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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.

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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

Introduction

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 trytophan 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) (Jęśko 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.

125 ## Materials & Methods

126 **Animals**

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

135 **NR supplementation**

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

145 **Body weight and food intake determinations**

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

149 **Liver weight and fat pat mass measurements**

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

155 **Blood biochemical assays**

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

164 **Histological analysis**

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

170 Scoring for steatosis (severity and extension) was performed in a blinded and independent
171 method by two observers as described before (Gariani and Menzies, 2016). The analysis used a
172 scale of 0-4, where 0 referred to absent of vacuolation in the hepar, 1 referred to 2 or 3 vacuoles
173 per hepatic cord per lobule, 2 referred to less than 50% of the lobule has fatty vacuolation, 3
174 referred to more than50% of the lobule has fatty vacuolation, and 4 corresponded to nearly the
175 entire lobule has fatty infiltration. Moreover, the focal extention was referred to 1, multifocal
176 was referred to 2, and almost total diffuse was referred to 3.

177 **Immunohistochemical analysis**

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

186 **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 $P < 0.05$.

215 ## Results

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

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

225 **NR favoured lipid homeostasis and hepatic steatosis in aged mice**

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

240 **NR improved hepatic fibrosis in aged mice**

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

244 **NR alleviated inflammation infiltrated in liver of aged mice**

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

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

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

300 NAD is a substrate for multiple enzymes of sirtuin family and participates in multiple cellular
301 functions, including DNA repair, energy metabolism, and regulation the activity of the sirtuins
302 by transcriptional control (Hoxhaj and Ben-Sahra, 2019). It is well-established that aging and
303 fatty liver related dysfunction leads to a pronounced effect on decline of NAD concentration in
304 liver. *Fan et al.* have reported that the expression of heptic mRNA of regulating NAD

305 biosynthesis is greatly reduced in aged mice or challenged high-fat diet (HFD) mice (Fan and
306 Cui, 2018). Additionally, this phenomenon seems to be a toxic element, providing destructive
307 actions because a shortage of NAD links ageing to progressive liver damage. The beneficial
308 effects of NAD regiment on fatty liver have been reported, for instance in a liver-specific Sirt1
309 knockout mouse (Katsyuba and Mottis, 2018) and in an enzyme-dead NAMPT transgenic mouse
310 (Zhou and Yang, 2016). Thus, supplementation NAD pool may be an attractive therapy strategy
311 for liver damage related diseases in elderly individual. Notably, NR, this vitamin B3 analog, as a
312 precursor of NAD biosynthesis, is commonly used to boost NAD pool (Jiang and Zhou, 2019).
313 Here we showed that the replenishing of NR has beneficial effect on liver of aged mice. Our
314 study demonstrated that aged mice administrated of NR (250 mg/kg/day) for 3 months improved
315 lipid disordered. Moreover, NR treatment exhibited an amelioration in hepatic steatosis and
316 fibrosis that was matched by an augmented blood NAD concentration, implying a systemic NAD
317 replenishing in aged mice.
318 An extensive body of evidence indicates that chronic inflammation contributes to the
319 degenerative changes of full-length tissues in the context of aging. Even normal brain of aged
320 individual is characterized by increased inflammation and subsequently elevated pro-
321 inflammatory cytokines (Frank and Barrientos, 2006). As inflammation rose by age shows a
322 reduction in adequate NAD content in brain of the murine (Braidy and Guillemin, 2011).
323 Previous study has also demonstrated that genetic blockade of NAD synthesis exerts
324 inflammatory effects on the liver reflecting by activation NLRP-3 inflammasome pathway and
325 production of IL-18 and IL-1β (Jiang and Zhou, 2019). The other independent group shows that
326 pharmacological inhibition of de novo NAD synthesis strengthens transcription genes involved
327 in inflammation, including Desmin and Tgfb (Katsyuba and Mottis, 2018). Intriguingly,
328 increasing the NAD concentration leads to promote pro-inflammatory cytokine synthesis by
329 activated immune cells (Van Gool and Gallí M, 2009). Consistently, decreasing the NAD pool
330 causes innate immune disorder in age-associated diseases (Minhas and Liu, 2019). In the present
331 study, we found that the supplementation of NR obviously weakened Kupffer cell accumulation
332 accompanied by inhibiting expression of IL-1β and TNF-α. In the context of lipid accumulation,
333 macrophages are recruited into liver and pro-inflammatory cytokines subsequently produced in
334 liver of aged mice. This data probably is discrepancy with several previous reports, thus further

