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Melatonin Modulates Lipid Metabolism and Reduces Cardiovascular Risk in Apolipoprotein E-Deficient Mice Fed a Western Diet

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Abstract: Melatonin (MLT), a natural compound found in the animal and vegetable kingdom, participates in several physiological processes. MLT exerts antioxidant and anti-inflammatory activities, among others, but information about its action on lipid metabolism is still scarce. For this reason, mice deficient in apolipoprotein E (ApoE$^{-/-}$) fed a Western diet (WD) were intragastrically treated with different concentrations of MLT (2 and 9 mg/kg) for 12 weeks. The lipid parameters were quantified, and, since links between cardiovascular risk and immune function and oxidative stress have been established, we also analyzed the population of leukocytes and the oxidative stress status. Although there was no change in the weight of the mice, a significant reduction in low-density lipoprotein cholesterol (LDL-C) was observed in mice treated with the higher concentration of MLT tested in this study. Additionally, an improvement in cardiovascular risk indexes was observed. A reduction in the hepatic total cholesterol (TC) and LDL-C levels was also observed in the treated mice. Finally, a decrease in leukocytes and lymphocytes in particular, as well as an increase in the antioxidant status, were shown in MLT-treated mice. In conclusion, MLT is a promising candidate that could be considered as a possible functional ingredient capable of preventing cardiovascular risk.

Keywords: ApoE; cardiovascular diseases; hypocholesterolemia; LDL; leukocytes

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1. Introduction

Cardiovascular diseases (CVDs), including coronary, rheumatic and congenital heart, cerebrovascular, and peripheral arterial diseases, are the leading cause of mortality worldwide (being responsible for 32% of all global deaths in 2019) and a major contributor to disability [1]. Furthermore, CVDs remain a major cause of rising health care costs, and their prevalence nearly doubled from 271 million in 1990 to 523 million in 2019 [2]. The relationship between cholesterol and CVDs has been reported by several epidemiological studies [3–5]. Low-density lipoprotein cholesterol (LDL-C) levels, also called 'bad cholesterol' as it is the main cholesterol-carrying particle in plasma, are directly associated with the subsequent risk of CVDs, due to LDL-C's ability to be deposited in the intima vessels and thus cause obstruction [6]. In contrast, increasing high-density lipoprotein cholesterol (HDL-C) levels has been accepted as a therapeutic strategy to reduce the risk of death from CVDs because HDL-C transports cholesterol from tissues to the liver, reducing the serum values [7]. However, studies have shown that when conventional lipid parameters remain apparently normal or moderately high, lipid relationships such as the Castelli risk

index (CRI) I (CRI-I, considered as the ratio between total cholesterol -TC- and HDL-C) and II (CRI-II, considered as the LDL-C/HDL-C ratio), and the atherogenic index of plasma (AIP, considered as the logarithm of the ratio between triglycerides -TGs- and HDL-C) are diagnostic alternatives for the prediction of cardiovascular events [8–10] and the effectiveness of therapy [11]. Specifically, the CRI-I has been associated with the formation of coronary plaques [12,13], and the CRI-II has been shown to be a predictor of cardiovascular risk [14]. Regarding the AIP, previous studies have correlated high values of this index to the risk of cardiovascular incidence and all-cause mortality in patients with coronary heart disease [15–17].

Alternatively, an elevated count of white blood cells serves as a robust and autonomous indicator of the risk of heart-related issues in individuals of all genders, whether they have coronary heart disease or not. Furthermore, an increased quantity of leukocytes is linked to the occurrence of coronary heart disease, peripheral arterial conditions, and strokes [18]. Furthermore, alterations in lipid metabolism are closely related to alterations in the immune system and prevailing chronic systemic inflammation in people with a pathological increase in body fat [19]. Furthermore, a rise in lymphocyte count has been observed in conditions like obesity, diabetes, and cardiovascular disease. Lymphocytes are notably elevated in visceral fat, playing a significant role as a key controller of insulin resistance. [20]. For this reason, the relationship between nutritional status and immune functions has been widely studied over the years, associating malnutrition with pathological conditions [21,22]. In such studies, overweight mice fed a Western diet (WD) have shown an increase in the total number of leukocytes and lymphocyte cell numbers [23,24].

Furthermore, oxidative stress has been widely highlighted as a major risk factor in the development of the main cardiovascular diseases. This is an imbalance between the production of reactive oxygen species (ROSs) and antioxidant defenses caused by certain cardiovascular risk factors, such as diabetes, hypertension, smoking, and obesity [25]. At the cardiovascular level, oxidative stress is highly implicated in myocardial infarction, ischemia/reperfusion, and heart failure due to damage to the vascular system through lipid peroxidation, membrane damage, immune cell activation (proteases, nucleases, and protein kinases), structural remodeling, or inflammation [26].

