The preventive and curative effects of melatonin against abdominal aortic aneurysm in rats

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ABSTRACT

Objective: Oxygen free radicals are important components involved in the histopathologic tissue alterations observed during abdominal aortic aneurysms (AAAs). This study examined whether melatonin has protective or therapeutic effects against AAAs.

Methods: Sprague-Dawley rats were divided into four groups. A $CaCl_2$ model was used to induce AAA. Starting on the operation day (Mel+AAA+Mel group) or 4 weeks after the operation (AAA+Mel group), the rats received intraperitoneal melatonin (10 mg/kg/day) for 6 and 2 weeks, respectively. The control and AAA groups received vehicle for 2 weeks after the sham operation and AAA induction, respectively. Angiographic measurements were recorded at the beginning, week 4, and week 6 of the study. After decapitation, aorta tissues were taken for the measurement of malondialdehyde, 8-hydroxy-2′-deoxyguanosine, glutathione levels, and myeloperoxidase and caspase-3 activity. Matrix metalloproteinase (MMP)-2, MMP-9, tumor necrosis factor-α, and inducible nitric oxide synthase protein expressions were analyzed by Western blot technique. Aortic tissues were also examined by light microscopy.

Results: $CaCl_2$ caused an inflammatory response and oxidative damage indicated by rises in malondialdehyde and 8-hydroxy-2′-deoxyguanosine levels. Myeloperoxidase and caspase-3 activities were increased, but glutathione levels were reduced. On the one hand, MMP-2, MMP-9, tumor necrosis factor-α, and inducible nitric oxide synthase protein expressions were increased in the vehicle-treated AAA group. On the other hand, melatonin treatment reversed all of these biochemical indices and histopathologic alterations.

Conclusions: According to the data, although melatonin tended to reverse the biochemical parameters given on week 4, the preventive effect is more pronounced when given concomitantly with AAA induction because values were closer to the control levels. (J Vasc Surg 2018;67:1546-55.)

Clinical Relevance: Melatonin prevents abdominal aortic aneurysm formation thorough anti-inflammatory and anti-oxidative effects. The preventive effect of this powerful antioxidant can be attributed to its ability to balance oxidant-antioxidant status, inhibit neutrophil infiltration, and regulate inflammatory mediators, suggesting a future role in the treatment and prevention of abdominal aortic aneurysms.

Owing to the high rupture incidence, abdominal aortic aneurysm (AAAs) is a life-threatening disease.[1] Aneurysm is an irreversible, progressive, and degenerative illness that forms with abnormal expansion of a transverse diameter of ≥1.5 times than normal in any segment of the aorta.[2] AAA diagnosis is problematic because most aneurysms are asymptomatic until rupture. The rupture of aneurysm risk increases with large aortic diameter. Mortality after AAA rupture is ~75% for those who reach the hospital.[3] No therapy has been established for small AAAs.[4]

AAA is not a passive enlargement of vasculature but represents an inflammation and tissue degeneration common to many forms of chronic disease.[5] Matrix metalloproteinases (MMPs), calcium-dependent zinc endopeptidases, are known to contribute to the inflammatory process because increased expression of MMPs has been observed in various inflammatory situations and associated tissue injury.[6] Similarly, pathologic features of AAA include elastin degradation, increased oxidative stress, inflammation, and vascular smooth muscle cell apoptosis.[7]

Medical therapy in AAA patients aims to decrease the widening of the aneurysm diameter and rupture probability. Also, medical therapies, such as β-blockers, angiotensin-converting enzyme inhibitors, and statins, have no effect in decreasing aneurysm diameter but do decrease the probability of rupture.[8-10] In light of the knowledge to date, prevention of aneurysm development should be taken into account in the clinical setting. Because inflammation has been shown in the

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This project was supported by Marmara University Scientific Research Projects Commission (project no: SAG-C-TUP-12114-0355).

Author conflict of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a conflict of interest.

0741-5214

http://dx.doi.org/10.1016/j.jvs.2017.04.028

pathophysiology of AAA, the effects of anti-inflammatory medicines on AAA are being investigated. Current studies are focusing on natural antioxidants.[11]

Melatonin, the major product of the vertebrate pineal gland, functions as a modulator of sleep, sexual behavior, immune function, and circadian rhythms.[12] Melatonin has been shown to have potent receptor-dependent and receptor-independent actions. Receptor-dependent effects are mediated predominantly through the MT_1 and MT_2 G-protein coupled receptors, whereas pleiotropic receptor-independent effects of melatonin involve the reactive oxygen species (ROS) scavenging nature, activation and increased expression of several antioxidant enzymes, and the ability to increase the efficiency of the mitochondrial electron transport chain.[13] Furthermore melatonin reduces blood pressure and has an antiadrenergic action on myocardial contractility, which are mediated by its receptors in the heart and arteries.[14]

In the light of above findings, we aimed to investigate if melatonin could attenuate the development of aneurysms and regress dilated aneurysmal sac in an aneurysmal rat model by periaortic application of $CaCl_2$.

METHODS

The Marmara University Animal Care and Use Committee approved the experimental protocols in this study.

Experimental groups. Sprague-Dawley rats (300-350 g) supplied by the Marmara University Animal Center were housed in an air-conditioned room with 12:12 light/dark cycles, where the temperature (22° ± 2° C) and relative humidity (65%-70%) were kept constant.