335 evidences are still needed to confirm the relationship between NAD level and inflammatory
336 reaction.
337 In conclusion, we show the proof that age-related NAD deficiency causes pathologic changes and
338 inflammation infiltration in liver of aged mice. The replenishment of NAD, by treated with NR,
339 is able to protect against age-induced hepatic steatosis, which is possibly associated with an
340 improvement in reduction of pro-inflammatory cytokines, such as IL-1β and TNF-α. Our study
341 raises the possibility of NR to alleviate liver injure in aged individuals, suggesting the clinical
342 advantage of NR during Vitamin supplement therapy. Further investigations are warranted to
343 treat age-related liver diseases by NAD supplementation strategy.
344

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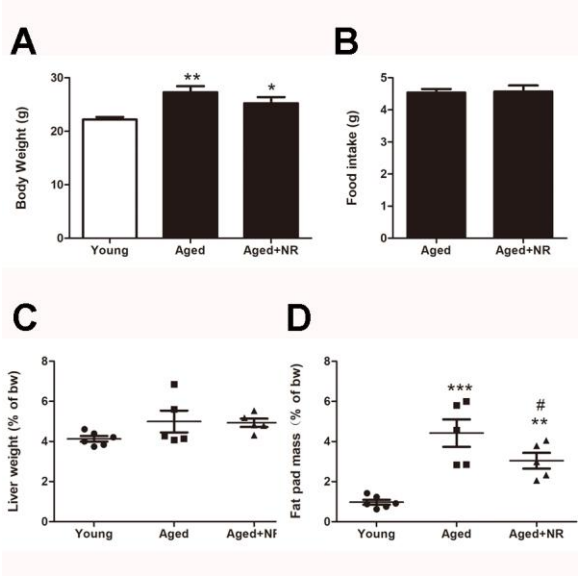


Figure 1 Effects of NR repletion on the body weight, food intake, relative liver weight and fat pad mass of aged mice. Compared with the young, aged C57BL/6 mice were treated with NR (400 mg/kg/day) for 3 consecutive months. A. Effect of NR repletion on the body weight of aged mice. B. Effect of NR supplementation on the food intake of aged mice. Changes of relative liver weight (C) and fat pat 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.