Melatonin (MLT) is considered a pleiotropic molecule due to its participation in various physiological processes. This particular indoleamine is primarily produced by the pineal gland in accordance with a daily cycle, reaching its highest levels at nighttime and the lowest levels during the daytime in humans [27]. In recent decades, this molecule has been shown to not be exclusively synthesized in the pineal gland but also in several peripheral tissues, such as the gastrointestinal tract, immune system cells, and skin cells [28,29]. Unlike studies on its antioxidant role, studies on the effect of MLT on the regulation of lipid metabolism are scarce. Previous studies have shown that MLT can reduce serum levels of TC, LDL-C, HDL-C, and TGs. However, the conclusions obtained are variable and depend on the methodology used [30–33]; thus, more studies are required to determine the benefits of MLT for the lipid profile [34].

Furthermore, the immunomodulatory role of MLT has been extensively studied in recent years, and its efficacy in controlling inflammatory processes has been demonstrated in different diseases [29,35–37]. Previous studies have reported that MLT therapy decreases the number of leukocytes in burn-induced Wistar rats [38] and controls the count of leukocytes and lymphocytes during intense effort in adolescent athletes [39]. However, there is no evidence of the anti-inflammatory role of MLT in an animal model of cardiovascular risk.

Given that growing evidence has shown that MLT might exert a cardiovascular protective effect through the control of lipid metabolism and anti-inflammatory and antioxidant status, the aim of this study was to evaluate the effect of MLT on (i) the plasmatic and hepatic lipid profile, (ii) white blood cell population, and (iii) anti-inflammatory and oxidative stress status in an experimental model of cardiovascular disease consisting of apolipoprotein E (ApoE) knockout mice ($ApoE^{-/-}$) fed a WD.

2. Materials and Methods

2.1. Study Design

Male ApoE$^{-/-}$ mice (kindly donated by Dr. Antonio Ordoñez and Dr. Raquel del Toro), at the age of four weeks, were kept in the animal facility at the Instituto de Biomedicina de Sevilla (IBiS) under typical conditions, which included a 12 h light and 12 h dark cycle, a temperature of 22 ± 2 °C, and humidity levels below 55%. These mice were provided with unrestricted access to both water and a Western diet (Test Diet 58v8, containing 45% energy from fat, as detailed in Supplementary Table S1). When the mice turned 6 weeks old, they were randomly divided into three groups and treated intragastrically with MLT daily (Sigma Aldrich, MO, USA) 2 mg/kg (WD + MLT (2 mg/kg), n = 12), 9 mg/kg (WD + MLT (9 mg/kg), n = 12) or the vehicle (=ethanol; WD, n = 10) for 12 weeks (Figure 1). The mice were closely monitored, controlled, and observed by the researchers themselves and by the technical staff of the IBiS Animal Facility. In addition, the veterinary manager of the animal facility checked the health status of the animals every week, not recording any side effects.

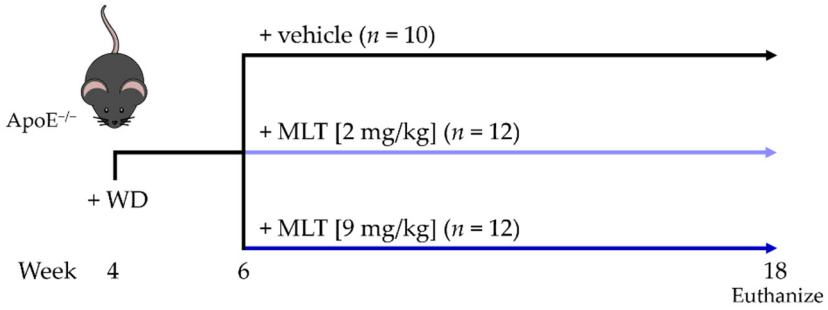

Figure 1. Experimental design of the study. ApoE$^{-/-}$, apolipoprotein E knockout mice; MLT, melatonin; WD, Western diet.

The MLT doses used in this study were chosen because they are equivalent doses of 10 mg and 50 mg MLT/day, respectively, in humans, calculated according to [40] (Figure 2). Daily food intake and individual body weight were measured weekly and recorded. At the endpoint, fasted animals were euthanized, and blood was collected in Minicollect EDTA tubes (Greiner Bio-one, Kremsmünster, Austria) by cardiac puncture. Subsequently, plasma was obtained by centrifugation (3000× g, 4 °C, 10 min) and stored at −20 °C until use. The animals were then perfused with phosphate-buffered saline (PBS) for 5 min using an FH100 peristaltic pump (Thermo Scientific, Vantaa, Finland), and the liver was collected and stored until use.