Rats were randomly divided into four groups of 10: sham operated control, abdominal aortic aneurysm (AAA), AAA+melatonin (Mel), and Mel+AAA+Mel. The rats in AAA+Mel group and Mel+AAA+Mel group received intraperitoneal melatonin (10 mg/kg/day) 4 weeks after the AAA induction and at the start of AAA induction, respectively. The dose of melatonin is based on our previous studies where melatonin exerted anti-inflammatory and antioxidant effects.[15-17] Melatonin was freshly prepared and given at the same time (1800 hours) every day. The control and AAA group received vehicle (1% alcohol in saline [1 mL/kg]) for 2 weeks after sham operation and AAA induction, respectively. Melatonin was purchased from Sigma-Aldrich (St. Louis, Mo).

AAA induction. A gauze presoaked in 0.5 mol/L $CaCl_2$ was directly applied to the adventitia of the infrarenal abdominal aorta for 15 minutes.[18] The abdominal cavity was washed with warm sterile saline before being closed with 2-0 silk suture.

Measurement of aortic size via fluorescence angiography. Angiography is used in the definitive diagnosis of AAA, so the aim of using angiography was to observe whether the aneurysm had formed. The formation of aneurysm and the diameter changes were evaluated by using the data derived from angiography. Aortic wall thrombus formation was examined histologically. All rats were scanned with florescence angiography at the beginning, at 4 weeks, and at the end of 6 weeks. To measure the infrarenal aorta diameter, rats were placed supine, anesthetized with ketamine (100 mg/kg) and chlorpromazine (30-50 mg/kg) and underwent laparotomy. Intestines were deviated on the right, and the abdominal aorta was isolated with blunt dissection.

After the infrarenal aorta and inferior vena cava were isolated, indocyanine green (0.25 mg/kg) was injected through the inferior vena cava, and then the abdominal aorta was scanned with florescence angiography using the SPY Elite Imaging System (NOVADAQ, Bonita Springs, Fla). The infrarenal aorta diameter was measured, and the dilation ratio (%) was calculated according to the following formula described by Morimoto et al[19]: dilatation ratio (%) = (maximal aneurysm diameter/native aortic diameter) × 100.

Western blot analysis. MMP2, MMP9, tumor necrosis factor (TNF)-α, and inducible nitric oxide synthase (iNOS) protein expression were measured by Western blotting. Samples were homogenized in cell lysis buffer, and protein concentrations were determined using the Bradford method.[20] Samples resolved by 4% to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis were transferred to polyvinylidene fluoride membrane, which was blocked with bovine serum albumin. The membrane was incubated overnight with primary antibody (1:500 dilution anti-MMP2 sc-10736, anti-MMP9 sc-10737, anti-iNOS sc-651, anti-TNF- α sc-1351, anti-β-actin sc-47778; Santa Cruz Biotechnology, Heidelberg, Germany) and washed with Tris-buffered saline containing 0.1% Tween-20. The membrane was washed and then incubated with horseradish peroxidase-conjugated secondary antibody for 2 hours. The blot was developed with chemiluminescence reagents and exposed to film. Data were analyzed using ImageJ Programme Optical Density Analysis Software (National Institutes of Health, Bethesda, Md). Signals were normalized with respect to β-actin.

Tissue 8-hydroxy-2′-deoxyguanosine levels measurement. Tissue samples were collected and genomic DNA from tissue was immediately extracted using commercial DNA extraction kit according to the manufacture's protocol (Invitrogen, Frederick, Md). Measurement of tissue 8-hydroxy-2′-deoxyguanosine (8-OHdG) levels was performed by competitive enzyme-linked immunosorbent assay using the OxiSelect Oxidative DNA Damage ELISA kit (Cell Biolabs, Inc, San Diego, Calif), as stated in the manufacturer's instructions.

Measurement of tissue caspase-3 activity. Caspase-3 activity assay was performed using the caspase-3 cellular activity assay kit (Calbiochem, San Diego, Calif),

