

The low protein diet affects the nonspecific inflammatory response of middle-aged and old mice through mTOR

J.-H. GUO, H.-Y. WANG, Q. ZHANG, M. FENG, L.-J. ZHANG

Department of Pathophysiology, Basic Medical College, Shanxi Medical University, Taiyuan, Shanxi, China

Jianhong Guo and Haoying Wang contributed equally to this work

Abstract. – **OBJECTIVE:** To explore the effect of low protein diet on nonspecific inflammatory changes in mice during aging and related mechanisms.

MATERIALS AND METHODS: Thirty-two 14-month-old female KM mice were randomly divided into 4 groups: control group, low protein group, high protein group, high protein + rapamycin group. Hematoxylin-eosin (HE) staining was performed to observe the pathological changes of the liver. Immunohistochemistry of liver sections was performed to detect the expression of CD68 protein. HE staining of colon sections was performed to observe intestinal lymphocyte infiltration. The percentage of spleen CD4⁺ T and CD8⁺ T cells was detected by flow cytometry. The mTOR expression in the liver was detected by Western blot and immunohistochemistry.

RESULTS: Compared with the control group, HE staining of liver tissue sections in high-protein group showed the cytoplasm of hepatocytes was loose and disordered, and the hepatic sinus was significantly expanded. Immunohistochemistry of the liver showed a significant increase in CD68 protein expression. Colorectal HE staining showed extensive lymphocyte infiltration. The number of CD4⁺ T and CD8⁺ T cells in spleen flow cytometry was significantly decreased ($*p < 0.05$). Western blot and immunohistochemistry detected a significant increase in mTOR expression in the liver ($*p < 0.05$, $*p < 0.05$). In the High Protein+Rapamycin group and Low-protein group, the time-dependent changes were reduced, the numbers of CD4⁺ T cell and CD8⁺ T cell in the spleen were significantly increased ($*p < 0.05$) and the expression of mTOR was significantly reduced ($*p < 0.05$).

CONCLUSIONS: Low-protein diet is beneficial for delaying the non-specific inflammatory changes of liver and intestines in middle-aged and aged mice, and this effect may be achieved through down-regulation of mTOR.

Key Words:

Middle-aged and old, Inflammatory response, mTOR, Low-protein diet.

Introduction

In recent years, aging has attracted more and more attention. It is characterized by the progressive decline of the time-related function of organisms leading to increased susceptibility to death¹. Many researches²⁻⁴ have suggested that aging is associated with significant changes in the immune system that can promote disease, physical weakness and death. Immunosenescence refers to aging-related decline of the antigen-specific immune response, which increases the susceptibility of the body to infection and cancer². Inflamm-aging, which can significantly impair patient health and increase mortality⁵, is another determinant factor affecting the aging and it is closely related to the incidence of diseases such as Alzheimer's disease, Parkinson's disease, and cancer⁶. Therefore, exploring the molecular events occurring in cells during the aging process is necessary to understand the pathogenesis of age-related diseases. At present, lifespan regulatory signaling pathways has been found mainly as follows: (1) the sirtuin family of Nicotinamide adenine dinucleotide (NAD)-dependent enzymes, the insulin/insulin-like growth factor (IGF) signaling (IIS) pathway; (2) the mechanistic Target of Rapamycin (mTOR) kinase and its downstream effectors⁷. The mTOR kinase, a serine/threonine protein kinase belonging to the phosphoinositide 3-kinase-related family

(PIP3), is highly conserved in the evolution of eukaryotes. In mammals, there are two different complexes of mTOR, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). Among them, the former can be inhibited by the immunosuppressive rapamycin, and the latter is relatively insensitive to rapamycin⁸. Studies have shown that mTORC1 is a metabolic sensor for nutrients, growth factors, energy and stress, and there are many downstream effectors, including regulation of ribosome biogenesis, autophagy, protein translation regulation, lipid synthesis, mitochondrial metabolism, pyrimidine synthesis⁹, and it can regulate the aging-related secretion phenotype¹⁰. However, studies on mTORC2 are relatively rare and mTORC2 has been found to be less sensitive to the acute effects of rapamycin. mTORC2 has been found to be associated with metabolic regulation, cytoskeletal dynamics, modulation of cell polarity and cell survival¹¹. In general, when mTOR acts as an energy sensor, it responds to rich nutrients, enhances certain anabolic processes after activation, and can inhibit catabolic processes to promote cell growth and proliferation⁹. On the contrary, when nutrients are limited, mTOR is suppressed and the opposite occurs. Although some studies have shown that mTOR plays a role in the process of aging, the specific mechanism remains unclear. Dietary restriction (DR) is a type of dietary intervention that reduces the intake of single or multiple nutrients without causing malnutrition¹². DR is the most robust environmental regulator that delays the aging in a variety of animals. Some authors have shown that DR can extend lifespan of yeasts, worms, fruit flies and even rodents¹³, and recently it has even been found to be effective to extend lifespan of primates¹⁴. DR also retards the progression of most age-related diseases, including cancer, neurodegenerative diseases, and cardiovascular disease¹³. In practice, DR manifests as a reduction in calorie intake, or limits the intake of one or more specific nutrients. Extensive evidence shows that mTOR, as a nutrition sensor, can extended life span by regulating multiple processes of metabolism under DR conditions¹⁵. Herein, we selected different protein diets as intervention factors to investigate the effects of non-specific inflammatory changes in middle-aged and aged mice and the potential molecular mechanisms by observing changes in liver Kupffer cells, intestinal lymphocytes, and splenic T cells.

