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The protective effect of nicotinamide riboside against age-induced hepatic disease in mice

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Background & Aims. Aging is one of the key triggers of non-alcoholic fatty liver disease (NAFLD). Yet, the pathomechanism of the age-associated NAFLD is not fully understood. Nicotinamide adenine dinucleotide (NAD), an ubiquitous coenzyme, has beneficial effects on aging. Here, we investigated the actions of NAD precursors nicotinamide riboside (NR) on the development of age-induced NAFLD. Methods. NR supplied food (2.5g/kg food) was applied to aged mice for three months. Changes of body weight, food intake, hepar weight and fat pat mass were measured. The serum concentrations of lipid content, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and NAD were determined by biochemical assays. Pathological assessment and immunohistochemistry analysis of hepatic tissues were used to evaluate the effect of NR on NAFLD development and inflammation infiltrated. Results. NR significantly reduced fat pat mass, lipid content and AST in aged mice, but didn't modify in terms of body weight, food intake, hepar weight and ALT in aged mice. Given normal chow, aged mice displayed decline of NAD concentration. In aged mice model, moderate NAFLD phenotypes, including steatosis and hepatic fibrosis (Masson's trichrome staining and TGF-β staining) were observed in liver. In addition, Kupffer cells accumulated and pro-inflammatory cytokines expression were more aggravated in hepatic tissues. Whereas, NR administration completely corrected these NAFLD phenotypes and inflammation infiltrated in liver. Conclusion. NR has benefits on age-associated lipid accumulation and hepatic steatosis, and the oral uptake of NR may be a promising strategy to prevent the progression of NAFLD.
The protective effect of nicotinamide riboside against age-induced hepatic disease in mice

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Abstract

**Background & Aims.** Aging is one of the key triggers of non-alcoholic fatty liver disease (NAFLD). Yet, the pathomechanism of the age-associated NAFLD is not fully understood. Nicotinamide adenine dinucleotide (NAD), an ubiquitous coenzyme, has beneficial effects on aging. Here, we investigated the actions of NAD precursors nicotinamide riboside (NR) on the development of age-induced NAFLD.

**Methods.** NR supplied food (2.5g/kg food) was applied to aged mice for three months. Changes of body weight, food intake, hepar weight and fat pat mass were measured. The serum concentrations of lipid content, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and NAD were determined by biochemical assays. Pathological assessment and immunohistochemistry analysis of hepatic tissues were used to evaluate the effect of NR on NAFLD development and inflammation infiltrated.

**Results.** NR significantly reduced fat pat mass, lipid content and AST in aged mice, but didn't modify in terms of body weight, food intake, hepar weight and ALT in aged mice. Given normal chow, aged mice displayed decline of NAD concentration. In aged mice model, moderate NAFLD phenotypes, including steatosis and hepatic fibrosis (Masson's trichrome staining and TGF-β staining) were observed in liver. In addition, Kupffer cells accumulated and pro-inflammatory cytokines expression were more aggravated in hepatic tissues. Whereas, NR administration completely corrected these NAFLD phenotypes and inflammation infiltrated in liver.

**Conclusion.** NR has benefits on age-associated lipid accumulation and hepatic steatosis, and the oral uptake of NR may be a promising strategy to prevent the progression of NAFLD.