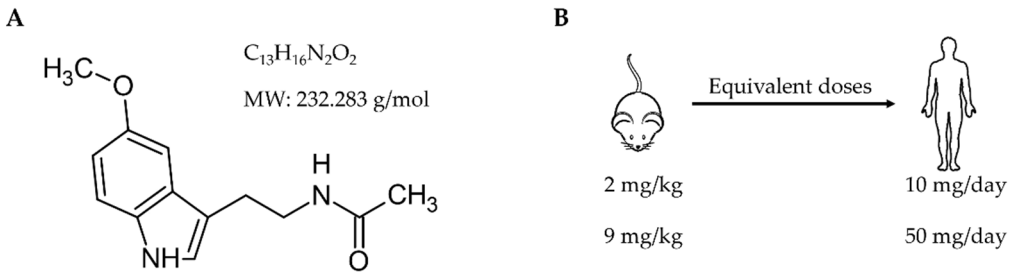

Figure 2. Chemical structure of melatonin ((**A**), CAS number: 73-31-4) and equivalent doses of it in mice and humans (**B**). MW, molecular weight.

Every experimental process adhered to the regulations established by Spanish law and conformed to the guidelines outlined in the EU Directive 2010/63/EU regarding animal experiments. Additionally, these procedures received approval from the Ethics Committee

at the Virgen Macarena and Virgen del Rocío University Hospitals, with reference number 21/06/2016/105.

2.2. Plasma and Hepatic–Lipid Profile

Plasma lipid parameters (including TC, TGs, LDL-C, and HDL-C) were measured with chemiluminescence immunoassay techniques using the COBAS e601 modular analyzer (Roche Diagnostic, Basel, Switzerland). In addition, the cardiovascular disease risk indexes CRI-I, CRI-II, and AIP were calculated according to [41]. On the other hand, 100 mg liver tissues were homogenized with a TissueRuptor (Qiagen, Hilden, Germany), and the hepatic TC, HDL-C, LDL-C, and TG concentrations were measured in the supernatants produced by Cobas Integra 400 (Roche Diagnostics, Indianapolis, IN, USA) at the 'Estación Biológica de Doñana' (EBD-CSIC, Seville, Spain).

2.3. White Blood Cell (WBC) Count

White blood cells were quantified in blood samples using the SYSMEX XE 5000 Hematology Analyzer (Sysmex Europe GmbH, Norderstedt, Germany) fluorescence flow cytometer.

2.4. Plasma ELISA

To confirm that MLT could act as an anti-inflammatory molecule, TNF was quantified by a commercial enzyme-linked immunosorbent assay (BD OptEIA™ Mouse TNF (Mono/Mono) ELISA Set, BD Biosciences, San Jose, CA, USA).

Briefly, the plasma of mice was incubated overnight with anti-mouse TNF antibody in a precoated 96-well plate. A biotinylated anti-mouse TNF antibody and streptavidin–HRP conjugate enzyme were used to detect the TNF cytokine. The addition of tetramethylbenzidine (TMB; Sigma-Aldrich, Saint Louis, MO, USA) led to the development of a color that was read at 450 nm with a CLARIOstar Plus microplate reader (BMG Labtech, Ortenberg, Germany) once the reaction was stopped by HCl.

2.5. Plasma Antioxidant Capacity

To test the role of MLT in the antioxidant capacity, the Trolox equivalent antioxidant capacity (TEAC) assay was performed. Briefly, 140 µL of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS, Sigma-Aldrich) radical solution was mixed with 10 µL of plasma. After 30 min of incubation at 30 °C, the ABTS radical content was quantified using a CLARIOstar Plus microplate reader (BMG Labtech) at 730 nm. Then, the values were extrapolated by a Trolox (Sigma-Aldrich) standard curve.

2.6. Statistical Analysis

The data are presented as the mean value accompanied by the standard error of the mean (SEM). Statistical analysis involved the use of non-parametric Mann–Whitney U tests or two-way ANOVA, followed by post hoc corrections, and statistical significance was established at p-values equal to or less than 0.05. The data underwent analysis using GraphPad Prism v.8 (GraphPad Software, San Diego, CA, USA).

3. Results

3.1. Melatonin Does Not Alter the Body Weight of Mice

As shown in Figure 3A, no significant differences in body weight (p = 0.815) were observed between the three experimental groups throughout the experiment. No differences were shown in basal or final body weight (Figure 3B,C) between the control group (basal: 21.29 ± 0.48 g; final: 26.32 ± 0.45 g) and the groups given 2 mg/kg MLT (basal: 20.38 ± 0.84 g, p = 0.594; final: 26.16 ± 0.82 g, p = 0.987) or 9 mg/kg MLT (basal: 20.37 ± 0.82 g, p = 0.571; final: 26.57 ± 0.87 g, p = 0.959). Additionally, the mice did not show significant differences in body weight gain when treated with MLT at 2 mg/kg (5.78 ± 0.39 g, p = 0.288) and 9 mg/kg (6.23 ± 0.39 g, p = 0.118) compared to the control group (5.03 ± 0.49 g) (Figure 3D).

