of aging was not observed [39].

A possible mechanism may be that antioxidant in the heart of aged rats have a considerable adaptability to respond to prooxidant exposure [41]. We suggest that cardiac antioxidant defense system is highly resistant to the damaging effect of oxidants as heart is one of the most important vital organs. Or synthesis of the cardiac antioxidative enzymes markedly increases in response to increased levels of ROS, so a compensatory increase in the activities or levels of some of the cellular antioxidant enzymes is detected. In fact, elevated GSH levels generally increase antioxidant capacity and resistance to oxidative stress [42]. During the aging process the activity of antioxidant enzymes, e.g. SOD, CAT, and

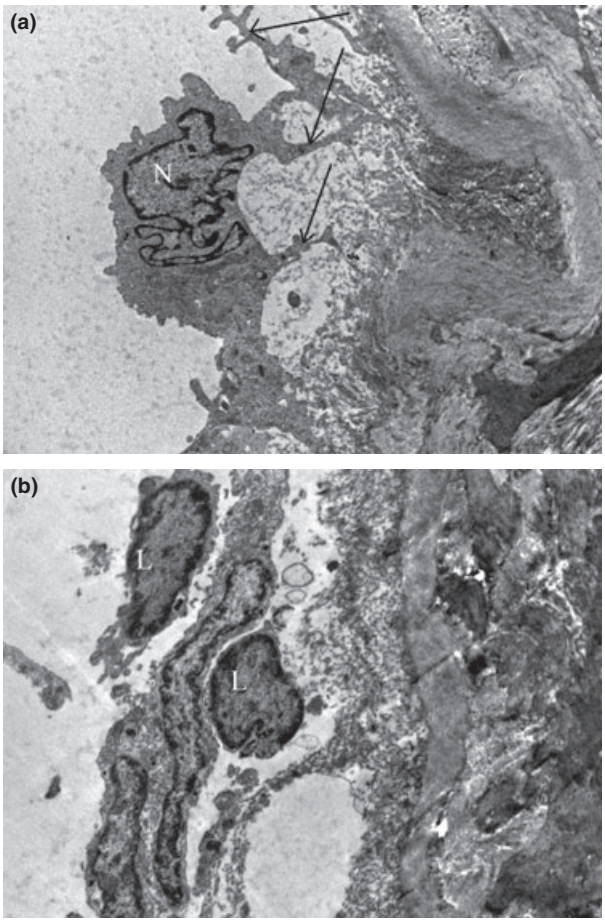


**Figure 2** Electron micrograph of thoracic aorta of aged rats. Sections are stained with uranyl acetate and lead citrate. (a) Irregular endothelial cell with irregular nuclei (N) and pseudopodia (arrows) is far from the basement membrane area ×6.400. (b) Subendothelial and luminal lymphocytes (L) are present ×6.800.

GSH-Px, depends on the factors such as race, gender, tissue, and subcellular localization of enzymes. During the aging process, ROS may also lead to the induction of some enzyme activity, which is explained as an adaptive phenomenon [43].

Recent studies demonstrate that the cardiovascular dysfunction associated with advanced aging is related to the local formation of reactive oxygen and nitrogen species in the myocardium and coronary vasculature [44,45]. Arterial endothelial dysfunction, which is increased with aging [46–48] is a key component of many cardiovascular disorders [49,50]. There is emerging evidence that age-associated endothelial dysfunction is related to the local formation of reactive oxygen and nitrogen species within and in the vicinity of the vascular wall [45,51,52]. Although we did not perform any method to evaluate the function of endothelium, we detected many abnormalities, which are probably inhibiting the function of endothelium. Prominent irregularity in endothelial cells and divergence of endothelial cells from basement membrane and neighboring cells were detected. Basement membrane was generally absent. Therapeutic approaches capable of preventing or reversing age-related endothelial dysfunction may thus help to reduce cardiovascular risk in the elderly.