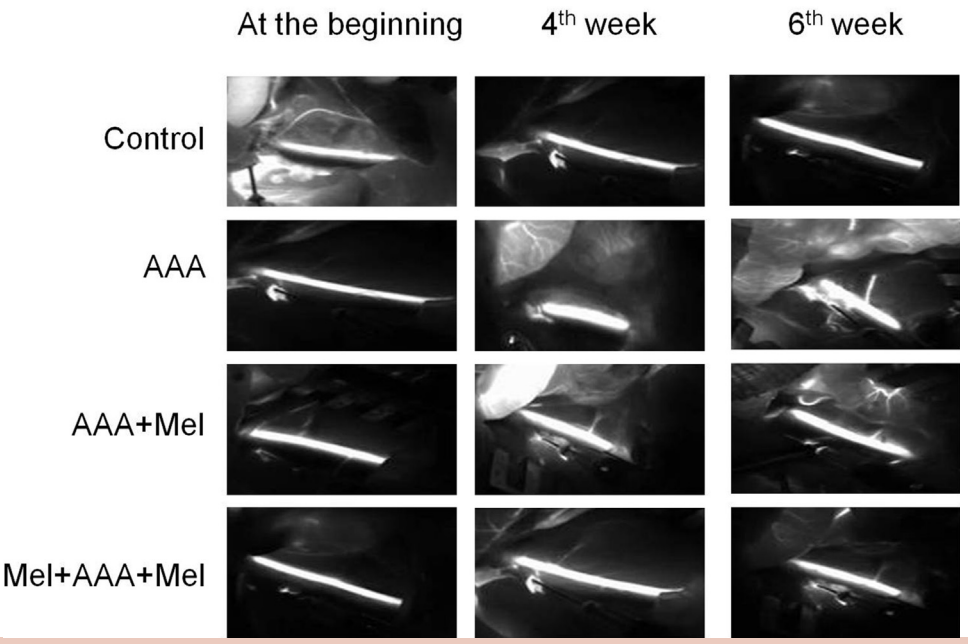

Fig 1. Fluorescence angiograms obtained at the beginning and at 4 and 6 weeks of aneurysm formation (original magnification × 300). *AAA*, Abdominal aortic aneurysm; *Mel*, melatonin.

according to the manufacturer's instructions. The colorimetric release of *p*-nitroaniline (pNA) from the Ac-DEVD-pNA substrate was recorded from 0 to 60 minutes at 405 nm using a specific activity of DEVD-pNA cleavage (pmol pNA/min) for each sample. The DEVD-pNA cleavage activity was calculated as pmol/min/mg protein. Protein concentration in tissue samples was determined using Bradford method.[20]

Measurement of tissue malondialdehyde and glutathione levels. Aortic tissue samples were homogenized with ice-cold 150 mmol/L KCl for the determination of malondialdehyde (MDA) and glutathione (GSH) levels. The MDA levels were assayed as products of lipid peroxidation by monitoring thiobarbituric acid reactive substance formation as described previously, and the results are expressed as nmol MDA/g tissue.[21] GSH measurements were performed using a modification of the Ellman procedure, and the results are expressed in μmol GSH/g tissue.[22]

Measurement of tissue myeloperoxidase activity. Myeloperoxidase (MPO) activity in aortic tissues was measured by a procedure similar to that described by Hillegas et al.[23] One unit of enzyme activity was defined as the amount of MPO present that caused a change in absorbance, measured at 460 nm for 3 minutes. MPO activity is expressed as U/g tissue.[23]

Histologic analysis. For the light microscopic investigations, aortic tissue samples were fixed with 10% formaldehyde, dehydrated in graded alcohol series, cleared in toluene, and embedded in paraffin. Tissue sections (5 μm) were stained with hematoxylin and eosin and examined under an Olympus BX51 photomicroscope (Tokyo, Japan).

Table. Dilatation ratio[a] for abdominal aortic aneurysm (*AAA*) and melatonin (*Mel*)-treated groups

Group	Week 4 Mean ± SEM, %	Week 6 Mean ± SEM , %
AAA	158.2 ± 9.6	173.8 ± 7.5
AAA+Mel	154.8 ± 6.6	132.4 ± 3.2[b]
Mel+AAA+Mel	115.4 ± 6.6[c,d]	113,0 ± 1.8[b,e]

SEM, Standard error of the mean.
[a]Dilatation ratio (%) = (maximal aneurysm diameter/native aortic diameter) × 100.
[b]$P < .001$ vs AAA (obtained at the same time point).
[c]$P < .01$ vs AAA (obtained at the same time point).
[d]$P < .01$ vs AAA+Mel group (obtained at the same time point).
[e]$P < .05$ vs AAA+Mel group (obtained at the same time point).

Statistics. Statistical analysis was performed using GraphPad Prism 4.0 software (GraphPad Software, La Jolla, Calif). Data are expressed as the mean ± standard error of the mean. Groups of biochemical data were compared by analysis of variance, followed by the Tukey multiple comparison tests. $P < .05$ was considered statistically significant. The Mann-Whitney *U* test was used to compare histologic scores.

RESULTS

Abdominal aorta fluorescein angiography results. Dilatation ratios were calculated using aorta diameters that were obtained using fluorescence angiography shown