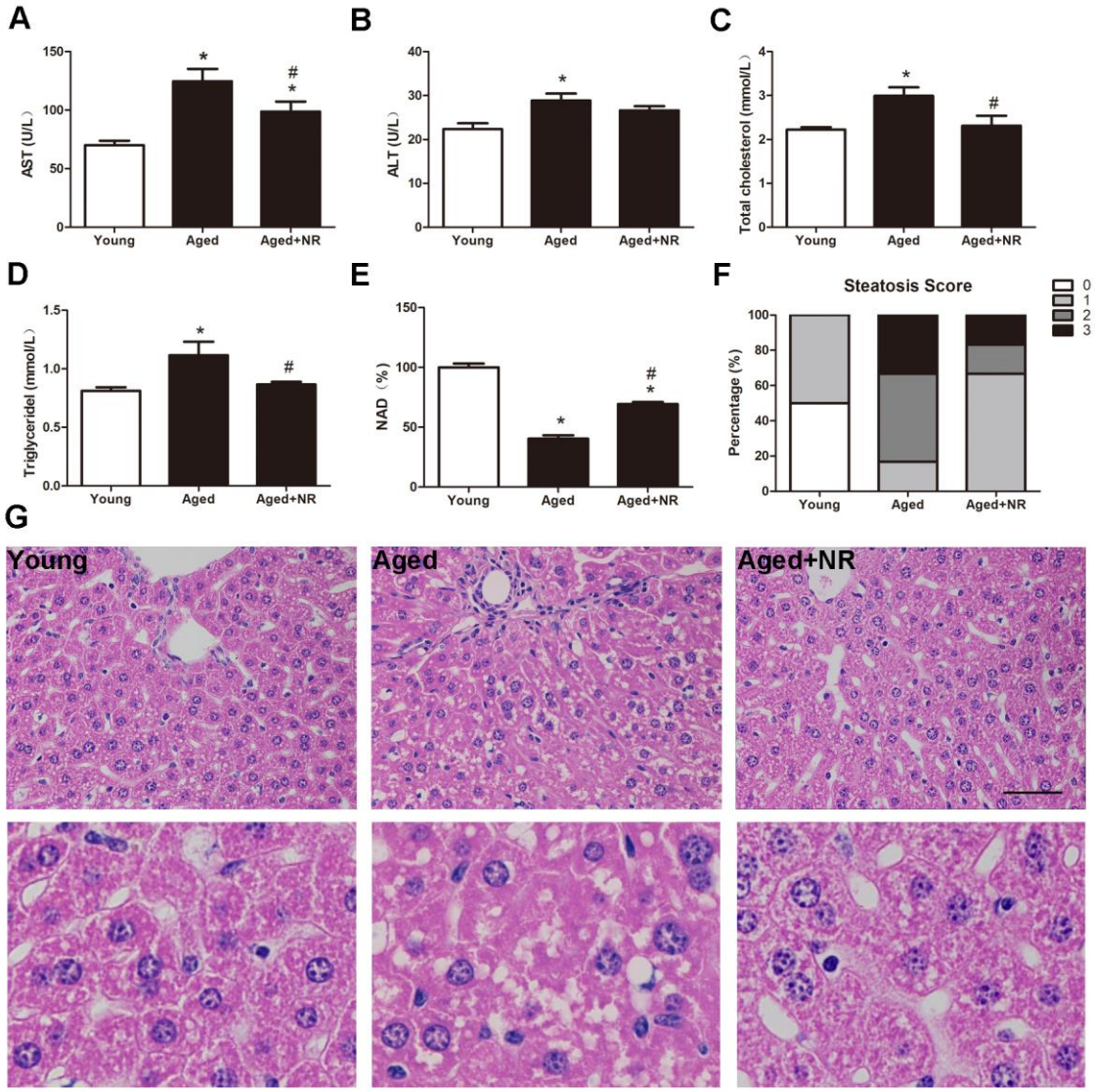


Figure 2 Effect of NR repletion on the development of the NAFLD in aged mice. The levels of triglyceride (A), total cholesterol (B), ALT (C), AST (D) and NAD (E) in serum of aged mice. F. hepatic steatosis was reduced by NR administration in aged mice. The percentage of classified livers in each of the 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 than50% 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.

