Original Article

Protective Effect of Melatonin on Age-induced Changes in Circadian Clock and Inflammatory Profile in Rat Liver

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Background: Aging is associated with a reduction in melatonin secretion and a deteriorating circadian system. In addition, aging is also defined by a chronic low-grade inflammation, also known as inflammaging. Sirtuin 1 (SIRT1) can affect clock function by binding with CLOCK/BMAL1 complexes and can be involved in the regulation of inflammation via deacetylation of high-mobility group box-1 (HMGB1) and inhibit the transcription of inflammation-related genes, NF-κB. Interestingly, melatonin, a hormone mainly secreted by the pineal gland, showed a variety of regulatory effects on circadian rhythm and inflammatory response in age-related liver disease.

Objective: It is of interest to examine the effects of melatonin on age-induced changes in the levels of circadian clock protein and inflammation-related factors in rat liver.

Materials and Methods: Rats were divided into 3 groups; young control group, aged control group, and aged rat treated with melatonin in drinking water (20 mg/L). After 2 months of melatonin administration, the liver tissues were collected. The levels of BMAL1, REV-ERBα, SIRT1, HMGB1, NF-κB, IL-6, and type I collagen, a marker of hepatic fibrosis, were analyzed by western blot analysis.

Results: The present results showed that aged rats exhibited significantly downregulated BMAL1, REV-ERBα, and SIRT1 but upregulated HMGB1, NF-κB, IL-6, and type I collagen in rat liver compared with the young control group. Melatonin treated in aged rats significantly attenuated the age-induced downregulation of BMAL1, REV-ERBα, and SIRT1 and attenuated the age-induced increase in HMGB1, NF-κB, IL-6, and type I collagen protein expression.

Conclusion: The present study suggested that the protective effect of melatonin on age-induced changes in clock-related inflammation in rat liver, including increasing the expression of clock and clock-controlled proteins, reducing the release of inflammatory profiles, and inhibiting fibrogenic response. These effects of melatonin may be involving the clock-related SIRT1/HMGB1/NF-κB axis.

Keywords: Aging; Melatonin; Inflammaging; Clock gene; Liver; SIRT1; HMGB1; NF-κB

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Organismal aging is characterized by a progressive impairment of homeostasis and decline in physiological function. Aging often shows disrupted day/night activity patterns and has potent effects on rhythmic expression of circadian clocks in both the suprachiasmatic nucleus (SCN), a circadian pacemaker, and peripheral tissues[1,2]. The molecular basis of the circadian clock is a transcriptional-translational autoregulatory feedback loop of core clock genes, e.g., Period (Per1-3), brain and muscle ARNT-like protein-1 (Bmal1), orphan nuclear receptor Rev-Erbα (Rev-erbα), circadian locomotor output cycles protein kaput (Clock), Cryptochrome (Cry1, 2), and their corresponding proteins[3]. Many of the output rhythms controlled by the circadian timing system are affected by aging, such as the decrease in amplitude of body temperature rhythms[4] and the altered circadian rhythms of the sleep-wake cycle and melatonin secretion[5,6]. Moreover, circadian rhythm disruption is associated with an increased risk of disorders, pathologies, and dysfunctions such as neuro-degeneration, diabetes mellitus, obesity, and metabolic syndrome[7,8]. Furthermore, aging is also defined by an imbalance between inflammatory and anti-inflammatory systems, which results in chronic low-grade inflammation, and this phenomenon has been termed as inflammaging[9].

The liver is a highly complex metabolic organ that is important for maintaining whole body homeostasis and plays a role in nearly every organ system. It has multiple functions that help support metabolism, digestion, immunity, vitamin storage, and detoxification. In a study of human livers, the incidence of chronic liver disease increases with age[10]. Numerous reports suggest that aging is associated with