Materials and Methods

Materials

A total of 32 female KunMing (KM) rats (65±5 g), aged 14 months, were obtained from Beijing Vital River Laboratory Animal Technology Co., (Beijing, China). The duration of treatment for these mice was 3 months. The experimental process complies with the standards of GB14922-94 Laboratory Animals-standards and monitoring for parasitology of the Quality and Technology Supervision Bureau of the People's Republic of China, Animal management regulations of the People's Republic of China and related Ethical requirements. Rapamycin (Beijing Huamaike Biotechnology Co., Ltd., Beijing, China), CD68 antibody (Abcam, Cambridge, MA, USA), mTOR antibody (CST, Danvers, MA, USA), CD3⁺, CD4⁺ CD8⁺ antibody (Multi Sciences Biotech Co., Ltd., Hangzhou, China), secondary antibody (Proteintech Group, Inc., Thermo Fisher Scientific, Waltham, MA, USA), Diaminobenzidine (DAB), Eosin Dye, Normal Goat Serum For Blocking (Solarbio Biotechnology Co., Ltd., Beijing, China).

Animal Modeling

Experimental Animal Grouping

Thirty-two rats were randomly divided into 4 groups: Control group (Ctrl, n=8), Low-Protein Group (LPG, n=8), High-Protein Group (HPG, n= 8), and High Protein + Rapamycin Group (HPRG, n=8). The control group was given standard feed and the remaining 3 groups were given special formula feed. The HPRG group were injected intraperitoneally with rapamycin every other day at a drug concentration of 0.2 mg/ml (the first 50 mg/ml rapamycin stock solution was prepared in dimethylsulfoxide (DMSO) and diluted with normal saline to the working concentration) in a dose of 2 mg/kg and the other 3 groups were injected intraperitoneally with equal volume of normal saline every other day.

Special Diets

The standard basic feed was purchased from Animal Houses of Shanxi Medical University, and its nutrient composition was obtained from Laboratory Animals Science. The special diets were specially configured. Under the Public Dietitians (knowledge base) and the pre-experimental results, the protein content of the low-protein

Table I. Three major nutrient levels per 100 g diet.

	Standard basal diet	Low protein diet	High protein diet
Protein (g)	18.00	14.40	21.60
Fat (g)	6.00	6.00	6.00
Carbohydrates (g)	58.60	62.20	55.00
Others (g)	17.40	17.40	17.40
Total (g)	100	100	100

diet was lower than the protein content of the standard basal diet by 20%, the protein content of the high-protein diet was higher than 20% of the protein content of the standard basal diet, and the total energy per diet remained the same (balanced by carbohydrates). According to the energy supply ratio of the three nutrients (fat 9 kcal/g, protein and carbohydrate are 4 kcal/g), the content of the three nutrients in each 100 g different diet could be calculated (Table I). The special diet consisted of standard basal feed powder, cornstarch and Yili high protein skimmed milk powder. The three major nutrient contents of cornstarch and skimmed milk powder were purchased from the Food Composition Inquiry Form (Public Dietitian (Level 4)) and the milk powder instructions were followed. The results are shown in Table II.

Image Acquisition and Analysis

The Olympus (Tokyo, Japan) optical photomicrography system was used to obtain histological examination charts. Image-ProPlus 6.0 software was used to detect the optical density of the image. GraphPad Prism software (La Jolla, CA, USA) was used to draw statistical charts.

Statistical Analysis

Statistical analysis was performed using SPSS 16.0 software (SPSS Inc., Chicago, IL, USA). One-Way ANOVA was used as the test method. The data was expressed as $\bar{x} \pm s$. $p < 0.05$ is significant for the difference.

Results

The Liver Tissue HE Staining

Low-protein diet can delay the time-related structural changes in the liver of middle-aged and old mice and has a protective effect. The high protein diet has the opposite effect, which can be inhibited by rapamycin. Compared with the Ctrl group, the LPG group had dense hepatocyte cytoplasm and well-aligned hepatocyte cords (Figure 1B). In the HPG group, a large number of hepatocytes were disorganized with cytoplasm loose, hepatic sinusoidal expansion and lymphocytic infiltration locally (Figure 1C). Rapamycin can inhibit the high protein diet-induced liver histological changes.