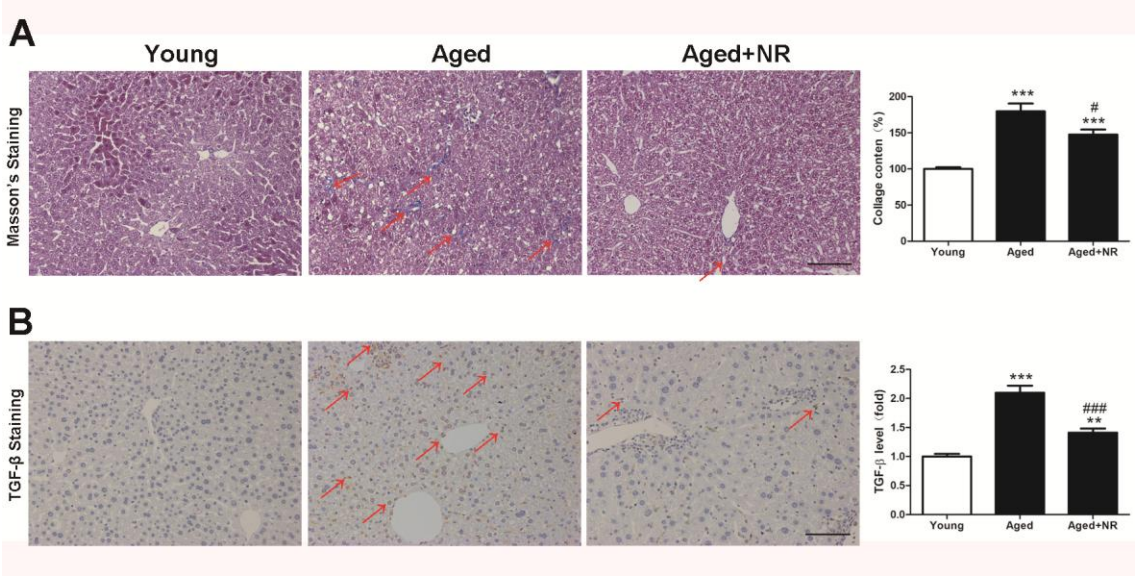
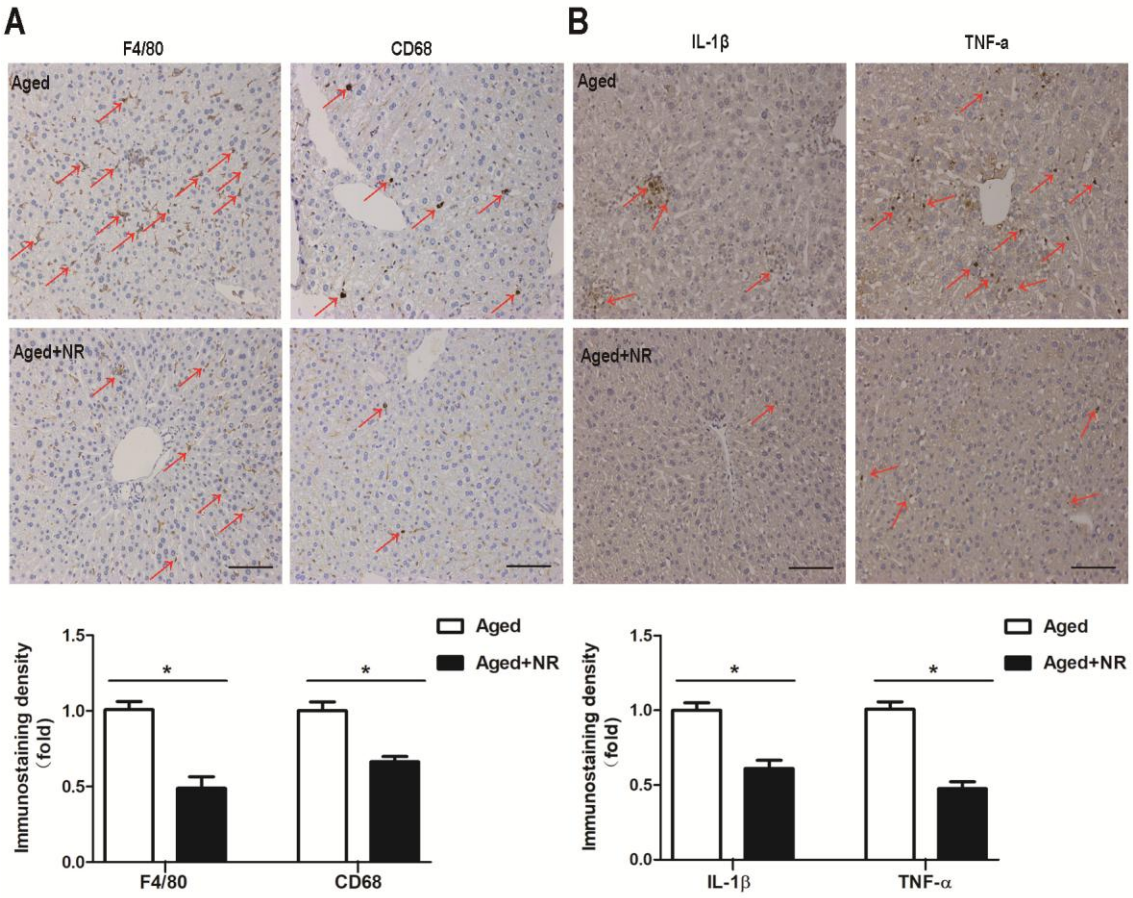


Figure 3 Effect of NR repletion on liver fibrosis in aged-relative NAFLD model. A. Masson's staining in livers from aged mice with NAFLD. Red arrows show positive blue staining for masson. B. Protein level of TGF-β was detected by immunohistochemistry. Red arrows show positive brown staining for TGF-β. 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.