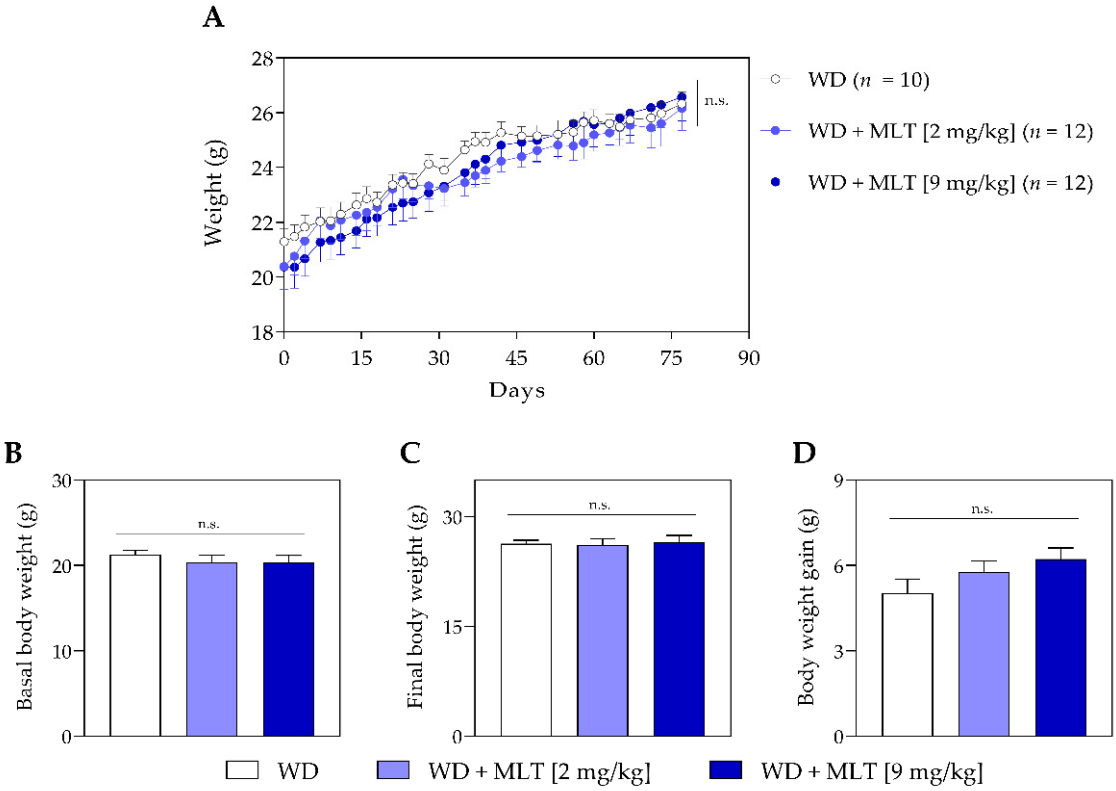

Figure 3. Body weight monitored over time (**A**), basal body weight (**B**), final weight (**C**), and body weight gain (**D**) in the in vivo experiments. Data were represented as mean ± SEM. n.s., not significant. MLT, melatonin; WD, Western diet.

3.2. MLT Improves the Plasmatic Lipid Profile and Reduces the Risk of Cardiovascular Disease

To investigate the lipid-lowering effect of MLT, the plasma lipid profile and the main cardiovascular risk indexes were analyzed. As shown in Table 1, a significant reduction was observed in LDL-C values (−24.6%, $p = 0.008$) in mice treated with 9 mg/kg MLT, compared to the control group. No significant differences were observed in the values of TC, TGs, or HDL-C between the MLT groups compared to the WD group ($p > 0.05$).

Table 1. Plasma lipid profile.

Biochemical Parameter	WD (mg/dL)	WD + MLT (2 mg/kg) (% of Control)	*p*-Value	WD + MLT (9 mg/kg) (% of Control)	*p*-Value
TC	506.10 ± 12.34	106.20 ± 8.81	0.548	87.12 ± 6.59	0.151
TG	99.38 ± 0.72	110.80 ± 14.89	0.706	105.20 ± 9.37	0.683
LDL-C	373.20 ± 36.81	112.20 ± 12.10	0.643	75.43 ± 4.39	0.008
HDL-C	113.70 ± 33.59	103.40 ± 5.41	0.548	111.00 ± 11.16	0.691

Results are expressed as a percentage of the control group and represent the mean ± SEM of each group. TC, total cholesterol; TGs, triglycerides; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; MLT, melatonin.