Immunohistochemical Detection of CD68 Expression in the Liver Tissues

The LPG group displayed a significant lower level of CD68 than the control group ($p < 0.05$) (Figure 2B). Significant high expression of CD68 in the HPG group ($p < 0.05$) (Figure 2C) can be inhibited by rapamycin (Figure 2D).

The Colon HE Staining

A large number of lymphocyte infiltrations were observed locally in the colonic mucosa of the HPG group (Figure 3C), and rapamycin could inhibit non-specific intestinal inflammatory changes by the high protein diet-induced (Figure 3D). In the LPG group, compared

Table II. Dietary formula per 1000 kcal energy.

Grouping	Standard basal diet	Low protein diet	High protein diet
Standard basis feed powder (g)	277.47	218.11	208.05
Corn starch (g)	—	52.35	—
Yili High Protein Skimmed Milk Powder (g)	—	—	68.95

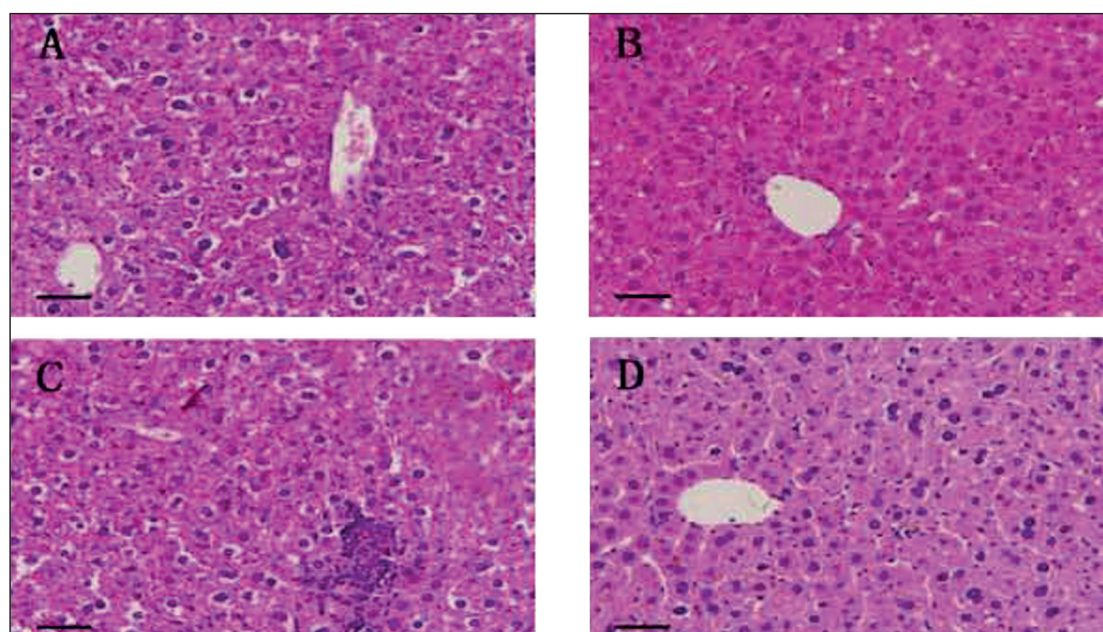


Figure 1. HE staining of representative liver sections ($\times 400$), *A*: Ctrl; *B*: LPG; *C*: HPG; *D*: HPRG.