**Subjects** Gastroenterology and Hepatology, Pharmacology

**Keywords** NAFLD, NAD, NR, Aged mice, Inflammation infiltrated
Non-alcoholic fatty liver disease (NAFLD), is a metabolic disorder that characterized by imbalanced in lipid metabolism and fatty acid accumulation in liver. Hepatocyte death, accompanied by inflammation, gradually leads to fibrogenesis, cirrhosis and ultimate hepatocellular carcinoma (Romeo, 2019). Advancing age, together with obesity and hypertriglyceridemia, is crux triggers of hepatic steatosis and progressive inflammation (Jadeja and Jones, 2019; Geisler and Renquist, 2017). Aging is a physiological process of all biological organisms decline, especially the liver. The prevalence of the NAFLD increases markedly with aging (Lee and Kim, 2007; Amarapurkar and Kamani, 2007). A clinical research, involved 589 consecutive liver biopsies, reveals that age over 30 is an independent risk factors for liver steatosis (Lee and Kim, 2007). Similarly aging is also evidenced to bring a higher mortality in old people with NAFLD (Frith and Jones, 2009). The age-related modification of structure and function of liver is supported by histologic proofs such as hepatic morphology disorder, hepatocyte polyploidization, and the reduced mitochondrial density that present even in defect of disease (Gan and Chitturi, 2011; Wu and Shen, 2019). Unfortunately, the mechanisms that underlie age-related hepatic dysfunction is not fully understood, hence the urge to explore valuable strategies to manage this chronically hepatic disease.

It is well established that aging process is partially reflected in mitochondria dysfunction, which would result in Nicotinamide adenine dinucleotide (NAD) depletion (Kang and Chung, 2013; Gomes AP, Price, 2013; Andreani and Bartolacci, 2018). NAD is involved in many cellular functions, which plays a crucial role in energy metabolism (Wątroba and Dudek, 2017). The salvaging synthesis pathway is the predominant manner for synthesis of NAD in mammalian cells. Nicotinamide riboside (NR) is considered as a NAD precursor for this pathway (Moon and Kim, 2018). Once it enters the cell, NR can convert to nicotinamide mononucleotide (NMN) by rate-limiting enzyme nicotinamide phosphoribosyltransferase (NAMPT). Then, NMN is further metabolized to NAD. Additionally, from tryptophan de novo biosynthesis of NAD is the other pathway, whereas limited by cell types (Yoshino and Baur, 2018). Decline of NAD level is certified to be related to age-associated diseases such as Parkinson's disease (PD) (Jeśko and Wencel, 2017) and Alzheimer's disease (AD) (Xie and Gao, 2019), which results from unbalance of NAD synthesis and consumption. Thus, regulating the NAD level of cells appears to be a promising strategy for repairing the cellular function. Better yet, NR is found in milk,
compositing NAD production as dietary source (Bieganowski and Brenner, 2004). In contrast to NR, other precursors of NAD biosynthesis, such as nicotinic acid (NA), nicotinamide or NMN, have been shown severe flushing or toxin in pre-clinical trials (Bogan and Brenner, 2008; Di Stefano and Nascimento-Ferreira, 2015). This highlights NR might be an important vehicular form for promotion NAD level of cells.

Supplementation of NAD has been shown beneficial outcomes on blood lipid and cholesterol profiles, even on improvement of metabolic disorder (Karpe and Frayn, 2004). Modulating of cellular NAD level can attenuate hepatic steatosis and inflammation in a mice model with methionine-choline-deficient diet (Katsyuba and Mottis, 2018). Evidence also displays that replenish NR in high-fat-high-sucrose dietary promote beneficial effects on NAFLD in C57BL/6 mice (Gariani and Menzies, 2016). In short, NAD has pronounced effects on hepatic homeostasis. Despite the advancing field, the potential of NAD replenish on aged liver is still unspecified.

In this study, we demonstrate that 18 months old C57BL/6 mice with common diet, exhibit dysfunction in NAD level and hepatic steatosis with moderate fatty infiltration. We also explore the NAD supplementation by enriched in NR dietary for mice may ameliorate age-induced NAFLD, including inflammation infiltration.
Materials & Methods

Animals
Female C57BL/6J mice in three-month old and fourteen-month old were purchased from Zhejiang Academy of Medical Sciences (Hangzhou, China), and used as young mice, aged mice and NR supplied aged mice respectively. All mice were maintained in a environmentally-controlled room (12 h light-dark cycle, 20-26 ℃, relative humidity 50%), fed a standard chow with free access to water. All animal experiment were performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Procedures were approved by the Institutional Animal Care and Use Committee of the Zhejiang Academy of Medical Sciences.