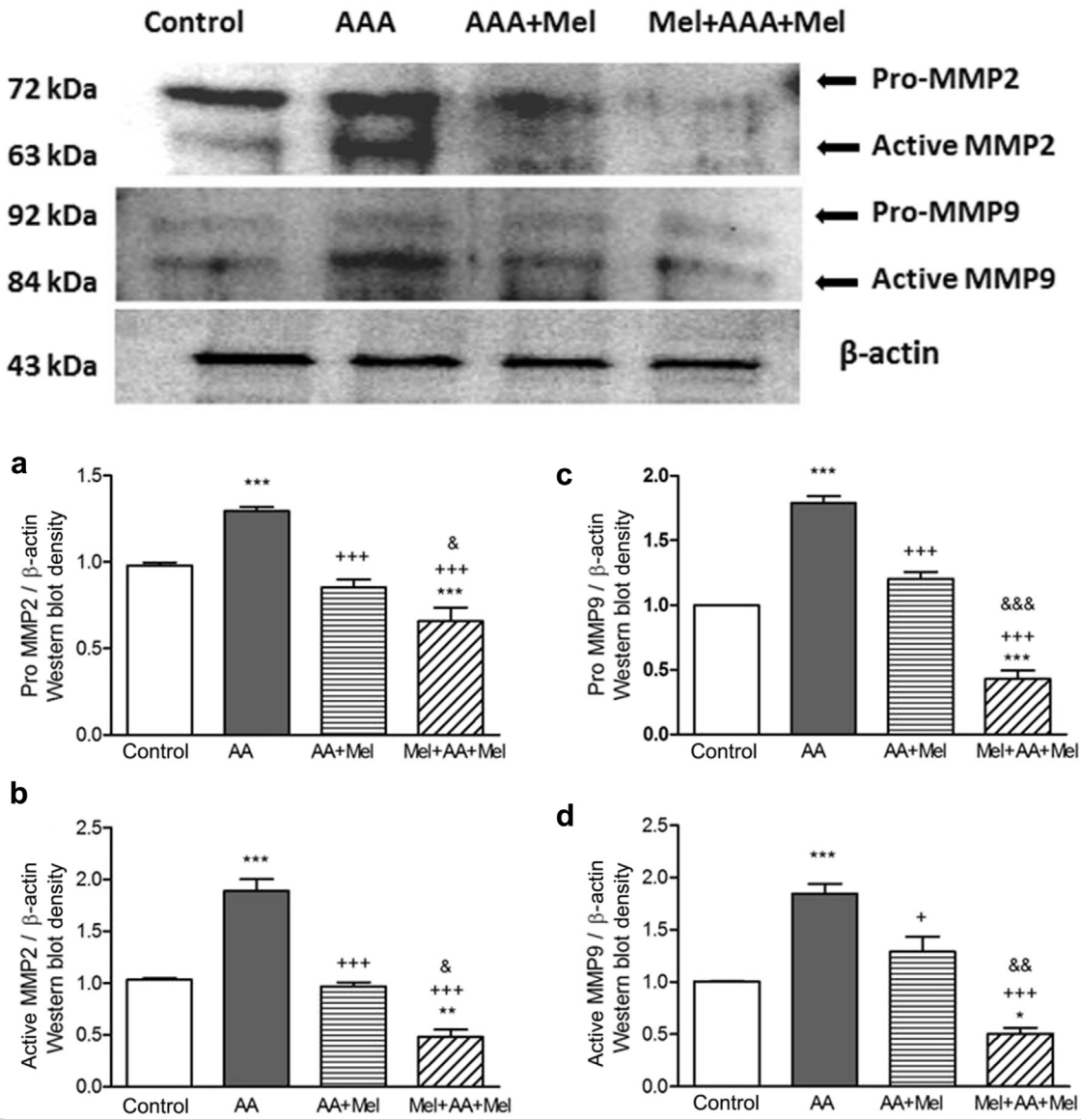

Fig 2. Top, Western blot of aortic tissue and qualitative analysis of **(a)** pro and **(b)** active matrix metalloproteinase (*MMP*) 2, and **(c)** pro and **(d)** active MMP9 in aortic tissues. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{*}P < .05$, $^{**}P < .01$, $^{***}P < .001$ vs control group; $^{+}P < .05$, $^{+++}P < .001$ vs vehicle-treated AAA group; $^{\&}P < .05$, $^{\&\&}P < .01$, $^{\&\&\&}P < .001$ vs AAA+Mel group.

in Fig 1. The dilatation ratios of the Mel+AAA+Mel group at 4 and 6 weeks and of the AAA+Mel group at 6 weeks were significantly lower than that of the AAA group. Furthermore, the aortic dilatation ratio of the Mel +AAA+Mel group was significantly lower at 4 and 6 weeks compared with the AAA+Mel group (Table).

Western blot analysis of MMP-2, MMP-9, TNF-α, and iNOS. Semiquantitative Western blot analysis revealed that MMP-2 and MMP-9 protein expression was significantly increased in aortic tissues in the AAA group and that melatonin treatment significantly reversed these increases ($P < .001$). However, when melatonin was given concurrently with $CaCl_2$, the decrease in both MMPs was more pronounced ($P < .01$; Fig 2). Similarly, the increased protein expressions of TNF- α and iNOS were reduced in the melatonin treatment groups, demonstrating both the therapeutic and prophylactic effect of melatonin ($P < .001$; Fig 3).

Caspase-3 activity and 8-OHdG levels. As a result of oxidative stress-induced apoptosis in the $CaCl_2$-induced AAA rats, caspase-3 activity in infrarenal aortic tissues was significantly higher than in that of controls. However, although both melatonin treatments significantly reduced the enzyme activity ($P < .05$ to $P < .001$),