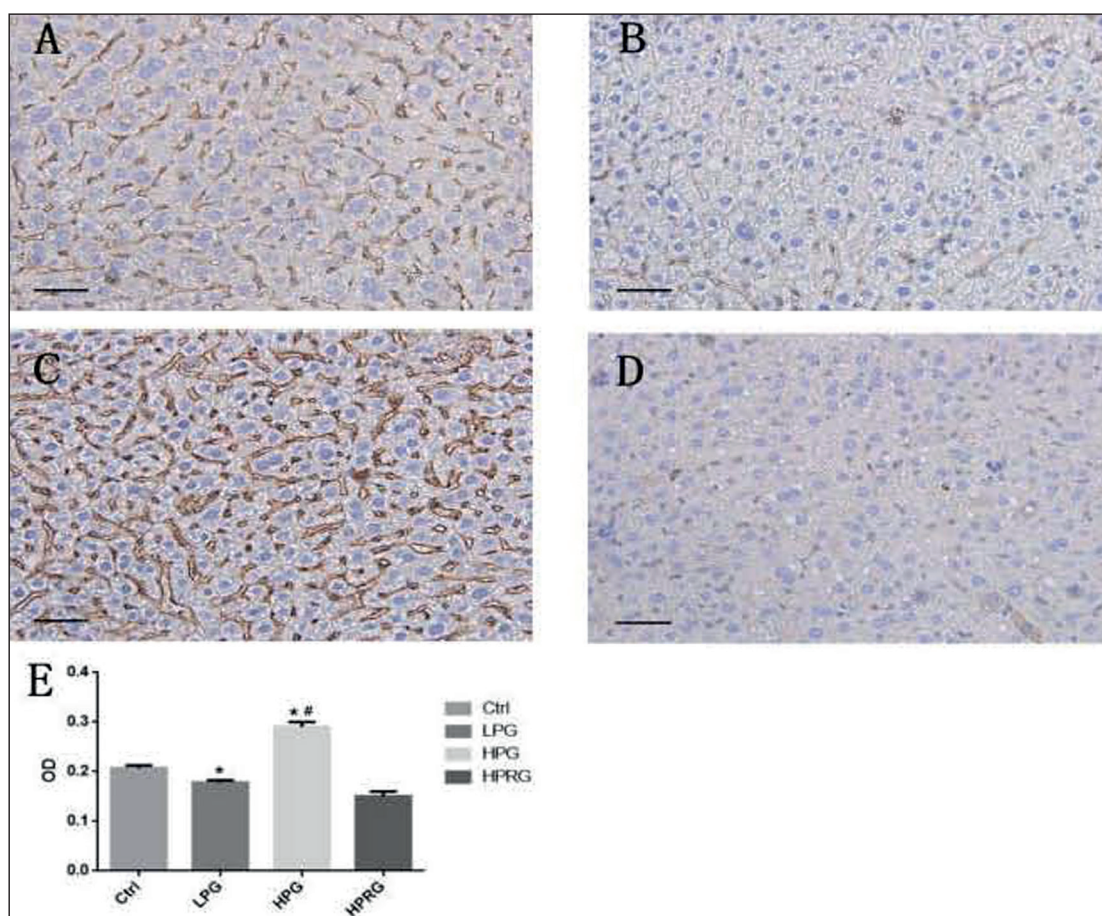


Figure 2. Liver CD68 expression was assessed by immunohistochemistry $\times 400$, scale = 500 μm , *A*: Ctrl; *B*: LPG; *C*: HPG; *D*: HPRG; *E*: Statistical plots: Each slice is randomly selected from five fields of view. Then, $t = 240/\text{Log}$ (gray value is measured, the average value is calculated, and it is converted into an OD value [ODue]). Finally a statistical graph is generated ($*p < 0.05$).