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515 **Figure 4** Effect of NR repletion on inflammatory infiltration of age-relatived NAFLD model. A.
516 Immunohistochemistry staining in liver of aged mice showed the effect of NR supplementation
517 on Kupffer cell accumulatiom. Red arrows show positive brown staining for F4/80 or CD68. B.
518 Protein levels of IL-1β and TNF-α were detected by immunohistochemistry. Red arrows show
519 positive brown staining for IL-1β or TNF-α. 200×; Scale bars, 100μm. Values are mean ± SEM,
520 (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

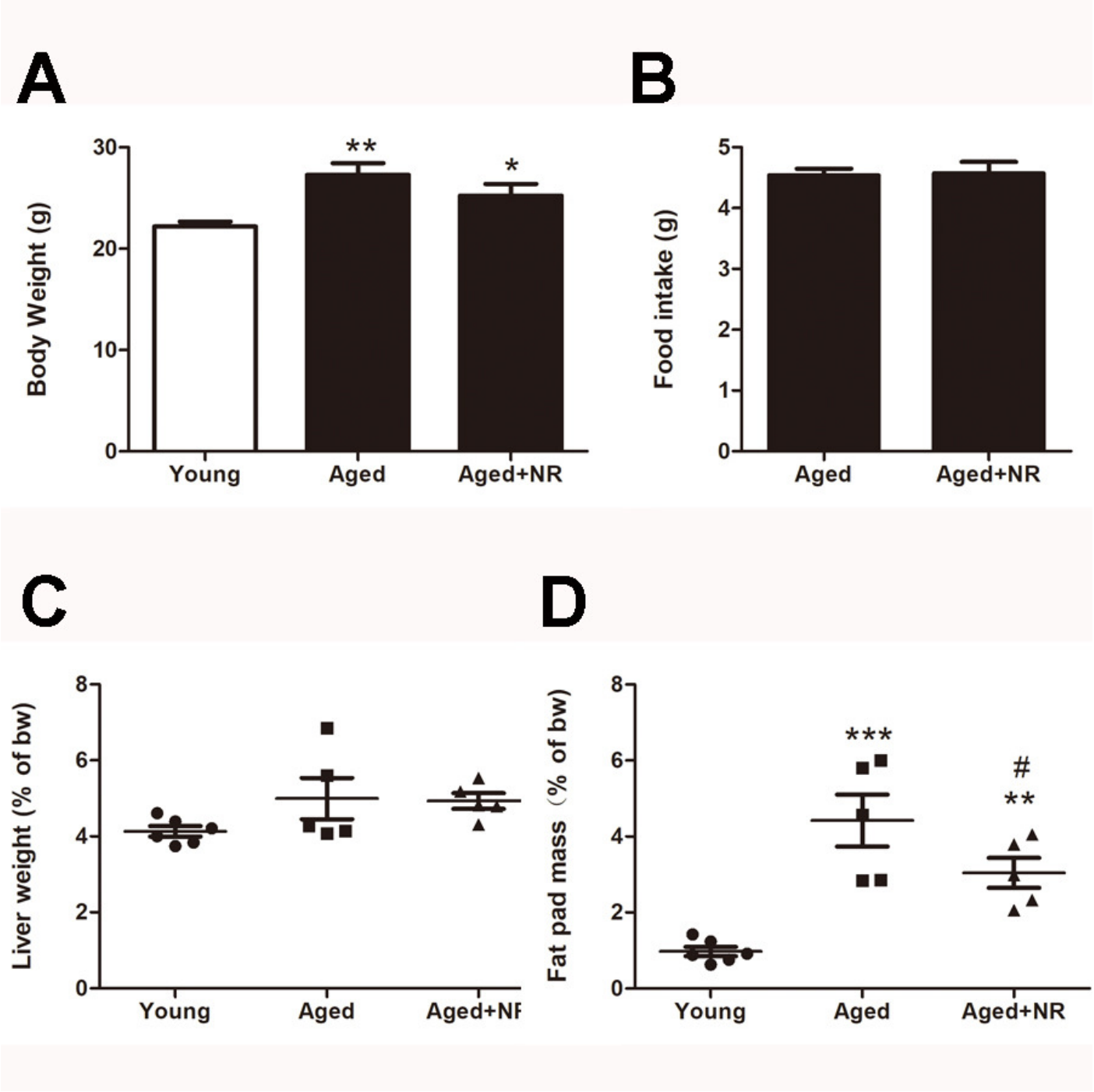

Figure 2

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

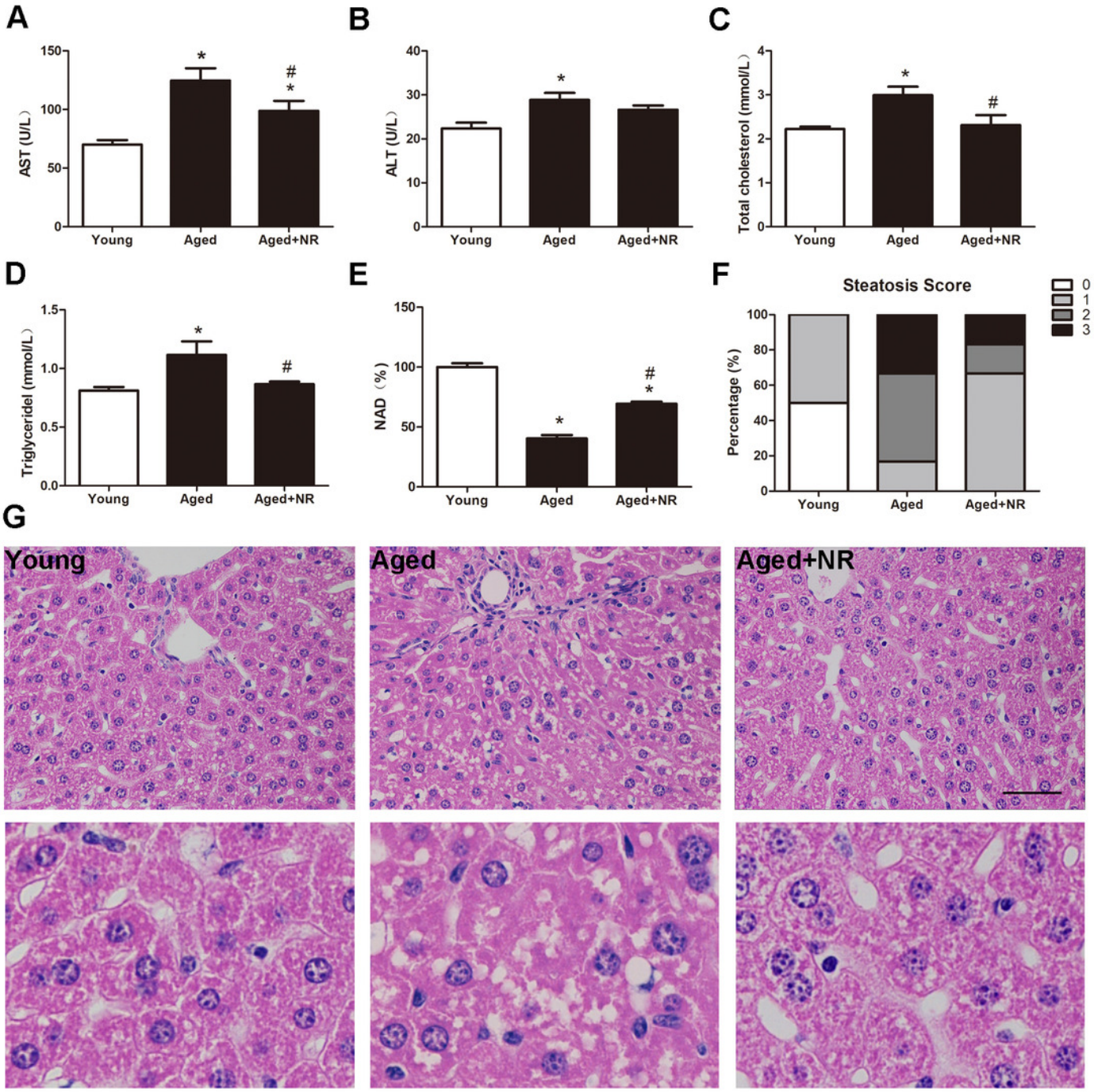

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Figure 3