NR supplementation
The mice were divided to three groups: (1) Young mice (Young); (2) Aged mice (Aged); and (3) NR treated aged mice (Aged + NR). NR (Baikai Chemical Technology Co., Ltd, Hangzhou, China) was mixed into the pellets with the concentration of 2.5 g/kg (Zhejiang Academy of Medical Sciences, Hangzhou, China). Food consumption of aged mice for ten days were measured and the average food intake was estimated at 160 g/kg. According to the food intake, the aged mice orally treated with NR around 400 mg/kg/day. The food containing NR was supplied from fifteen-month old and four-month old for C57BL/6J mice and lasted for three months until sacrificed. Mice in the Young and Age groups were received common food correspondingly. After 3 months, mice were used for ex vivo studies.

Body weight and food intake determinations
Body weight changes of each group were measured at the end of NR administration. Additionally, the food intake of aged mice was measured in cages every 3-5 days. The average daily amount of each mouse was calculated.

Liver weight and fat pat mass measurements
Mice of three groups were euthanized by chloral hydrate (800 mg/kg) injection intraperitoneally, after an overnight fasting period. Mice were transcardially perfused with 4 ℃ saline. The hepar and total fat pat were quickly removed, carefully cleaned, and blotted dry. Then the collected samples were measured carefully. The ratios between tissue mass and body weight were calculated respectively.

Blood biochemical assays
Blood samples were acquired from inferior vena cava of anesthetized mice. About 0.7 ml blood was harvested. Samples were still standing at room temperature for 40 min and at 4 °C for 2 h, then followed by 3000 rpm centrifugation for 10 min. The supernatant was used for blood biochemical index measurement. According to the manufacturer's instructions, triglyceride (TG), total cholesterol (TC), alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents in serum were quantified by an automatic biochemistry analyzer (Backman). Blood NAD concentration was determined by a commercial NAD Quantitation Colorimetric Kit (K337-100, Biovision, San Francisco, CA, USA).

**Histological analysis**

Those isolated livers, taken from the same lobe, were fixed in 4% paraformaldehyde for 8 hours and then were embedded in paraffin for histological processing. Samples were cut into thin section (5 μm) and stained with hematoxylin and eosin (H&E) to assess histopathology, and Masson's trichrome for collagen evaluations. Images were obtained at 200 magnifications under the inverted phase-contrast microscope (Leica Microsystems, Wetzlar, Germany).

Scoring for steatosis (severity and extension) was performed in a blinded and independent method by two observers as described before (Gariani and Menzies, 2016). The analysis used a scale of 0-4, where 0 referred to absent of vacuolation in the hepar, 1 referred to 2 or 3 vacuoles per hepatic cord per lobule, 2 referred to less than 50% of the lobule has fatty vacuolation, 3 referred to more than 50% of the lobule has fatty vacuolation, and 4 corresponded to nearly the entire lobule has fatty infiltration. Moreover, the focal extention was referred to 1, multifocal was referred to 2, and almost total diffuse was referred to 3.

**Immunohistochemical analysis**

For immunohistochemical analysis, thin sections blocked by 5% goat serum followed by incubating in specific primary antibodies. PBS was applied to wash the sections for 3 times. Then, samples was stained with horseradish peroxidase-conjugated secondary antibodies and visualized by substrate DAB. Images were taken with a microscope (Leica, 200×) under same acquisition settings for each section. The primary antibodies were used as follow: TGF-β (MAB240-100, R&D System, 1:600 dilution), F4/80 (LS-C96373-100, Lifespan, 1:1000 dilution), CD68 (ab125212, Abcam, 1:600 dilution), IL-1β (SRP8033, Sigma, 1:1000 dilution), TNF-α (ab6671, Abcam, 1:600 dilution).