In addition, cardiovascular risk was evaluated in each group by calculating the indexes CRI I, CRI II, and AIP. Although no differences in CRI I or II were observed at the lowest concentration (2 mg/kg) (104.50 ± 12.27% and 110.80 ± 15.81%, respectively, $p > 0.05$), they were reduced at the highest concentration of MLT (9 mg/kg) (CRI I: 80.35 ± 7.04%, $p = 0.016$ and CRI II: 74.93 ± 6.27%, $p = 0.016$) (Figure 4). In addition, mice treated with MLT showed a reduction in the AIP of 51.74% and 42.31% at both concentrations of MLT tested ($p < 0.05$), respectively (Figure 4).

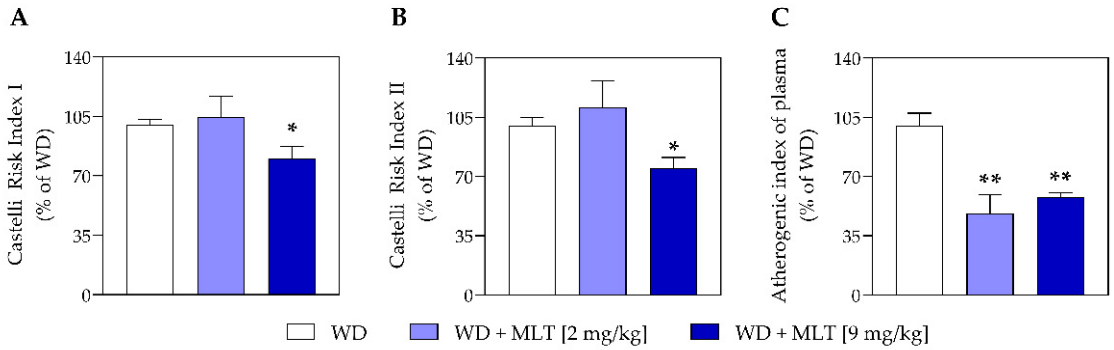

Figure 4. Evaluation of cardiovascular disease risk through Castelli risk index I (TC/HDL-C) (**A**) and II (LDL-C/HDL-C) (**B**) and atherogenic index of plasma (Log(TG/HDL-C)) (**C**). Results are expressed as a percentage of the control group and represent the mean ± SEM of each group. * $p \leq 0.05$, ** $p \leq 0.01$ vs. WD group; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MLT, melatonin; TC, total cholesterol; TGs, triglycerides; WD, Western diet.

3.3. MLT Treatment Decreases Hepatic Lipids

As shown in Figure 5, the levels of TC (Figure 5A) and LDL-C (Figure 5C) were reduced by 21.57 and 14.17%, respectively, after treatment with 9 mg/kg MLT, without significant differences in 2 mg/kg MLT-treated mice. On the other hand, TG (Figure 5B) and HDL-C (Figure 5D) levels remained unchanged for both groups treated with MLT.

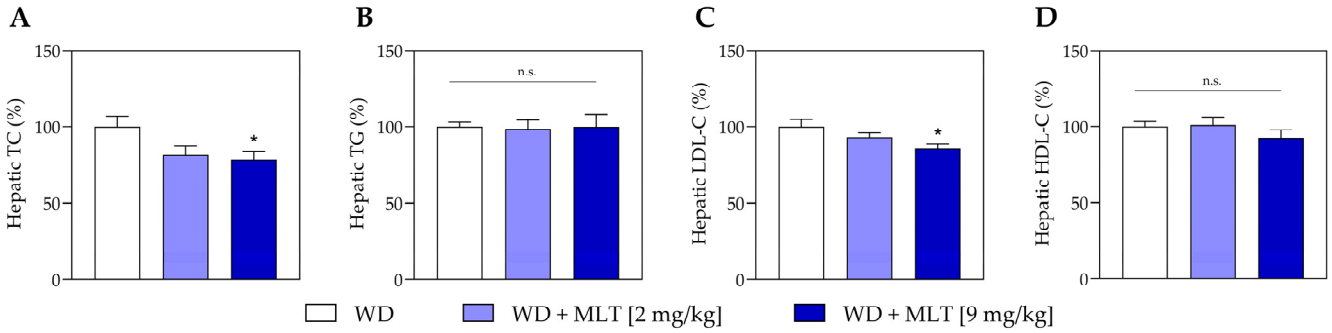

Figure 5. Hepatic TC (**A**), TG (**B**), LDL-C (**C**), and HDL-C (**D**) content in the three experimental groups. Results are expressed as a percentage of the control group and represent the mean ± SEM of each group. * $p \leq 0.05$ vs. WD. n.s., not significant. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; MLT, melatonin; TC, total cholesterol; TGs, triglycerides; WD, Western diet.