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

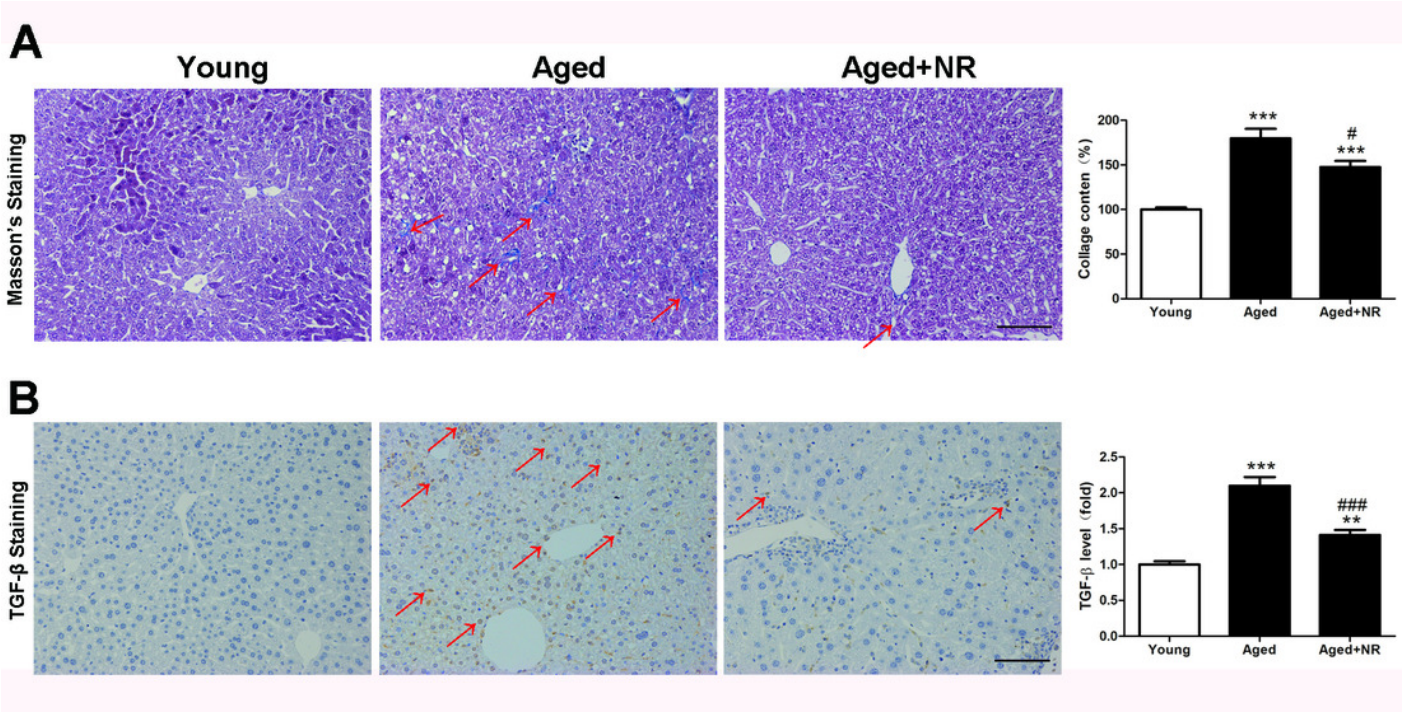

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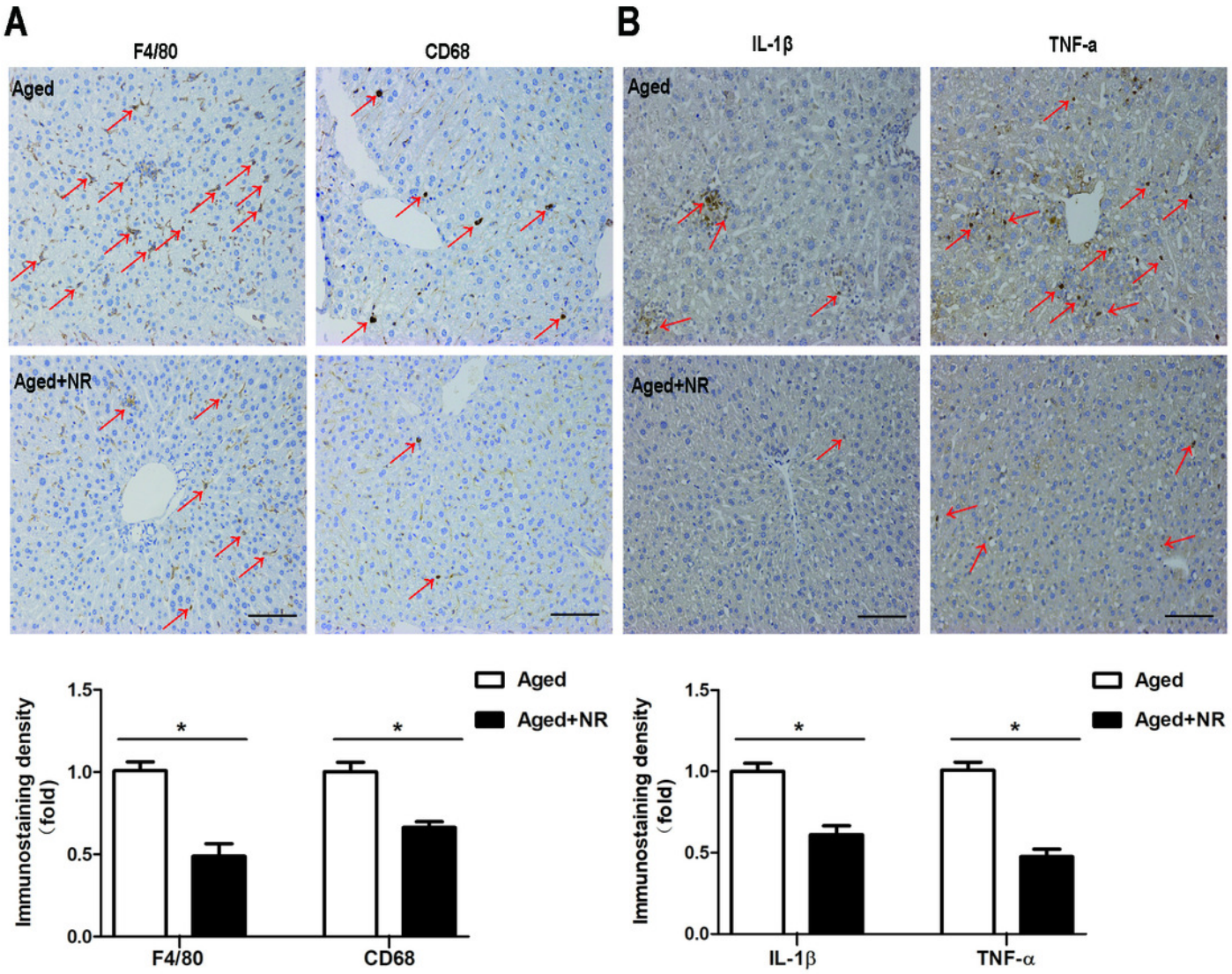
Figure 4

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