**Statistical analyses**
Data were expressed as mean ± SEM. Values from different groups were analyzed using one-way ANOVA followed by Newman-Keuls multiple comparison test. Statistical analysis was done in GraphPad Software (Prism Version 5.01). Statistical significance was considered as $P < 0.05$. 
Results

Changes of body weight, food intake, relative liver weight and fat pat mass in NR treated aged mice

After 3 months of NR supplied, body weight in aged mice with NR repletion was a little lower than the aged mice (25.2 ± 1.2 vs 27.3 ± 1.1, P > 0.05), although no significant difference was shown (Figure 1A). There was also no significant difference in food intake or liver to body weight ratio between NR supplied aged mice and aged mice. (Figure 1B and 1C). As shown in Figure 1D, ageing was sufficient to induce fat pat mass to body weight ratio increased compared with young. While the ratio was greatly decreased in aged mice with NR repletion when compared to the aged mice (3.0 ± 0.4 vs 4.4 ± 0.7, P < 0.05).

NR favoured lipid homeostasis and hepatic steatosis in aged mice

To answer whether the NR supplied could improve the susceptibility to development of NAFLD in aged mice, we used 15 months old mice with 3 months feeding of NR. The TG and TC contents were significant elevated in aged mice when compared to young mice. NR supplied aged mice significantly reduced both TG (0.87 ± 0.02 vs 1.12 ± 0.12, P < 0.05, Figure 2A) and TC level (2.31 ± 0.23 vs 2.99 ± 0.19, P < 0.05, Figure 2B). The results were in agreement with improved fat accumulation, suggesting NR protects against age-induced lipid disorders. As a result, serum elevation in ALT and AST of aged mice indicated an impaired of liver. While the level of AST was greatly attenuated with NR (98.8 ± 8.56 vs 124.7 ± 10.56, P < 0.05, Figure 2D) without ALT (Figure 2C). These results matched by changes in NAD concentration (Figure 2E). Moreover, H&E staining presented hepatocellular irregularity shaped and severity of steatosis in aged mice (Figure 2G). The histology score in aged mice was greatly improved by NR supplied (Figure 2F). These observations suggested that aging could promote the lipid accumulation and the ensuing development of NAFLD. And NR was demonstrated to prevent age-induced hepatic steatosis.

NR improved hepatic fibrosis in aged mice

To further determined the influence of NR on development of NAFLD in aged mice, Masson's trichrome and TGF-β staining were performed. NR weaken hepatic collagen and fibrosis, as revealed by less Masson's trichrome staining and TGF-β staining (Figure 3A, 3B).

NR alleviated inflammation infiltrated in liver of aged mice
We investigated the influence of NR on hepatic inflammation in aged mice. Immunohistochemical staining for F4/80 and CD68 indicated that accumulated Kupffer cells were much more in liver of aged mice compared with young mice. NR supplied obviously reduced the number of Kupffer cells (Figure 4A). In agreement with NR-induced improvement in macrophagocyte infiltration, there was also a significant down-regulation of pro-inflammatory cytokines IL-1β and TNF-α expression in liver from aged mice (Figure 4B).
Discussion

The principal findings arose from the present study. First, the supplementation of NR ameliorated lipid homeostasis and hepatic steatosis in aged mice. Second, the supplementation of NR reduced collagen deposition and hepatic fibrosis in liver from aged mice. Finally, we showed that NR treatment decreased Kupffer cells infiltrating as well as lowered IL-1β and TNF-α expression in liver from aged mice.