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multiple changes in liver morphology and its function, such as a decline in volume, hepatic blood flow, and the ability to metabolize many substances[10-12]. Inflammaging is the basic mechanism that sustains progressive liver dysfunction and leads to an accelerate aging of this organ[13,14]. Previous studies have long shown that inflammatory markers, such as high levels of interleukin-6 (IL-6)[15], coagulation factor, and fibrinogen[16,17], are increased in aged individuals. Circadian regulation of the inflammatory response has been well documented[18], some evidence showed a role for the circadian clock in regulating the mammalian immune response. For example, Bmal1-deficient mice have lower B-cell counts compared to wild-type mice[19], and Per2 mutant mice produce lower levels of proinflammatory cytokines[20]. However, inflammation also affects clock gene expression in the periphery. The administration of lipopolysaccharide (LPS), a powerful mediator of systemic inflammation, suppresses the expression of clock genes in the liver[21]. Moreover, sirtuin 1 (SIRT1), a histone deacetylase, can modulate the circadian clock by regulating CLOCK/BMAL1 activity and has been shown to impact on nuclear factor kappa B (NF-κB) levels[22]. At the molecular level, NF-κB can mediate the effects of cytokines on circadian rhythms and represses CLOCK/BMAL1-dependent genes[23].

The hallmarks of aging impact on the different types of liver cells including hepatocytes, hepatic stellate cells (HSCs), and macrophages in the liver (also known as Kupffer cells). Several studies have shown that increased expression of alpha-smooth muscle actin (αSMA), type I collagen, markers of HSC activation, and collagen deposition in the liver of aging rats[10-12]. In old rodent liver, there is increased production of cytokines that contribute to the inflammaging and downregulation of SIRT1, which might influence cellular senescence[24]. In addition, high-mobility group box-1 (HMGB1), a key protein participating in the pathogenesis of chronic liver disease, also acts as a proinflammatory cytokine that contributes to multiple injuries, including those from the liver, such as fibrosis, hepatocellular carcinoma, and non-alcoholic fatty liver disease (NAFLD)[25]. HMGB1 can activate NF-κB, which results in the production of the proinflammatory cytokines in hepatocytes and Kupffer cells, while induces a fibrogenic response by regulation of type I collagen production in HSCs[26].

Interestingly, Melatonin (N-acetyl-5 methoxytryptamine), a hormone mainly secreted in the pineal gland, is believed to be an anti-aging hormone and can extend the life span[27,28]. It is well-established that endogenous melatonin production and pulsatile release diminish in elderly persons[6]. Hence, the reduction in melatonin with age may be a factor in enhanced oxidative damage and an increased inflammatory response observed in the elderly population. In addition, melatonin showed a variety of regulatory effects on circadian rhythm, oxidative stress, apoptosis, and immune function[27,28]. Previous study has shown that 4-week melatonin supplementation in the drinking water can reduce oxidative stress in the liver of aging (24 months old) rats[11]. Although protection effects of melatonin against inflammatory liver injury have been studied in senescence-accelerated mice[29] and chronic liver disease[30], no information exists on whether melatonin may influence the mechanistic links between the circadian clock and liver inflammation through the SIRT1/HMGB1/NF-κB axis. Thus, in the present study, the authors attempted to investigate the effects of long-term melatonin administration during nighttime for 2 months on age-induced changes in the levels of circadian clock proteins and inflammatory profile in rat liver.

Materials and Methods

Animals

Young adult male Wistar rat (2 months) and old rat (8 and 9 months old; retired breeders) were purchased from the National Laboratory Animal Center of Mahidol University, Salaya, Thailand. All animals were caged in temperature (22±2°C) and humidity-controlled rooms with ad libitum access to food and water under a 12 h light (200 lux)/12 h dark (2 lux dim red light) conditions (light on at 7 AM). The study was carried out in accordance with experimental protocols approved by the Animal Ethics Committee of the Faculty of Medicine, Srinakharinwirot University (No. COA/AE-005-2564).

Drug treatment

At the age of 22 months (aged group), the rats were randomly divided into 2 groups (n=4), i.e., melatonin-treated group and aged control group. In additional, 2 months old rats (n=4) were used as a young control group. All rats were kept individually in standard laboratory cages. The melatonin-treated group was given daily melatonin (Sigma-Aldrich, St. Louis; MO) dissolved in tap water during nighttime (7 PM to 7 AM) for 2 months. Melatonin was prepared three times a week by dissolved in ethanol and added to the drinking water to a final concentration of 20 mg/L (final concentration of ethanol, 0.01%). The aged control and young control groups were given tap water with 0.01% ethanol as drinking water during nighttime. The weight and the consumption of water containing melatonin were measured in each rat. The daily melatonin intake for each rat was approximately 1.3 mg/kg, which was expected to rise 20 to 30 times normal plasma melatonin[31]. Rats in all experimental groups were euthanized by exposure to a lethal dose of CO_2 and were then decapitated during the daytime (between 1 PM and 2 PM). The anteromedial lobe of the liver was dissected, quickly frozen in dry ice, and stored at -80°C until western blot analysis.