Lipid peroxidation, a process induced by free radicals, leads to oxidative deterioration of polyunsaturated lipids. Under normal physiologic conditions, only low levels of lipid peroxides occur in body tissues. The excessive generation of free radicals leads to peroxidative changes that ultimately result in enhanced lipid peroxidation [35]. MDA, a secondary product of lipid peroxidation, is used as an indicator of tissue damage [53]. Aging has been reported to be associated with increased disruption of membrane lipids leading to subsequent formation of peroxide radicals [54]. A higher plasma lipid peroxidation was found in aged rats when compared to the younger ones [55]. Tissue MDA levels were significantly higher in both the heart ($P < 0.05$) and aorta ($P < 0.001$) of aged rats. A decrease in cellular antioxidative enzyme activities is associated with an increase in lipid peroxidation in oxidative stress definition. The accumulation of ROS and eventual lipid peroxidation is because of the inefficient antioxidative defense system to scavenge ROS. We demonstrated cellular damage not only by increased MDA levels but also by prominent electron microscopic alterations. We come to the conclusion that not to detect any decrease in antioxidative enzyme activities or levels do not represent the absence of an injury caused by ROS to cellular membrane systems. Sivonova et al. [56] suggest that selective measurement of antioxidant capacity does not provide relevant information on the overall antioxidant status. Moreover, total antioxidant capacity is determined not only by concentration/activity of individual antioxidants but also by synergistic action.

The main consequences of aging process are a progressive change in the morphological and functional characteristics of the cardiovascular system and an increase in the number of age-related cardiovascular disorders. The main morphological changes during aging happen in the structure of cardiac tissue, in the conduction system, and in vessels. Hydroxyl radicals have damaging effect of on protein structure