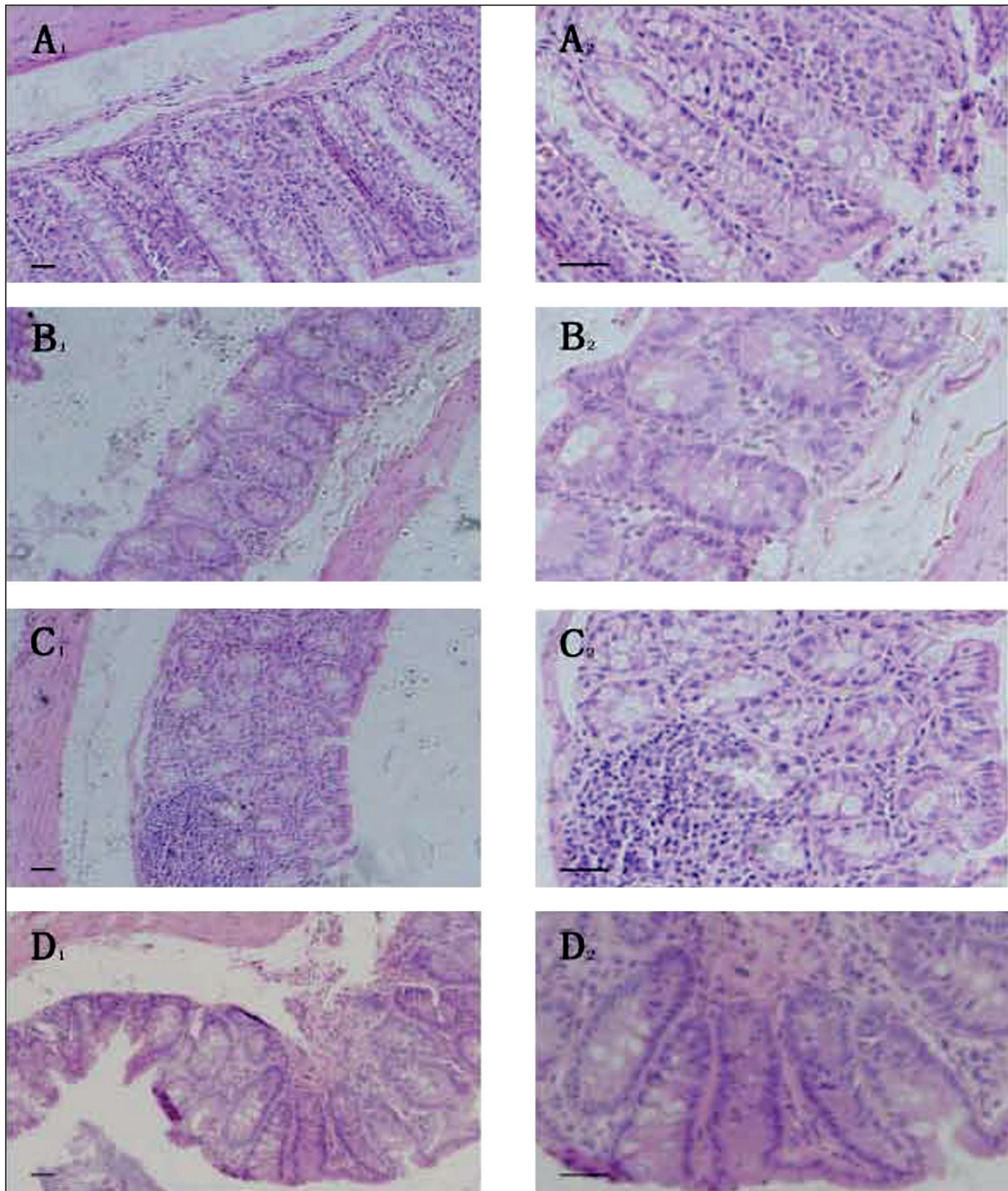


Figure 3. Colon HE staining 400 $\times$, **A:** Ctrl; **B:** LPG; **C:** HPG; **D:** HPGR. Figure 7.

with the control group, the secreted cells of the mucosal gland were significantly decreased, the gland structure was intact, and no obvious lymphocyte infiltration was observed (Figure 3B).

Flow Cytometry Detection of CD4⁺ T, CD8⁺ T Cell in the Spleen

The number of CD4⁺ T cells in LPG group was significantly higher than that in control group ($p < 0.05$) (Figure 4B). The high-protein diet had no

significant effect on CD4⁺ T cells (Figure 4C), but the number of CD4⁺ T cells in the HPGR group increased significantly (Figure 4D). The effect of different protein diets on CD8⁺ T cells in spleens of middle-aged and old mice was similar to that of CD4⁺ T cells (Figure 5).

The Expression of mTOR Protein in Liver

Immunohistochemical and Western blot analysis showed that, compared with the Ctrl group, the expression of mTOR in the LPG group was significantly reduced ($p < 0.05$) (Figure 6B, Figure 7). mTOR expression in the HPG group was significantly increased ($p < 0.05$) (Figure 6C, Figure 7), but it can be inhibited by rapamycin (Figure 6D, Figure 7).

Discussion

Modern medicine believes that aging, caused by the gradual deterioration of a series of physiological functions, is a multi-system and multi-organ degenerative disease which can directly lead to increased risk of multiple diseases and deaths¹⁶. We found that the hepatocytes increased the volume with cytoplasm loose and hepatic sinusoidal expansion in the Ctrl group, but there are still some binuclear hepatocytes at the replication stage, suggesting that the liver of old mice still has strong regeneration capacity. Morphological function studies suggest that, compared with other organs, the lifespan of the liver is much longer than the lifespan of indivi-