Prevalence of NAFLD increases dramatically with age, although this disease appears in different age groups (Zhou and Li, 2019). There are strong evidences suggesting steatohepatitis and fibrosis are associated with aging, which results in a higher mortality in elderly individuals with NAFLD (Argo and Northup, 2009; Ooi and Mgaith, 2018). Researchers have identified several mechanisms underlying the age promotion the morbidity of NAFLD. Physiological changes characterize aging may trigger the development of components of the metabolic disturbance. For example, the functional decrease in the lysosomal degradative pathway of autophagy appears to be remarkable in aged individual, which may encourage lipid accumulation in the liver (Martinez-Lopez and Athonvarangkul, 2015; Chi and Tsai, 2019). Furthermore, the level of oxidative stress, inflammation and DNA damage increase with aging, and these excessive elevations have also been implicated as mediators of NAFLD pathogenesis. Previous study have reported that the histological grade of steatosis similarly increased in aged mice compared with young and middle mice (Fontana and Zhao, 2013). Recently, the deteriorate morphology and function of livers have also been observed in natural aging rat models (Minhas and Liu, 2019). In this study, our results indicated that 18-month-old C57BL/6J mice exhibited an impaired lipid homeostasis including body weight gain, fat accumulation and serum TG and TC increase. These mice showed great susceptibility to development of NAFLD, reflected in steatosis with moderate fatty infiltration. Liver is a vital regulator of metabolism. Therefore, it is important to maintain hepatic function of elderly. Nevertheless, the little data are currently available in molecular mechanism for aging-related NAFLD.

NAD is a substrate for multiple enzymes of sirtuin family and participates in multiple cellular functions, including DNA repair, energy metabolism, and regulation the activity of the sirtuins by transcriptional control (Hoxhaj and Ben-Sahra, 2019). It is well-established that aging and fatty liver related dysfunction leads to a pronounced effect on decline of NAD concentration in liver. Fan et al. have reported that the expression of heptic mRNA of regulating NAD
biosynthesis is greatly reduced in aged mice or challenged high-fat diet (HFD) mice (Fan and Cui, 2018). Additionally, this phenomenon seems to be a toxic element, providing destructive actions because a shortage of NAD links ageing to progressive liver damage. The beneficial effects of NAD regiment on fatty liver have been reported, for instance in a liver-specific Sirt1 knockout mouse (Katsyuba and Mottis, 2018) and in an enzyme-dead NAMPT transgenic mouse (Zhou and Yang, 2016). Thus, supplementation NAD pool may be an attractive therapy strategy for liver damage related diseases in elderly individual. Notably, NR, this vitamin B3 analog, as a precursor of NAD biosynthesis, is commonly used to boost NAD pool (Jiang and Zhou, 2019). Here we showed that the replenishing of NR has beneficial effect on liver of aged mice. Our study demonstrated that aged mice administrated of NR (250 mg/kg/day) for 3 months improved lipid disordered. Moreover, NR treatment exhibited an amelioration in hepatic steatosis and fibrosis that was matched by an augmented blood NAD concentration, implying a systemic NAD replenishing in aged mice.

An extensive body of evidence indicates that chronic inflammation contributes to the degenerative changes of full-length tissues in the context of aging. Even normal brain of aged individual is characterized by increased inflammation and subsequently elevated pro-inflammatory cytokines (Frank and Barrientos, 2006). As inflammation rose by age shows a reduction in adequate NAD content in brain of the murine (Braidy and Guillemin, 2011). Previous study has also demonstrated that genetic blockade of NAD synthesis exerts inflammatory effects on the liver reflecting by activation NLRP-3 inflammasome pathway and production of IL-18 and IL-1β (Jiang and Zhou, 2019). The other independent group shows that pharmacological inhibition of de novo NAD synthesis strengthens transcription genes involved in inflammation, including Desmin and Tgfb (Katsyuba and Mottis, 2018). Intriguingly, increasing the NAD concentration leads to promote pro-inflammatory cytokine synthesis by activated immune cells (Van Gool and Gallí M, 2009). Consistently, decreasing the NAD pool causes innate immune disorder in age-associated diseases (Minhas and Liu, 2019). In the present study, we found that the supplementation of NR obviously weakened Kupffer cell accumulation accompanied by inhibiting expression of IL-1β and TNF-α. In the context of lipid accumulation, macrophages are recruited into liver and pro-inflammatory cytokines subsequently produced in liver of aged mice. This data probably is discrepancy with several previous reports, thus further
evidences are still needed to confirm the relationship between NAD level and inflammatory reaction.