3.4. MLT Reduces Lymphocytosis

To determine the immune status after MLT treatment, the total number of leukocytes, as well as their subpopulations (lymphocytes, monocytes, and granulocytes) was quantified in the plasma sample.

As shown in Table 2, although there were no significant differences in the number of leukocytes in the mice treated with 2 mg/kg MLT compared to the control group ($p > 0.05$), the daily ingestion of 9 mg/kg MLT significantly reduced the number of leukocytes (−50.36%, $p = 0.011$). In particular, MLT treatment reduced the lymphocyte populations by 56% ($p = 0.030$), while the monocyte and granulocyte subpopulations were not altered by the MLT treatment.

Table 2. Hemogram.

Cells ($\times 10^3/\mu L$)	WD	MLT (2 mg/kg)	MLT (9 mg/kg)
Leukocytes	1.49 ± 0.21	1.14 ± 0.16	0.74 ± 0.15 *
Lymphocytes	0.99 ± 0.18	0.76 ± 0.14	0.43 ± 0.13 *
Monocytes	0.069 ± 0.024	0.067 ± 0.021	0.062 ± 0.032
Granulocytes	0.37 ± 0.06	0.31 ± 0.03	0.33 ± 0.04

Results are expressed as the number of cells ± SEM of each experimental group. *, $p \leq 0.05$ with respect to the control group. MLT, melatonin; WD, Western diet.

3.5. MLT Reduces the Number of TNF Pro-Inflammatory Cytokines and Improves the Antioxidant Capacity

The immunomodulatory effect of MLT was corroborated by quantifying the pro-inflammatory cytokine TNF in the plasma of mice. As shown in Figure 6A, MLT treatment reduced the TNF concentration in a dose-dependent manner, with a statistical difference for the treatment with MLT 9 mg/kg being observed (a reduction of 52.6%, $p = 0.044$, with respect to the WD group). The antioxidant capacity of MLT was measured by the ABTS radical scavenging assay in plasma. Figure 6B shows a significant increase in the TEAC values in plasma of mice treated with MLT 9mg/kg. Specifically, the treatment increased the TEAC values by 58.5 ± 29.70% compared to the control group (WD). No differences were observed for 2 mg/kg MLT-treated mice.

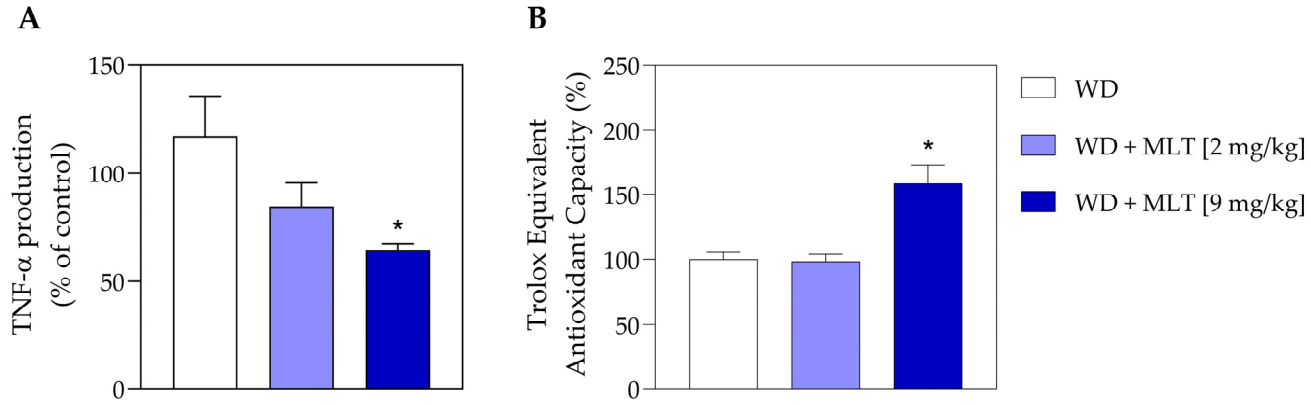

Figure 6. Effect of MLT treatment on plasma anti-inflammatory and antioxidant status evaluated by an enzyme-linked immunosorbent assay (ELISA) (**A**) and the Trolox equivalent antioxidant capacity (TEAC) assay (**B**). Results are expressed as a percentage of the control group and represent the mean ± SEM of each group. * $p \leq 0.05$ with respect to the Western diet (WD) group. MLT, melatonin.