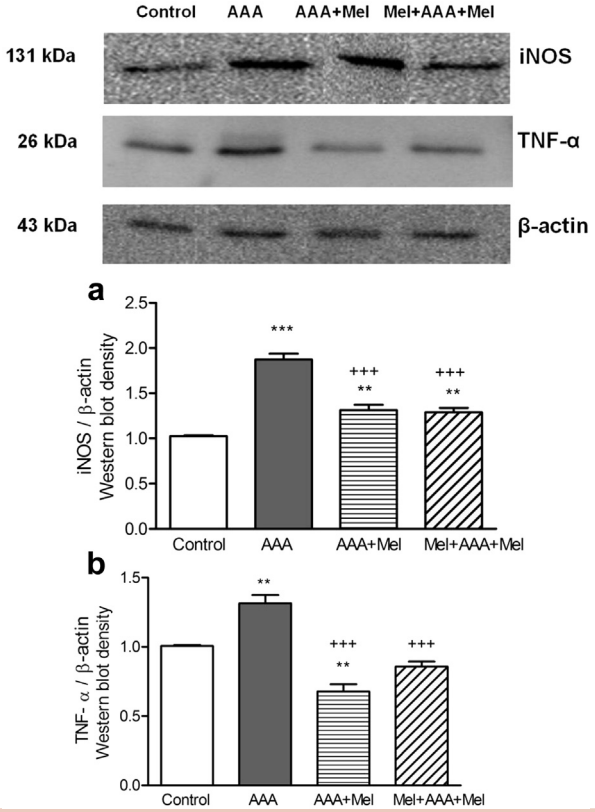

Fig 3. Top, Western blot of aortic tissue and qualitative analysis of **(a)** inducible nitric oxide synthase (*iNOS*) and **(b)** tumor necrosis factor (*TNF*)-α. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{**}P < .01$, $^{***}P < .001$ vs control group; $^{+++}P < .001$ vs vehicle-treated AAA group.

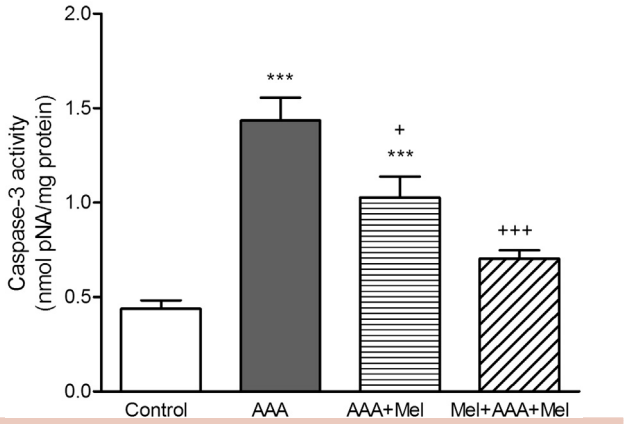

Fig 4. Caspase-3 activities in aortic tissues of groups. Each group consists of ten rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{***}P < .001$ vs control group; $^{+}P < .05$, $^{+++}P < .001$ vs vehicle-treated AAA group.

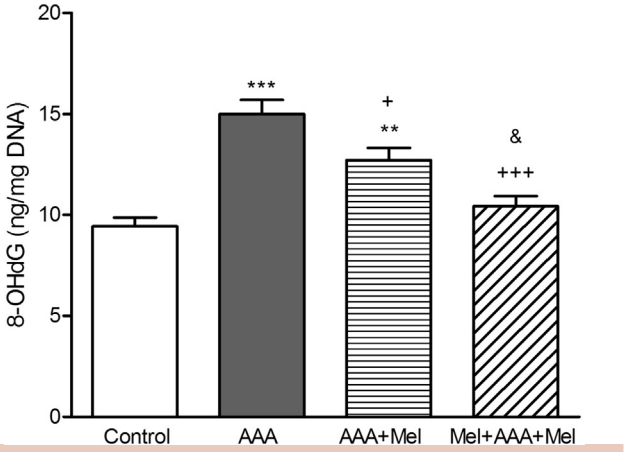

Fig 5. Oxidative DNA damage measured by 8-hydroxy-2′-deoxyguanosine (*8-OHdG*) in aortic tissues of the study groups. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{**}P < .01$, $^{***}P < .001$ vs control group; $^{+}P < .05$, $^{+++}P < .001$ vs vehicle-treated AAA group; $^{\&}P < .05$ vs AAA+Mel group.

caspase-3 activity was still higher in the AAA+Mel group ($P < .001$; Fig 4).

An index of oxidative DNA damage, 8-OHdG levels were significantly increased ($P < .001$) in the aortic tissues of rats with AAA. Melatonin treatment in the AAA+Mel and Mel+AAA+Mel groups resulted in decreased 8-OHdG levels ($P < .05$ and $P < .001$, respectively). However, a comparison of the two melatonin treatment groups showed 8-OHdG levels in the Mel+AAA+Mel group were significantly lower ($P < .05$) than in the AAA+Mel group (Fig 5).

Aortic MDA and GSH levels and MPO activity. As expected, $CaCl_2$ caused a significant increase in MDA levels and MPO activity ($P < .001$), along with a concomitant decrease in endogenous GSH levels ($P < .001$), whereas melatonin treatment reversed these changes ($P < .05$ to $P < .001$), demonstrating antioxidant and anti-inflammatory activity. Furthermore, the inhibition of MPO activity by melatonin in the Mel+AAA+Mel group was significantly higher ($P < .05$) compared with the AAA+Mel group (Figs 6-8).

Histology. Control aorta revealed a regular morphology with intima, media, and adventitia (Fig 9, *a*), whereas in the AAA group, there was detachment in the media layer that was a result of the formed thrombosis (Fig 9, *b*), and leukocyte accumulation was observed in the media and adventitia layers. In the Mel+AAA+Mel group, the reduced concentration of the leukocytes in the media layer and reduced layout of collagen and elastic fibers in the intima layer were prominent (Fig 9, *c*). Regeneration of the media layer was observed in the AAA+Mel group, but there was a thrombus that detached the media layer (Fig 9, *d*).