Western blot analysis

The liver tissues were lysed in radio-immunoprecipitation assay buffer (RIPA buffer) (Santa Cruz Biotechnology, Inc) with a sonicator. Tissue and cell debris were removed by centrifugation at 12,000 x g for 15 minutes at 4°C. The supernatants were collected for use in western blot analysis. The protein concentration was determined by using the Qubit™ protein assay kit (Invitrogen,

Paisley, UK) with the Qubit® 2.0 Fluorometer. Thereafter, lysate proteins were denatured at 100°C for 5 minutes in sample buffer (62.5 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 2% mercaptoethanol, and 0.01% bromophenol blue). After that, lysate proteins were separated by 12% sodium dodecyl sulfatepolyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride (PVDF) membranes. The PVDF membrane was stained with Ponceau Red solution to detect the transfer efficacy. After washing with Tris-buffer saline that contained 0.1% Tween-20 (TBST), the membrane was incubated in blocking buffer (5% nonfat milk in TBST) for 1 hour at room temperature. The membrane was then incubated with a mouse monoclonal antibody against actin (Millipore, MA, USA; 1: 10,000), mouse monoclonal antibody against BMAL1, mouse monoclonal antibody against HMGB1, rabbit monoclonal antibody against REV-ERBα (Abcam, Cambridge, UK; 1: 1,000), mouse monoclonal antibody against SIRT1, rabbit monoclonal antibody against NF-κB p65 (Cell signaling, Danvers, MA, US; 1: 1,000), mouse monoclonal antibody against IL-6, or goat polyclonal antibody against COL1A1 (Santa Cruz, CA, USA; 1: 1,000) diluted in TBST at 4°C for overnight. After washing with TBST, these membranes were incubated with 1: 10,000 horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, HRP-conjugated goat anti-rabbit IgG (Abcam, Cambridge, UK), or HRP-conjugated donkey anti-goat IgG antibody (Santa Cruz, CA, USA) for 1.5 hours at room temperature. Finally, protein bands were visualized by clarity western enhanced chemiluminescence (ECL) substrate (Bio-Rad, Hercules, CA, USA) and quantitated using the Scion Image program (National Institutes of Health, Bethesda, MD). The values of protein bands were normalized by these of actin from the same samples.

Statistical analysis

All data were expressed as the mean±SEM. One-way analysis of variance (ANOVA) and Tukey's post hoc tests were used in this study to examine differences in the data. The significance levels were set at p-values less than 0.05.

Results

Effects of melatonin on age-induced alterations in clock proteins expression in the liver

To determine the effect of aging and melatonin on the alteration of the circadian clock, the core clock protein, BMAL1 and REV-ERBα in rat liver, was examined by western blot analysis. BMAL1 ($p<0.01$) and REV-ERBα ($p<0.05$) protein significantly decreased in the aging liver when compared to the expression in the young rat. The melatonin-treated group significantly increased the expression of BMAL1 (Figure 1A) and REV-ERBα (Figure 1B) protein in aged mice compared with the aged untreated group ($p<0.05$).

Effects of melatonin on age-induced alterations in SIRT1 expression in the liver

Because the SIRT1 regulatory pathway can modulate circadian clock, HMGB1, and NF-κB expression, the authors determine the effect of melatonin on SIRT1