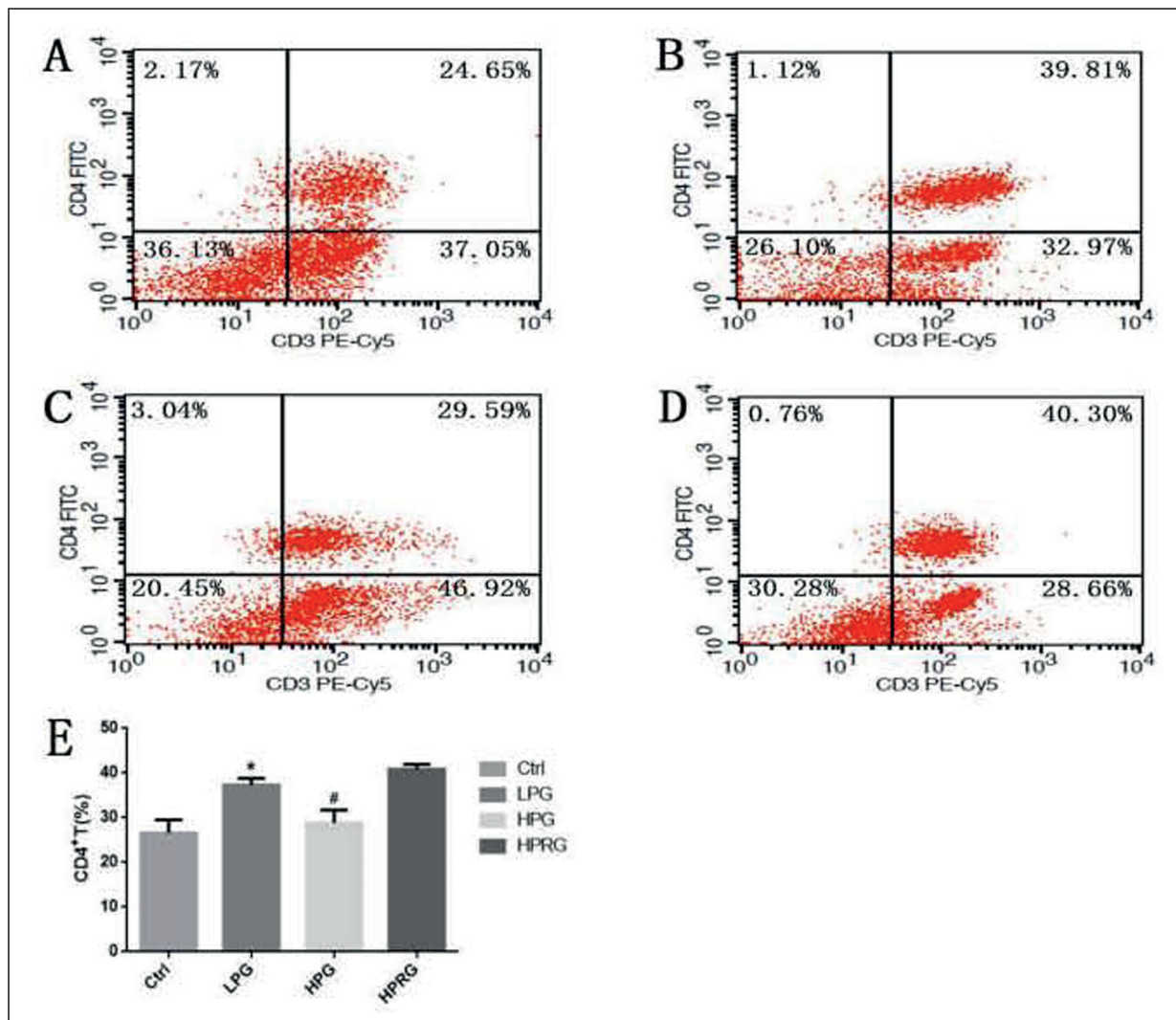


Figure 4. Percentage of splenic CD4⁺ T cells was measured by Flow cytometry, **A:** Ctrl; **B:** LPG; **C:** HPG; **D:** HPGR; **E:** Chart: * $p < 0.05$.