DISCUSSION

$CaCl_2$ caused an increase in oxidative damage, apoptosis, and MMPs along with an increase in TNF-α

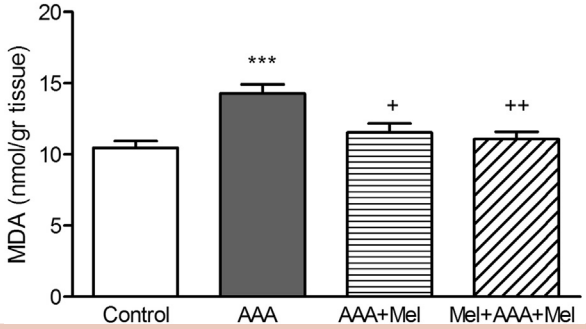

Fig 6. Malondialdehyde (*MDA*) levels in aortic tissues of groups. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{***}P < .001$ vs control group; $^{+}P < .05$, $^{++}P < .01$ vs vehicle-treated AAA group.

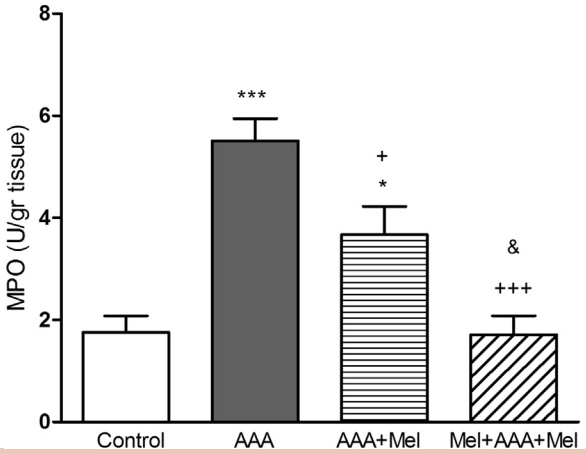

Fig 7. Myeloperoxidase (*MPO*) activity in aortic tissues of groups. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{*}P < .05$, $^{***}P < .001$ vs control group; $^{+}P < .05$, $^{+++}P < .001$ vs vehicle-treated AAA group; $^{\&}P < .05$ vs AAA+Mel group.

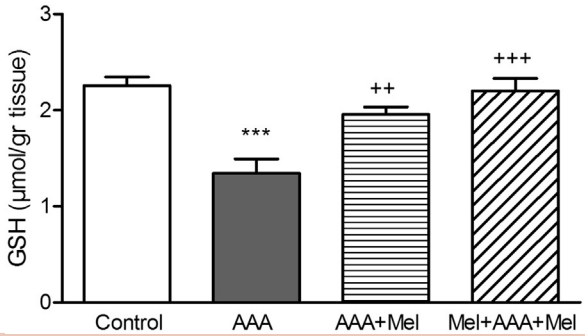

Fig 8. Glutathione (*GSH*) levels in aortic tissues of groups. Each group consists of 10 rats. The *range bars* show the standard error of the mean. *AAA,* Abdominal aortic aneurysm; *Mel,* melatonin. $^{***}P < .001$ vs control group; $^{++}P < .01$, $^{+++}P < .001$ vs vehicle-treated AAA group.

and iNOS expression in the aortic tissues, and GSH was depleted. However, melatonin treatment effectively reversed these changes, suggesting that melatonin might be an appropriate preventative treatment for AAA.

Various etiologic factors cause the formation of AAA. An imbalance between ROS generation and antioxidant capacity, and increased inflammation, just like in many other chronic diseases, also play a role in AAA.[24] MMPs are known to be important in the regulation of inflammatory mediators, with effects on physical barriers. Entry of leukocytes into inflammatory areas takes place via MMP-mediated degradation of matrix proteins. In the case of an aneurysm, MMPs, arising from activated vascular cells or inflammatory cells, lead to elastic lamina degradation.[6,25] Furthermore , elastic lamina cannot be restored once degraded because of the inability of adult cells to remodel elastic fibers.[26] Accordingly, to reverse the aneurysmal state of the aorta to its healthy state, the inflammatory enzyme activity should be decreased to facilitate elastic lamina regeneration along with prevention of degradation. Thus, control of inflammation is essential to stop the progression of an aneurysm. Because scavenging of free radicals is thought to reduce the inflammatory response, antioxidants are believed to be beneficial against the aneurysmal state.

$CaCl_2$-induced aneurysm models have been widely accepted.[18] MMP activities in the aortic tissues of rats with $CaCl_2$-induced aneurysms were seen to increase just like in clinical cases.[27] In the study of Shang et al,[28] vitamin C decreased the aneurysm diameter by 25.8% in an elastase infusion AAA model. Wang et al[29] similarly showed the protective effects of quercetin in aneurysm formation and decreased aneurysmal sac diameter through inhibition of MMP and oxidative stress. Furthermore, resveratrol, a potent antioxidant agent, decreased oxidative stress, inflammatory response, and extracellular matrix destruction in $CaCl_2$-induced AAA models.[30] However, rats lacking MMP9 cannot form an aneurysm.[27]

MMP2 and MMP9 levels were evaluated in our study to show melatonin's effectiveness in reducing the inflammatory response. An increase in the expression of both pro and active MMP2 and MMP9 of the AAA group demonstrated tissue injury, whereas expression was reduced in the melatonin-treated groups, in which the reduction of MMPs in the Mel+AAA+Mel group was more pronounced, indicating prevention of inflammation coinciding with aneurysm. No study to date has showed melatonin's effect on aneurysms. However, Wang et al[31] were able to demonstrate that inflammatory cytokines, including MMP9, were elevated in the aorta endothelial tissue in pinealectomized rats. Our results are in agreement with the Wang et al[31] study, because in our study, melatonin treatment effectively reduced MMP expression.