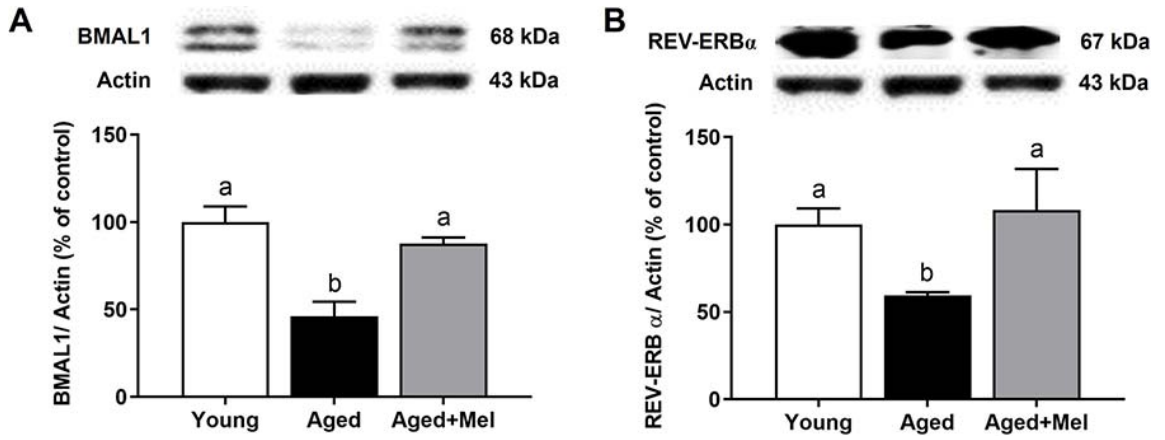

Figure 1. The effect of melatonin on clock protein expression in the aging rat liver. BMAL1 (A) and REV-ERBα (B) protein levels in the liver of young control group (Young), aged control group (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel) were measured by western blot analysis. Representative bands from the different groups are shown. The protein band was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences among groups: all $p<0.05$ except differences in BMAL1 expression between Young and Aged group for which $p<0.01$.

expression in the liver of the aged rat. SIRT1 protein levels in the aged liver significantly decreased ($p<0.001$) when compared to the young control group. Melatonin significantly increased ($p<0.001$) SIRT1 protein levels compared with those seen in the aged control group (Figure 2).

Effects of melatonin on age-induced alterations in HMGB1 expression in the liver

The authors determined whether melatonin could downregulate HMGB1 protein, a sterile inflammatory mediator, in the aging liver. The levels of HMGB1 were significantly increased ($p<0.001$) when compared to those seen in the young control group. Melatonin was able to significantly reduce ($p<0.01$) the levels of HMGB1 proteins compared with the expression levels in the aged control group (Figure 3).

Effects of melatonin on age-induced alterations in NF-κB expression in the liver

NF-κB, a transcription factor that plays a key role in the inflammatory response, was investigated in the present study to determine whether melatonin can restore aged-induced changes in proinflammatory cytokine through modulating the NF-κB signaling pathway. The results showed that NF-κB protein levels in aged liver were significantly increased ($p<0.01$) when compared to those seen in the young control group. The melatonin-treated group significantly decreased ($p<0.05$) NF-κB protein expression when compared to the levels seen in the aged control group (Figure 4).

Effects of melatonin on age-induced alterations in the levels of proinflammatory cytokine in the liver

To determine the effect of melatonin on age-induced inflammation in the liver, the proinflammatory cytokine IL-6 was examined by western blot analysis. In the aging liver, IL-6 levels were significantly increased ($p<0.001$) when compared to those seen in the young control group. Melatonin was able to significantly reduce the levels of IL-6 ($p<0.001$) proteins compared with the expression levels in the aged

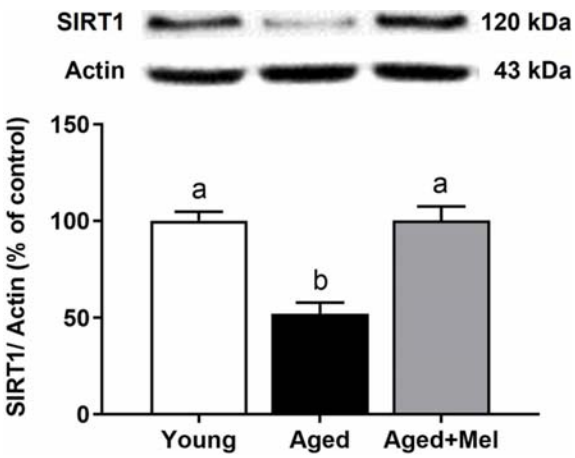

Figure 2. The effect of melatonin on sirtuin1 (SIRT1) protein expression in the aging rat liver. The levels of SIRT1 protein in the liver were compared among three groups of young control (Young), aged control (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel). Representative bands from the different groups are shown. The protein band from western blot was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences ($p<0.001$) among groups.