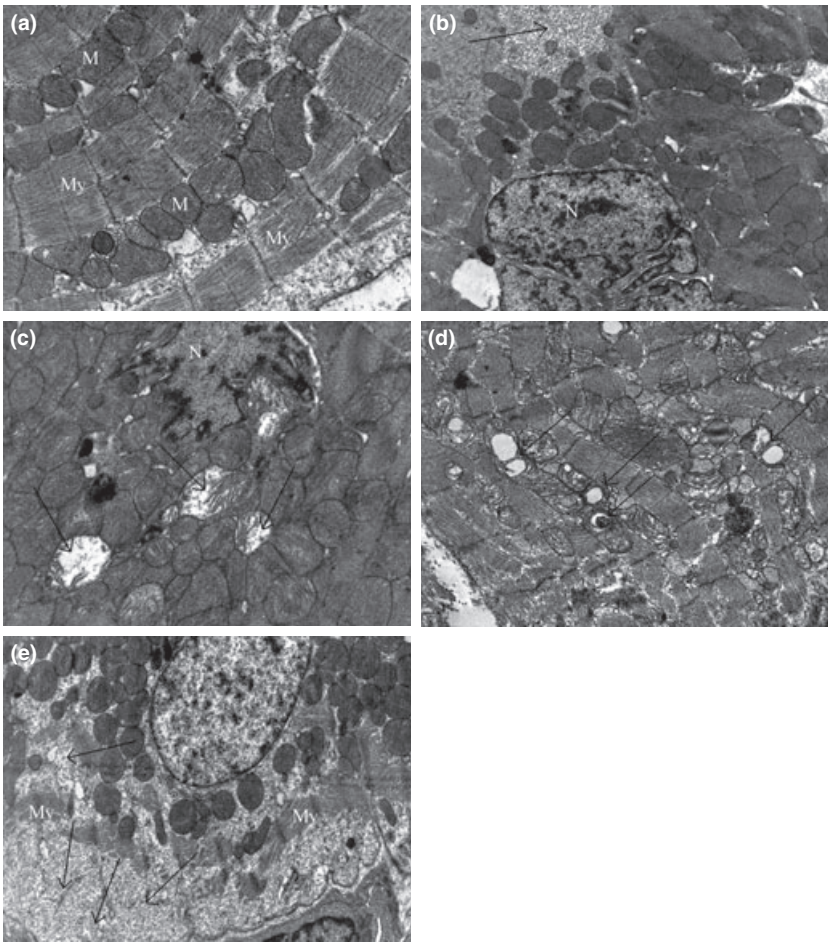


**Figure 3** Electron micrographs of cardiac myocytes from young and aged groups. Sections are stained with uranyl acetate and lead citrate. (a) Cardiac myocyte of a young rat. Many mitochondria (M) between myofilaments (My) are observed. Regular cross striation is obvious ×12.500. (b) Cardiac myocyte of an aged rat. Nuclear irregularity (N) and myofilament disorganization are observed. Myofilament-free cytoplasmic matrix is seen (arrow) ×8.000. (c) Cardiac myocyte of an aged rat. Nuclear irregularity (N), mitochondrial edema, and cristae loss (arrows) are observed ×12.500. (d) Cardiac myocyte of an aged rat. Mitochondrial degeneration is clear. Bizarre mitochondrial configuration with many vacuoles and dense bodies is observed (arrows) ×10.000. (e) Cardiac myocyte of an aged rat. Prominent myofilament (My) loss (arrow) and disorganization are seen ×8.000.

in cardiac mitochondria, myofibrils [57], and sarcoplasmic reticulum [58] in adult rats. Babusikova et al. [59] have shown that oxidative damage to proteins and lipids of cardiac sarcoplasmic reticulum increases during in vitro-generated oxidative stress in senescent rats. It has been suggested that the accumulation of oxidant-induced damage in interfibrillar mitochondria may be a major contributing factor to the age-related alterations in myocardial function [60]. ROS in cells are formed as a result of defects in coupled electron transport within mitochondria. The overproduction of ROS could cause a wide spectrum of oxidative damage to various cellular components, which would lead to cell death, or elicit apoptosis by inducing changes in mitochondrial membrane permeability [24,61]. We observed prominent mitochondrial degeneration, myofilament disruption and disorganization in cardiac myocytes of aged rats. Overproduction of ROS (and the inability to scavenge this excess) leads to damaging lipid peroxidation and relatively more diffusible (but still potent) reactive intermediates, which affect widespread protein targets and amplifies mitochondrial abnormalities [62]. In our opinion, mitochondrial degeneration is the most important evidence representing cellular oxidative damage.

Lipofuscin accumulation in the nondividing cells of the brain and heart is very prominent and is, in fact, regarded as a biomarker of aging. Lipofuscin granules are characterized by a single membrane envelope, enclosing yellowish-brown material that can autofluoresce. Lipofuscin is regarded as a product of lysosomes – organelles containing hydrolytic enzymes to degrade proteins, lipids, and damaged organelles. As production of lysosomal enzymes decline with age and as lysosomes engulf increasingly cross-linked proteins and lipids that are resistant to enzyme degradation, dysfunctional lysosomes accumulate in cells as lipofuscin granules [63]. Growing evidence reveals that autophagy is