TNF-α plays a key role in the inflammatory response through release of proteases that weaken the aortic

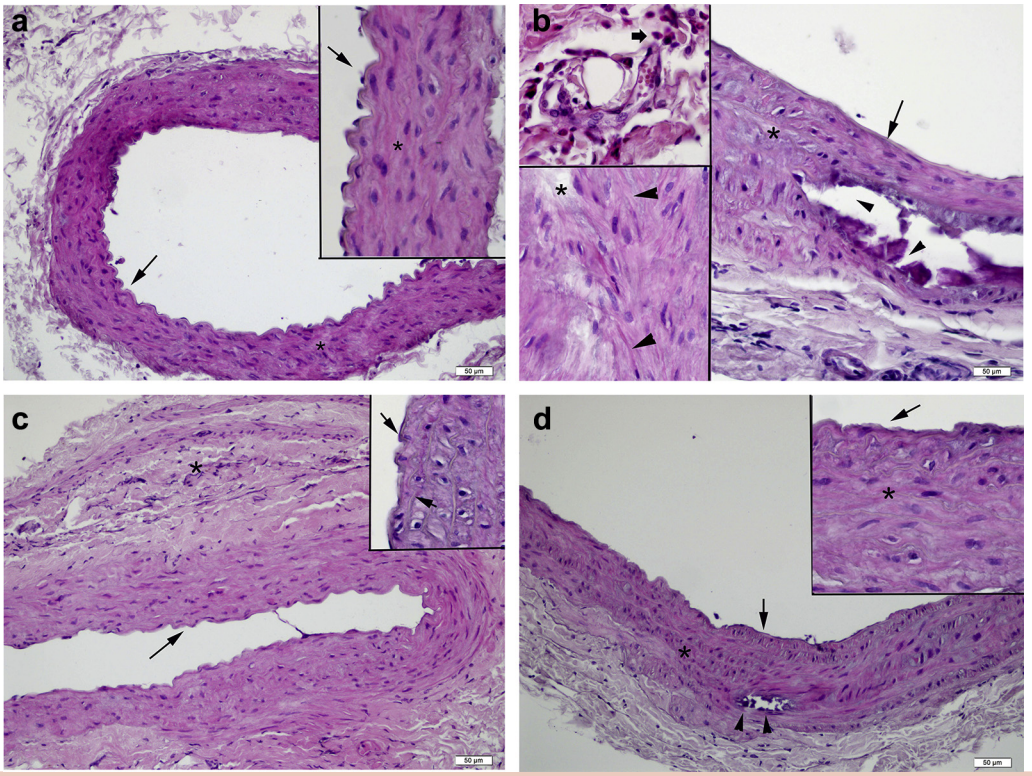

Fig 9. **a,** Control group: regular layout of intima (*arrow*) and media layers (*). **b,** Aortic aneurysm group (AAA): the undulation of lamina elastica was observed as a linear morphology in the intima layer (*arrow*), the detachment in the media layer as a result of thrombosis (*arrowheads*), edema in the media (*), the displaced elastic fibers (*arrowheads, lower inset*), and leukocytes (*bold arrow, upper inset*). **c,** Melatonin (Mel) + AAA group: the reorganization of undulation of lamina elastica (*arrow*) and rearrangement of elastic fibers in intima and media layers (*arrowheads, inset*), adventitia (*). **d,** AAA+Mel: although the overall morphology revealed regeneration there were minimal detachments in the media because of thrombosis (*arrowheads*), the linear morphology of elastic lamina demonstrated undulation (*arrow*), and there was regeneration of elastic fibers in the media (*).

matrix and cause dilation; indeed, TNF-α antagonists and TNF-α binding protein have been shown to prevent the formation of AAA.[32] Antioxidants that have anti-inflammatory properties, such as edevarone, vitamin C, and resveratrol, have also been shown to attenuate aneurysm formation by decreasing TNF-α levels.[19,28,30] In a rat model of chronic intermittent hypoxia, Hung et al[33] demonstrated that melatonin treatment depressed nicotinamide adenine dinucleotide phosphate-oxidase expression and that proinflammatory mediators protected vascular endothelium and ameliorated systemic hypertension. Similarly, melatonin prevented inflammatory cell infiltration and stenosis of the carotid artery lumen induced by exposure to cigarette smoke, demonstrating its therapeutic ability against vascular injury.[34]

Our previous studies proved melatonin had protective effects on the cardiovascular system through anti-inflammatory properties.[35] In our study, one inflammatory determinant of aneurysm, TNF-α levels, were significantly elevated in aortic tissue of rats with AAA. However, melatonin given concomitantly with $CaCl_2$ reduced $CaCl_2$-induced inflammation and protected against aneurysm development. Similarly, $CaCl_2$-induced inflammation causes an increase in iNOS, whereas melatonin treatment also reduced these inflammatory responses.