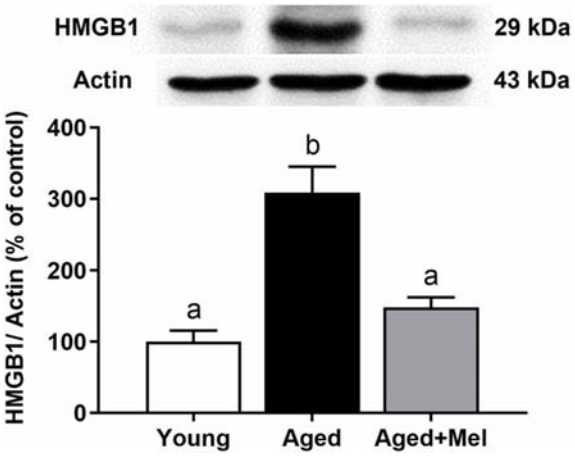

Figure 3. The effect of melatonin on HMGB1 protein expression in the aging rat liver. The levels of HMGB1 protein in the liver were compared among three groups of young control (Young), aged control (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel). Representative bands from the different groups are shown. The protein band from western blot was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences in HMGB1 expression between Young and Aged group ($p<0.001$) and between Aged and Aged+Mel group ($p<0.01$).

control group (Figure 5).

Effects of melatonin on age-induced alterations in the levels type I collagen in the liver

To determine whether melatonin can restore the aged-induced hepatic fibrosis, the severity of hepatic fibrogenesis was estimated. Thus, the levels of extracellular matrix (ECM) protein and type I collagen in the liver can be used as the markers to assess hepatic fibrosis. The results showed that the levels of type I collagen in the aged group were significantly increased ($p<0.05$) when compared to those seen in the young control group. The melatonin-treated group significantly decreased ($p<0.05$) type I collagen protein expression when compared to the levels seen in the aged control group (Figure 6).

Discussion

The aging liver exhibits several characteristics consistent with inflammation-induced hepatic injury, and many studies have examined the effect of aging on the inflammation status in the mammalian tissues[10-12]. In rat liver, aging has been associated with enhanced oxidative stress and inflammatory profile[11-13]. In the present study, it has been shown that melatonin suppresses age-induced changes in clock-related inflammation through SIRT1/HMGB1/NF-κB axis. Several studies have suggested that the molecular clock may play a fundamental role in controlling the inflammatory response[18-21,32]. Moreover, the evidence indicates that a rhythm in the susceptibility to LPS correlates with increased production of proinflammatory cytokines with activation of REV-ERBα, a BMAL1 target, attenuating inflammation, in particular through repression of IL-6[32].

In these experiments, the authors also investigated the possibility that the expression of these core clock proteins, BMAL1 and REV-ERBα, might be involved in aged-induced alterations in inflammation response. The levels of BMAL1 were markedly decreased in the aged control group. It has been observed that the absence of the Bmal1 gene results in not only abolishment of its circadian function but also reduced expression in many metabolic genes. For example,

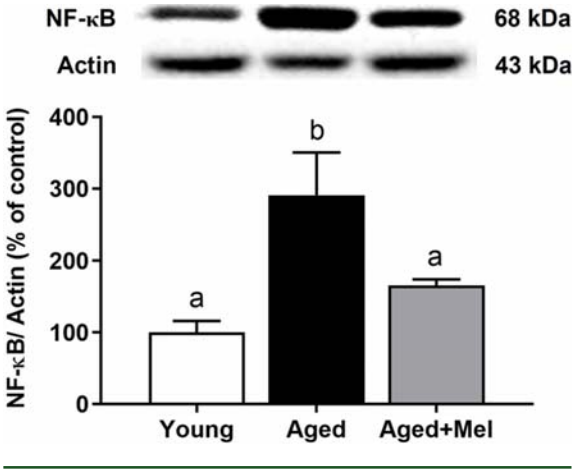

Figure 4. The effect of melatonin on NF-κB protein expression in the aging rat liver. The levels of NF-κB protein in the liver were compared among three groups of young control (Young), aged control (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel). Representative bands from the different groups are shown. The protein band from western blot was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences in NF-κB expression between Young and Aged group ($p<0.01$) and between Aged and Aged+Mel group ($p<0.05$).