4. Discussion

This study highlights the cardioprotective effect of MLT therapy in an ApoE$^{-/-}$ mouse model characterized by a greater susceptibility to cardiovascular accidents. In fact, ApoE$^{-/-}$ mice display poor lipoprotein clearance with subsequent accumulation of cholesterol ester-enriched particles in the blood, which promotes the development of atherosclerotic plaques [42].

The present study shows, for the first time, that treatment with MLT for 12 weeks reduces the LDL-C concentration and cardiovascular risk indexes in WD-fed ApoE$^{-/-}$ mice. Additionally, MLT treatment decreases the plasma levels of lymphocytes and improves the plasma antioxidant status. The effects of MLT were not related to the body weight gain of the mice, which remained unchanged for the three experimental groups.

High plasma concentrations of TC, TG, and LDL-C, as well as low plasma concentrations of HDL-C, among other things, are risk factors for the onset and progression of CVDs [25,41]. Similarly, a previous study showed that an increase of 1.0 mmol/L in LDL-C was associated with an increased absolute risk of myocardial infarction in individuals

aged 70–100 years [43]. On the contrary, an increase of 1 mg/dL in HDL-C reduces the risk of coronary heart disease by 2% in men and 3% in women [44]. It is remarkable that in the present study, 12 weeks of MLT treatment at 9 mg/kg reduced plasma LDL-C by −24.6%, while HDL-C levels were not altered. Furthermore, a strong association has been shown between CVDs, metabolic dysfunction-associated fatty liver disease (MAFLD), and the accumulation of liver fat (steatosis) [45]. In this sense, the present study shows for the first time that treatment with 9 mg/kg MLT reduced the liver total cholesterol and LDL-C in WD-fed ApoE$^{-/-}$ mice, which is in agreement with previous studies performed in rats [46,47]. Although these previous studies showed the effect of MLT treatment on serum cholesterol and LDL-C concentration [48–50], none of these were performed in a specific model of hypercholesterolemia and cardiovascular disease. In fact, these previous studies were focused on the effect of MLT on a specific increase in LDL-C caused by nicotine administration [51], cigarette smoke [52], diabetes induction [53], or a diet modification [31,54].

In addition, MLT reduces the CRI I, CRI II, and AIP, which are used as optimal indicators of cardiovascular risk [41]. Bhardwaj et al. have reported that CRIs can contribute significantly to the estimation of the risk of coronary artery disease, especially when the absolute values of the plasma lipid parameters do not change markedly [55]. Furthermore, Quispe and colleagues proposed that the CRI I should be considered for additional risk assessment in the primary prevention population, specifically in high-CV-risk individuals, such as patients with diabetes [56]. Regarding the AIP ratio, this is a novel indicator of dyslipidemia, and patients with coronary artery disease (CAD) have significantly higher values compared to healthy controls [57]. Furthermore, the AIP has been described as an independent predictor of CAD [57]. Although it was previously shown that daily treatment of rats fed a high-cholesterol diet with intraperitoneal MLT at 12.5 mg/kg decreased the CRI II [31], there are no previous studies related to the effect of MLT on the CRI I and AIP. Thus, to our knowledge, this is the first study to describe the potential of this molecule to reduce the CRI I and II, and AIP in a mouse model with hypercholesterolemia and cardiovascular risk.

Taking into account the present results, together with previous studies, we suggest that MLT could be a good candidate to prevent the development of CVDs and treat pathologies that cause an imbalance in the lipid profile, such as metabolic syndrome and obesity, among others.

In addition, the role of MLT in the modulation of the immune system has been extensively studied over the last years in different contexts, such as in autoimmune diseases [58], infections [59], and even pathologies associated with metabolic syndrome, including neuroinflammation [60]. Mice fed with WD have an increased number of blood leukocytes and lymphocytes [23]. According to these data, elevated cholesterol levels are widely known to predispose individuals to a pro-inflammatory state through a systemic increase in leukocytes and, to a greater extent, the lymphocytes and soluble mediators secreted by these cells [61]. Furthermore, a previous study has shown that body fat affects the number of circulating leukocytes and lymphocytes in children [24]. In numerous studies, the capacity of MLT to modulate the immune response in different diseases has been described, as well as the association of this molecule with low blood levels of leukocytes and/or lymphocytes [29,62]. Winklewski et al. showed that MLT treatment significantly decreases the number of leukocytes and lymphocytes in ethanol-intoxicated mice [63], and other authors have shown that MLT treatment reduces the number of lymphocytes in animals with zymosan-induced peritonitis [64]. Furthermore, recent studies show the ability of MLT to not only control the number of immune cells such as leukocytes and/or lymphocytes in various pathological situations, but also to modify its pro/anti-inflammatory profile, favoring resolution of the disease [35,58,65]. However, there is no evidence of the effect of MLT on blood leukocyte and/or lymphocyte levels in mice fed a high-fat diet. This study is the first to report the effect of MLT on the levels of lymphocytes and leukocytes in ApoE$^{-/-}$ mice fed with a WD. Furthermore, since a decrease in the population of