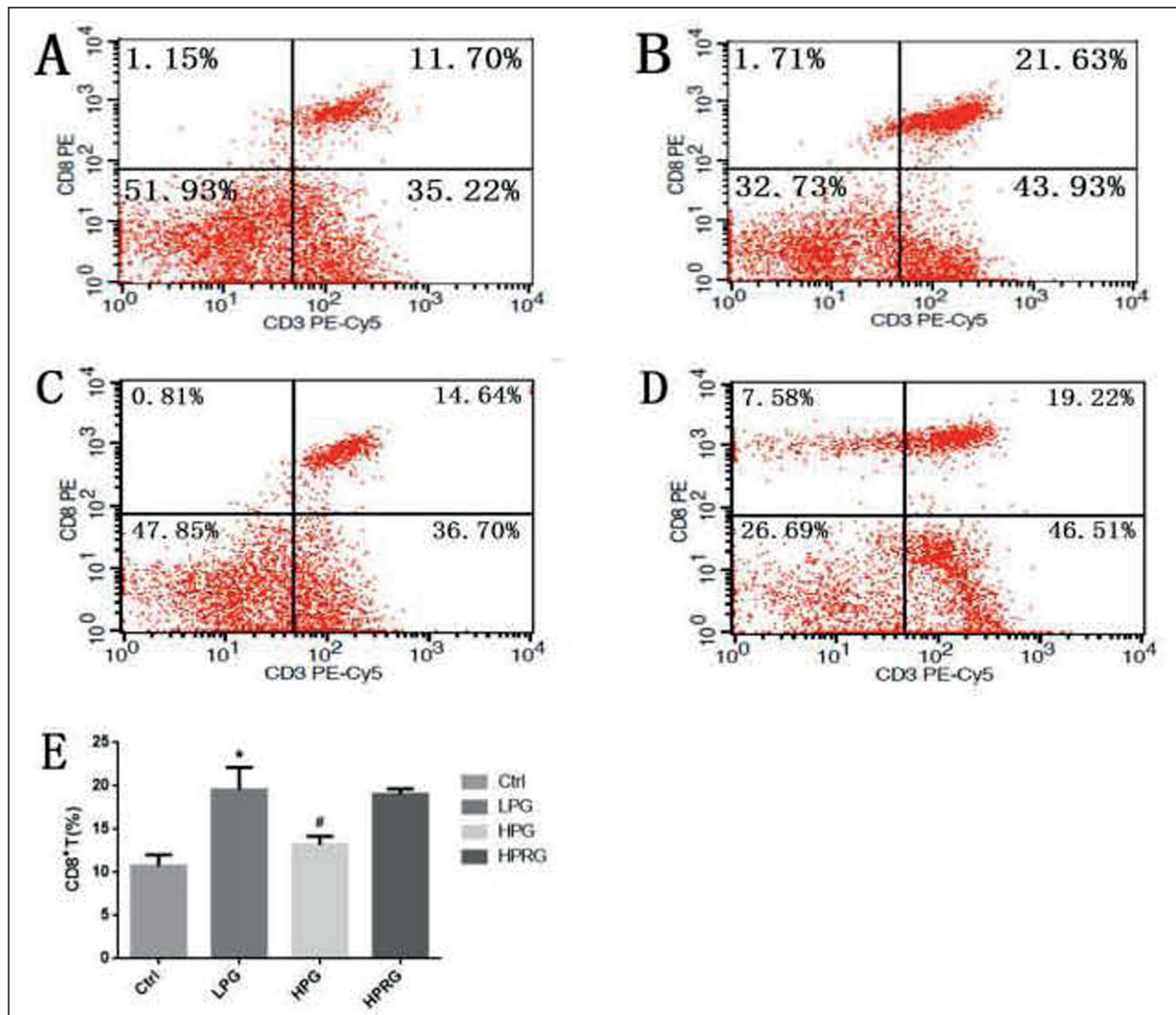


Figure 5. Percentage of CD8⁺ T cells in the spleen was measured by Flow cytometry, **A:** Ctrl; **B:** LPG; **C:** HPG; **D:** HPRG; **E:** Chart: * $p < 0.05$.

dual organisms and the main reason is that the liver has a long-lasting regenerative capacity¹⁷. By dietary administration of different protein concentrations, we found that, compared with the Ctrl group, the LPG group had dense hepatocyte cytoplasm and well-aligned hepatocyte cords, and the HPG group had disorganized structure and local lymphocyte infiltration. This suggests that the low-protein diet may have protective effects on liver tissue, while the high-protein diet may increase organ damage and accelerate aging. This conclusion, especially the effect of protein restriction on extending lifespan of lower organisms, is consistent with the results of caloric restriction currently confirmed by many research institutes¹⁵. In order to

further explore the possibility of non-specific inflammatory reactions in middle-aged and old mice, on the one hand, we measured the CD68 (mononuclear macrophage-specific molecular markers) of liver, to detect the change of the kupffer cell number. On the other hand, we detect infiltration of lymphocytes from the colon wall. Macrophages have roles in almost every aspect of an organism's biology ranging from development, homeostasis, to repair through to immune responses to pathogens. Both macrophages and lymphocytes belong to chronic inflammatory cells and are an important component of innate immunity. They play a central role in inflammatory responses and body defense. They can perceive a wide range of stimuli in