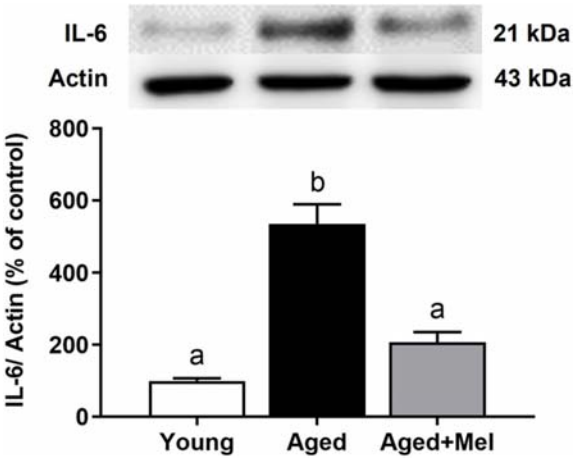

Figure 5. The effect of melatonin on IL-6 protein expression in the aging rat liver. The levels of IL-6 in the liver were compared among three groups of young control (Young), aged control (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel). Representative bands from the different groups are shown. The protein band from western blot was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences ($p<0.001$) among groups.

Bmal1 knock-out mice show impaired glucose homeostasis and energy balance[33]. Moreover, BMAL1 is both necessary and sufficient to promotes the expression of lipid synthesis enzymes in the liver[34]. With respect to the immune system, Bmal1 knock-out mice exhibit impaired B cell development and reduced numbers of B cell in blood and spleen[18,19]. Consistent with BMAL1 results, REV-ERBα levels were decreased in the aging liver. Moreover, REV-ERBα also plays a crucial role in metabolic pathways, including hepatic lipid metabolism and energy homeostasis, as well as inflammatory pathways[32]. Recently, it was reported that BMAL1 and REV-ERBα could regulate the inflammatory response by down-regulating NF-κB-mediated transcription in inflammatory cells[34]. As expected, melatonin upregulated the expression of BMAL1 and REV-ERBα in the aging liver. Corresponding to previous studies that melatonin was able to restore the reduction of the circadian clock in the aging rat[5,35]. Melatonin affects the expression of Bmal1 gene expression through regulation of RORα and REV-ERBα expression[35,36]. Interestingly, a previous study revealed the effect of melatonin in the reversion of age-induced alterations of clock genes in the SCN, a master clock. Therefore, it is possible that the effect of melatonin in the expression of liver clock proteins in this study probably results from the resynchronization of clock genes in the SCN, followed by the synchronization of the peripheral oscillations, including the liver clock, by the SCN clock[36]. Furthermore, melatonin, via regulation of various transcription factors, including NF-κB and nuclear receptors (RORα and REV-ERBα), may differentially regulate the expression of other clock and clock-controlled genes in the liver.

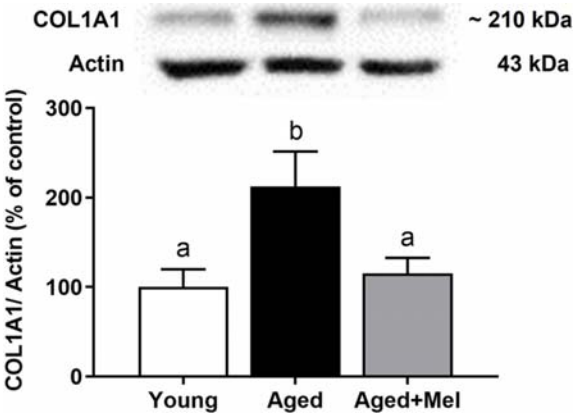

Figure 6. The effect of melatonin on type I collagen (COL1A1) protein expression in the aging rat liver. The levels of type I collagen in the liver were compared among three groups of young control (Young), aged control (Aged), and aged rats treated with melatonin in drinking water (20 mg/L) for 2 months (Aged+Mel). Representative bands from the different groups are shown. The protein band from western blot was quantified via densitometry and normalized by actin. The ratios were calculated as a percentage of the respective value of the young control group. The values represent mean±SEM (n=4). Different letters above error bars indicate significant differences ($p<0.05$) among groups.