lymphocytes in the blood, as well as in the plasma levels of TNF, was observed in this study, we could say that MLT contributes to the decrease in the subpopulation of Th1 lymphocytes, being characterized by the production of TNF and involved in inflammatory processes [66]. Also, M1 pro-inflammatory macrophages are responsible for the production of TNF. Our results indicate a slight decrease in monocytes, which could contribute to the decrease in M1-phenotype macrophages and therefore to the decrease in TNF. This is in agreement with previous results which demonstrated that MLT promotes the polarization of macrophages to an M2-type anti-inflammatory profile [67]. In this way, MLT has been shown to reduce TNF levels in the blood in women with polycystic ovary syndrome, patients with COVID-19, and anemic patients with chronic kidney disease, among other populations [68–70].

Finally, oxidative stress is widely known for its role in the generation and development of cardiovascular diseases. ApoE$^{-/-}$ mice have been shown to have a high basal pro-oxidative status compared to C57BL/6 mice due to the antioxidant role of ApoE [71]. In the present work, oxidative stress was also increased through the intake of a WD. Furthermore, it has been widely demonstrated that the consumption of fat-rich diets increases oxidative stress, and in turn, increases the risk of developing cardiovascular disease [72]. In the present investigation, the intake of 9 mg/kg MLT daily for 12 weeks was capable of alleviating the oxidative effects caused by the consumption of the WD, with no improvements being observed when the daily concentration consumed was 2 mg/kg MLT. Due to the close relationship between oxidative stress and cardiovascular diseases, it is concluded that the consumption of MLT, in addition to leading to the previously mentioned effects, would reduce the risk of cardiovascular disease, through the reduction in oxidative stress. The fact that MLT controls the number of systemic lymphocytes as well as the lipid parameters in WD-fed ApoE$^{-/-}$ mice, a murine model of hypercholesterolemia, atherosclerosis, and metabolic syndrome, indicates that this molecule could be used to restore the lipid and anti-inflammatory imbalance generated in the development of cardiovascular events.

In addition, it is important to note that chronobiotic MLT is not the same as MLT, which is administered in high doses to treat different diseases. Both have different treatment guidelines to obtain the greatest effectiveness without harming the patient's health. Specifically, therapy with high doses of MLT has been tested in patients with Charcot–Marie–Tooth neuropathy (70 mg/day for 6 months) [73], in multiple sclerosis (25 mg/day for 6 months) [74], and neoplastic cachexia (20 mg/day for 3 months) [75], among other diseases, and in all of them, there was evidence of its effectiveness as an anti-inflammatory or antioxidant without showing side effects that could compromise the patients' health. Furthermore, a series of articles reinforce the hypothesis that increasing inflammatory responses leads to the suppression of nocturnal MLT production and that MLT administration to control inflammatory processes could at the same time compensate for this loss of nocturnal MLT [76–79]. In line with this, it would also be interesting to delve deeper into the possibilities offered by chronotherapy in MLT treatment in the future, the objective of which is to understand the impact that biological rhythms have on the response to a given therapy to optimize its action, maximize the benefits, and minimize possible adverse effects.

As a limitation of our study, the effect of MLT globally on the number of leukocytes and lymphocytes was analyzed, but not the effect that MLT could have on the different immune subpopulations. In fact, different subsets of white blood cells play different roles, and some are even opposite [80,81]. However, there are previous studies in which MLT therapy in an inflammatory context decreases the number of CD4 cells and macrophages, and more specifically the potentially pathogenic Th1 cells (characterized by the production of TNF), while promoting the regulatory responses mediated by Tregs [35,58]. Regarding macrophage subpopulations, MLT favors polarization from M1 (characterized by the production of TNF) towards the M2 profile, promoting an anti-inflammatory environment [67]. Our results support the hypothesis that MLT could reduce these pro-inflammatory subpopulations (Th1 cells and M1 macrophages) due to a decrease in TNF levels.

Given all the above and that MLT is a pleiotropic molecule with a wide range of functions, we suggest that the consumption of MLT could also control other key risk factors, such as oxidative stress, involved in these pathologies.

5. Conclusions

MLT could be considered a functional ingredient to prevent or treat the development of CVDs derived from high cholesterol intake, since in addition to controlling plasma and liver TC and LDL-C levels and reducing CV risk indexes, it decreases systemic inflammation and oxidative stress due to excessive fat consumption. However, more research is needed to decipher the molecular mechanisms by which MLT exerts these actions and to develop clinical trials to determine the effect of MLT in patients with cardiovascular risk.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/nutraceuticals4020016/s1, Table S1: Western diet composition.

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