In conclusion, we show the proof that age-related NAD deficiency causes pathologic changes and inflammation infiltration in liver of aged mice. The replenishment of NAD, by treated with NR, is able to protect against age-induced hepatic steatosis, which is possibly associated with an improvement in reduction of pro-inflammatory cytokines, such as IL-1β and TNF-α. Our study raises the possibility of NR to alleviate liver injure in aged individuals, suggesting the clinical advantage of NR during Vitamin supplement therapy. Further investigations are warranted to treat age-related liver diseases by NAD supplementation strategy.

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References


Figure 1 Effects of NR repletion on the body weight, food intake, relative liver weight and fat pad mass of aged mice. Compared with the young, aged C57BL/6 mice were treated with NR (400 mg/kg/day) for 3 consecutive months. A. Effect of NR repletion on the body weight of aged mice. B. Effect of NR supplementation on the food intake of aged mice. Changes of relative liver weight (C) and fat pad mass (D) caused by NR treatment. Values are mean ± SEM, (n=5-6 per group). *P<0.05, **P<0.01, ***P<0.001 vs Young mice, #P < 0.05 vs aged mice.
Figure 2 Effect of NR repletion on the development of the NAFLD in aged mice. The levels of triglyceride (A), total cholesterol (B), ALT (C), AST (D) and NAD (E) in serum of aged mice. F. hepatic steatosis was reduced by NR administration in aged mice. The percentage of classified livers in each of the four steatosis categories in different groups was as follows: 0, no vacuolation; 1, 2 or 3 vacuoles; 2, less than 50% of fatty vacuolation; 3, more than 50% of fatty vacuolation. G. Representative images stained with H&E of liver tissues of aged mice (400×; scale bar, 50μm); box regions are shown at higher magnification under the original pictures. Values are mean ± SEM, (n=5-6 per group). *P<0.05 vs Young mice, #P < 0.05 vs aged mice.
Figure 3 Effect of NR repletion on liver fibrosis in aged-relative NAFLD model. A. Masson's staining in livers from aged mice with NAFLD. Red arrows show positive blue staining for masson. B. Protein level of TGF-β was detected by immunohistochemistry. Red arrows show positive brown staining for TGF-β. 200×; Scale bars, 100μm. Values are mean ± SEM, (n=5-6 per group). **P<0.01, ***P<0.001 vs Young mice, #P < 0.05, ###P<0.001 vs aged mice.
Figure 4 Effect of NR repletion on inflammatory infiltration of age-related NAFLD model. A. Immunohistochemistry staining in liver of aged mice showed the effect of NR supplementation on Kupffer cell accumulation. Red arrows show positive brown staining for F4/80 or CD68. B. Protein levels of IL-1β and TNF-α were detected by immunohistochemistry. Red arrows show positive brown staining for IL-1β or TNF-α. 200×; Scale bars, 100μm. Values are mean ± SEM, (n=5-6 per group). *P<0.05.
Figure 1

Effects of NR on the body weight, food intake, relative liver weight and fat pad mass in aged mice
Figure 2

Effect of NR repletion on the development of the NAFLD in aged mice

A

B

C

D

E

F

G

Young

Aged

Aged+NR
Figure 3

Effect of NR repletion on liver fibrosis in aged-relative NAFLD model
Figure 4

Effect of NR repletion on inflammatory infiltration of age-related NAFLD model

A

- F4/80
- CD68

Aged

Aged+NR

B

- IL-1β
- TNF-α

Aged

Aged+NR

Immunostaining density (fold)

F4/80 CD68 IL-1β TNF-α

Aged Aged+NR

*