Inflammatory cells are known to produce ROS. Accumulation of highly reactive ROS triggers subsequent damaging events, including formation of 8-OHdG and lipid peroxidation.[36] In the present study, the levels of 8-OHdG were increased markedly in the aortic tissues of rats with aneurysm, suggesting that DNA is one of the targets for $CaCl_2$-induced aortic tissue damage. Our results are supported by the study of Morimoto et al,[19] who showed increased numbers of 8-OHdG-positive cells in aneurysm wall through immunohistochemical analysis. They also showed a significant decrease in 8-OHdG by treatment with the antioxidant agent edevarone.[19]

Similarly, alogliptin, a dipeptidyl peptidase-4 inhibitor and also riboflavin, by decreasing ROS generation, inhibit oxidative DNA damage (8-OHdG-positive cells) in the aortic wall through their antioxidant effects.[24,36] In

agreement with these studies, in the present study, melatonin, a powerful free radical scavenger, significantly reduced 8-OHdG levels alongside a reduction in aneurysm formation.

Melatonin's ability to penetrate into the cell nucleus makes it much more effective in protecting DNA against oxidative damages than other antioxidants.[37] Very limited knowledge is available in the literature on melatonin's protective effect against oxidative DNA damage in aortic tissues in vivo. However, an in vitro study of peroxynitrite-induced injury in aortic smooth muscular cells showed melatonin decreased the resulting DNA damage.[38]

In addition to the aforementioned indicators, owing to the role of apoptosis in the pathogenesis of aneurysms, we analyzed caspase-3 levels as an apoptotic marker in the aortic tissues.[30] Our study demonstrated that the $CaCl_2$-induced aneurysm is associated with increased caspase-3 levels and that melatonin treatment effectively reduced these levels.

Free radical-mediated lipid peroxidation causes degradation of polyunsaturated fatty acids of cell membranes, leading to breakdown. As a result, membrane fluidity and permeability changes along with enhanced rates of protein degradation, which leads to cell lysis. Our study showed that $CaCl_2$ caused oxidative injury in aortic tissues because MDA, an index of lipid peroxidation, was significantly increased in the AAA group. However, MDA levels significantly decrease when melatonin is given as both preventative and therapeutic treatment. These results are in agreement with our previous studies of melatonin's effects on MDA in free radical-mediated oxidative injury. In our previous studies, increases in MDA levels in aortic tissues induced by cholesterol accumulation, chronic nicotine administration, and diabetes were decreased by melatonin treatment.[16,17,39]

MPO is an essential enzyme for normal neutrophil function, and when neutrophils are stimulated by various stimulants, MPO and other tissue-damaging substances are released from these cells. MPO is thus an index of neutrophil infiltration.[40] In this study, MPO activity was increased after $CaCl_2$ application, demonstrating the role of neutrophils during aneurysm formation, and melatonin treatment markedly reduced this inflammation through its anti-inflammatory effects. In accordance with the biochemical measures, histologically evident extensive leukocyte infiltration in the vehicle-treated AAA group was significantly reduced in the melatonin-treated groups.

Depletion of tissue GSH causes an increase in susceptibility of the cell to oxidative attack. As seen in the present study, $CaCl_2$-induced oxidative stress reduced tissue GSH levels, and melatonin replenished these antioxidant levels. In addition to its free radical scavenging activities, melatonin increases its efficiency by stimulating the antioxidative enzymes GSH peroxidase and GSH reductase, which are critical constituents of the GSH-redox cycle. Moreover, its stimulatory effect on γ-glutamylcysteine synthetase, an important player in both the synthesis and recycling of GSH in the cell, has an essential role in the maintenance of GSH.[41]

Our study has limitations. First, this study lacked a sham-operated group where effects of melatonin alone could have been investigated. This setup will be included in our future studies to reveal the effects of melatonin alone on aortic diameter.

CONCLUSIONS

This study demonstrates for the first time that melatonin, by reducing $CaCl_2$-induced inflammation and oxidative damage in aortic tissues, reduced aneurysm formation. Furthermore, the preventive effect of this powerful antioxidant can be attributed to its ability to balance oxidant-antioxidant status, to inhibit neutrophil infiltration, and to regulate the inflammatory mediators, which were all based on its antioxidant and anti-inflammatory activities. The observed beneficial effects of melatonin were not mediated by its receptors; however, those effects were based on its antioxidant and anti-inflammatory activities, which are dependent on its molecular structure and properties. Consequently, melatonin has no toxicity, and clinical research should be conducted in individuals who have risk factors for AAA development. Thus, melatonin treatment seems to be a promising supplement for the prevention of AAA development in patients with high risk.

AUTHOR CONTRIBUTIONS

Conception and design: GT, Sİ, GŞ, OD, SA, AÇ
Analysis and interpretation: GT, GŞ, ÖÇ
Data collection: GT, GŞ, ÖÇ, ŞÇ,
Writing the article: GT, Sİ, GŞ, ÖÇ, ŞÇ
Critical revision of the article: GT, Sİ, GŞ, OD, SA, AÇ
Final approval of the article: GT, Sİ, GŞ, ÖÇ, ŞÇ, OD, SA, AÇ
Statistical analysis: GT, GŞ, ÖÇ
Obtained funding: GT, Sİ
Overall responsibility: GT
GT and Sİ contributed equally to this article and share co-first authorship.

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Submitted Sep 10, 2015; accepted Jun 5, 2016.