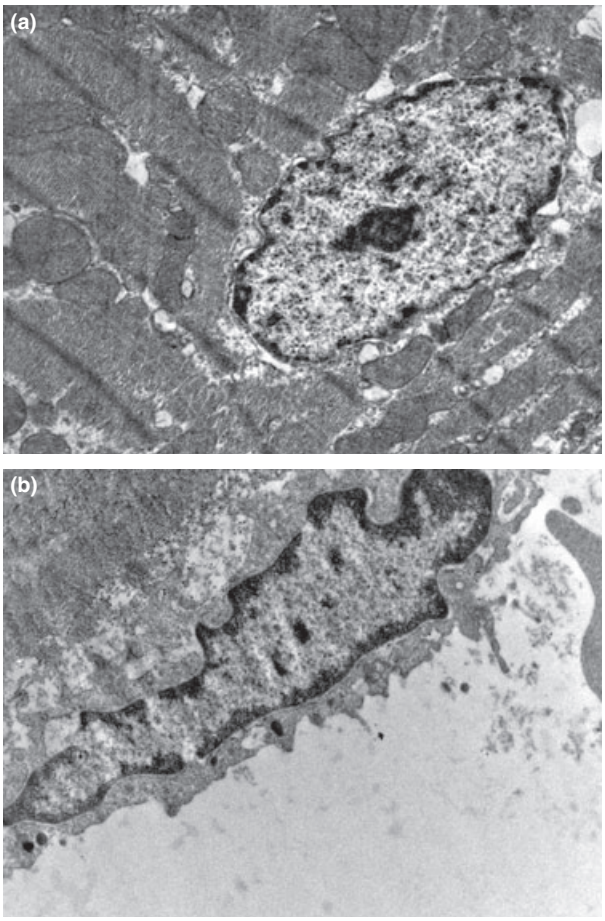


**Figure 4** Electron micrographs taken from melatonin and caffeic acid phenethyl ester (CAPE)-administered rats. Sections are stained with uranyl acetate and lead citrate. (a) Cardiac myocyte of a CAPE-administered rat. Ultrastructural picture is normal ×10.000. (b) Thoracic aorta of a melatonin-administered rat. Ultrastructural picture is nearly normal ×16.000.

involved in the progression or prevention of many human diseases [64]. We observed lipofuscin accumulation within the cardiac myocytes of aged rats.

With advancing age, the large arteries dilate [65–67], their collagen content increases, elastin becomes frayed [62,65], and internal elastic lamina becomes fragmented [62]. In our study, elastin fibers were disorganized and disrupted in aged rats. An increased elastase or gelatinase activity with aging may contribute to both elastic membrane break and elastin reduction in its content with aging. In our study, melatonin ameliorated the age-related elastic fiber decrease but CAPE failed to.

With recent years, many investigators have suggested that pineal gland and melatonin are involved in the process of both aging and age-related diseases. These theories stem form the importance of melatonin in a number of biologic functions and the fact that melatonin production in organism is gradually lost throughout life [68–70]. Also, melatonin directly neutralizes a number of free radicals and ROS, and it stimulates several antioxidative enzymes such as SOD, GSH-Px, and GSH, which increases its efficiency as an antioxidant [69,71,72]. The evidence from the last 10 years suggests that melatonin influences the cardiovascular system. The presence of vascular melatoninergic receptors/binding sites has been demonstrated; these receptors are functionally linked with vasoconstrictor or vasodilatory effects of melatonin. Melatonin can contribute in cardioprotection of the rat heart, following myocardial ischemia [73]. The beneficial effect of chronic pharmacological intervention with melatonin, which reduces the deteriorative and functional oxidative changes in cardiac mitochondria with age, has been reported [10]. Higher concentrations of melatonin $(>10)^{-5}$ M) protect arterial wall integrity against inflammation, oxidative stress, and ischemia/reperfusion injury [74–76], especially in large arteries, such as the aorta [74]. Our study has revealed that melatonin significantly reduces both cardiac and aortic tissue MDA levels. Additionally, we detected potent stimulatory effects of melatonin on the activities, or the levels of all of the antioxidant enzymes. Mean levels of GSH, and activities of CAT, SOD, and GSH-Px were higher in melatonin-administered aged rats even than those of young rats. Ultrastructural pictures of the heart and aorta of melatonin-administered aged rats were also not much different than those of young animals.

Another aim of this study was to investigate the effect of CAPE on the heart and aorta of aged rats. To our knowledge, this is the first study evaluating the effect of CAPE on cardiovascular system of aged rats. CAPE is a structural relative of flavonoids, and it is one of the major components of honeybee propolis, a product that for its broad spectrum of activities has been largely used as a folk medicine. Similar to flavonoids, CAPE has demonstrated several biologic and pharmacologic properties, such as anti-inflammatory [77], anticarcinogenic [78], and antioxidant activities [79,80]. Recently, Ozguner et al. [81] reported the protective effect of CAPE on mobile phone-induced and free radical-mediated oxidative heart impairment in rats. Our study has revealed that CAPE significantly reduces both cardiac and aortic tissue MDA levels. Additionally, we detected potent stimulatory effects of CAPE on the activities or the levels

of all of the antioxidant enzymes. Mean levels of GSH, and activities of CAT, SOD, and GSH-Px were higher in CAPE-administered aged rats even than those of young rats. Ultrastructural pictures of the heart and aorta of CAPE-administered aged rats were also not much different than those of young animals.

Taking the results together, we suggest that long-term supplemental administration of CAPE and melatonin is beneficial in delaying age-related cellular damage in cardiovascular system.

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