In addition, the authors found that SIRT1 was downregulated by aging, and the reduction could be prevented by melatonin administration. With this evidence, another research has also shown that melatonin increased SIRT1 expression in a variety of models[30,35]. Several lines of evidence have shown that SIRT1 is involved in both aging and circadian clock regulation. SIRT1 affects clock function by binding with CLOCK/BMAL1 complexes and deacetylating BMAL1 protein[22]. Nevertheless, another research has shown that CLOCK/BMAL1 enhancer complexes also bind to the SIRT1 promoter to enhance its expression in the liver as well as Rev-erbα knock-out mice exhibited reduced expression of the SIRT1 gene in skeletal muscle[22]. Consequently, the present findings suggest that SIRT1 reduction by aging may be due to the change in BMAL1 and REV-ERBα. However, the full relationship between these clock proteins and SIRT1 in the liver remains to be explored. Furthermore, SIRT1 can be involved in the regulation of inflammation via deacetylation and inhibit the transcription of inflammation-related genes[22,24]. It has been shown previously that Sirt1 overexpression can improve a healthy lifespan and delayed aging in mice[35].

Concerning about inflammaging, NF-κB induces the expression of various proinflammatory factor genes and then followed by the release of many proinflammatory cytokines, including IL-6 that is shared across age-related pathologies having a strong chronic inflammatory status[9,12,14]. Furthermore, previous studies have demonstrated the inhibitory effect of SIRT1 on the NF-κB-dependent inflammatory pathway. Overexpression SIRT1 or activation of SIRT1 show beneficial effects on the liver through suppressing the transcriptional activation by NF-κB, resulting in the reduction of the proinflammatory cytokines, such as IL-6 and TNFα[12,35]. Conversely, SIRT1 deficiency in macrophages induces NF-κB activity in the liver, hence inducing hepatic inflammation[10,35]. In agreement with previous work, the present study showed that melatonin, by upregulation of SIRT1 and downregulation of NF-κB expression, attenuates age-induced hepatic inflammatory response by reducing the production of proinflammatory cytokine IL-6.

In addition, HMGB1, a highly conserved non-histone chromosome binding protein, participates in the regulation of inflammation via activating NF-κB[25]. Previous studies indicated that SIRT1 can deacetylate HMGB1 to prevent its release, thereby attenuating the inflammatory response[26]. Strikingly, HMGB1 levels were upregulated in liver injury, including age-induced liver inflammation.

HMGB1 was elevated during liver fibrosis and its expression was closely correlated with the activation of NF-κB-dependent inflammatory signaling in hepatocytes and Kupffer cells as well as the regulation of type I collagen production in HSCs[26]. In this regard, it is of interest to note that HMGB1 may contribute to the response against tissue damage like in age-related liver disease[25,26]. The present results, therefore, revealed that the levels of type I collagen, a putative biomarker for hepatic fibrosis, are elevated in age-induced inflammatory livers. Fascinatingly, melatonin administration successfully prevented aged-induce liver fibrosis by downregulation of HMGB1 and reducing the production of type I collagen. Based on the present results, the authors have evidence to believe that melatonin may exert its anti-inflammaging in the liver via clock-related SIRT1/HMGB1/NF-κB axis. However, the exact mechanisms still require further investigation.

Conclusion

In summary, the present study suggested that the protective effect of melatonin on age-induced changes in clock-related inflammation in rat liver, including increasing the expression of clock and clock-controlled proteins, reducing the release of inflammatory profiles, and inhibiting fibrogenic response. The molecular biological studies have shown that the protective effect of melatonin may be involving the clock-related SIRT1/HMGB1/NF-κB axis. In further study, overexpression or gene knockout techniques will be applied to further elucidate the role of the SIRT1 pathway in melatonin anti-inflammaging.

What is already known on this topic?

The protective effects of melatonin against inflammatory liver injury have been studied in a variety of models. However, no information exists on whether melatonin may influence the mechanistic links between the circadian clock and liver inflammation through the SIRT1/HMGB1/NF-κB axis.

What this study adds?

Although the anti-inflammation properties of melatonin have been extensively studied, there are no quantitative data to establish the potential effects of melatonin on clock-related inflammaging in the rat liver. The present study indicated the effects of melatonin on age-induced changes in the levels of circadian clock protein, BMAL1 and REV-ERBα, inflammation-related factors, SIRT1, HMGB1, NF-κB, and IL-6, as well as type I collagen that could be used as a marker of hepatic fibrosis.

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Potential conflicts of interest

The authors declare no conflict of interest.

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