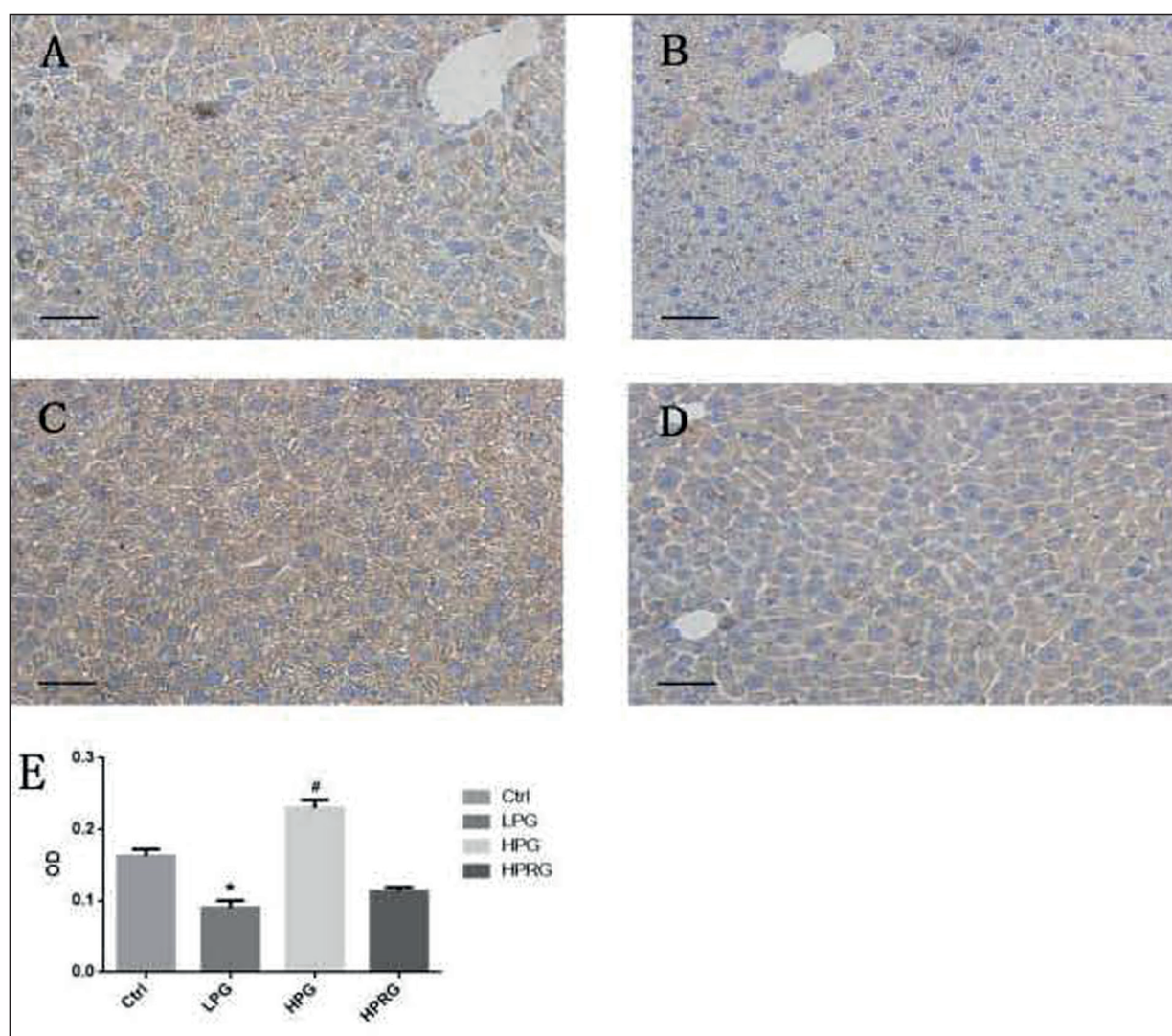


Figure 6. Liver mTOR expression was assessed by Immunohistochemistry $\times 400$, scale = 500 μ m, **A**: Ctrl; **B**: LPG; **C**: HPG; **D**: HPRG; **E**: Chart: Method of preparation as in Figure 2, $*p < 0.05$.

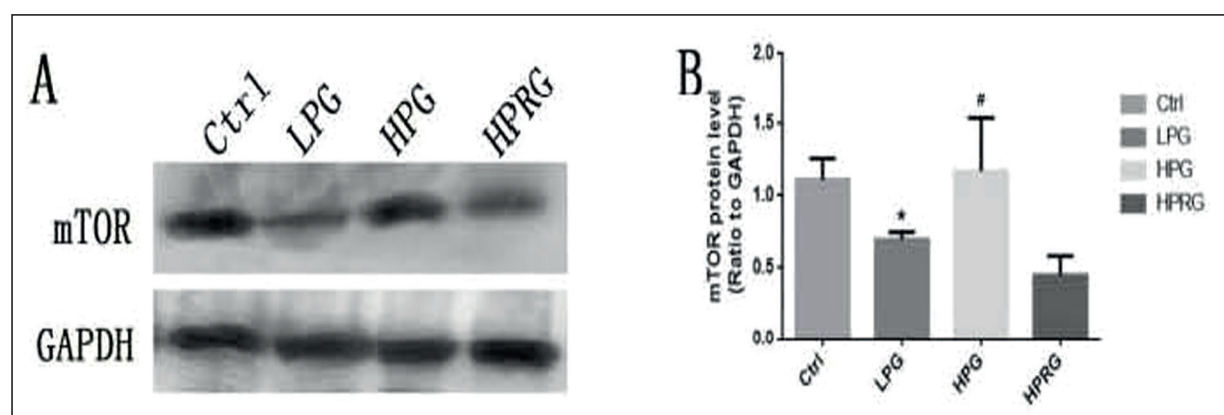


Figure 7. Expression of liver mTOR protein was assessed by Western blot, **A**: Ctrl; **B**: LPG; **C**: HPG; **D**: HPRG; **E**: Chart: Method of production same as Chart 4: $*p < 0.05$.

the environment and produce responses¹⁸. Kupffer cells, accounting for approximately 20% to 25% of the non-parenchymal cells of the liver under normal conditions, were the largest population of intrinsic macrophages in the body. As an important component of the innate immune system, Kupffer cells can be triggered by all kinds of endogenous and exogenous stimuli and play a key role in a variety of immune regulation processes¹⁹. Compared with the Ctrl group, the expression of CD68 in the liver of LPG group was significantly reduced and the lymphocyte infiltration in the colon wall was significantly reduced which suggested that the number of Kupffer cells in the liver and lymphocytes in the intestine increasing during the aging process, a wide range of non-specific inflammatory reactions may occur and low protein diet can reduce the corresponding inflammatory changes. Some researchers had referred to the hyperactivation of nonspecific immunity and the rise of pro-inflammatory factors during the aging process as inflamm-aging²⁰. This shows that the essence of aging is that the body's pro-inflammatory state increases with age²¹. The study found that high-protein diet increased the expression of CD68, suggesting that hepatic non-specific inflammatory reactions were aggravated, but it could be inhibited by rapamycin. To further study the changes of immune cells in aging and the effects of diet, we used flow cytometry to detect the content of T cells in spleen. T lymphocytes form an important cellular immune system in the body. According to their surface antigens, they can be divided into CD3⁺, CD4⁺ T cells and CD3⁺, CD8⁺ T cells subpopulations. The former is mainly helper T cells (Th), and the latter can be divided into cytotoxic T cells (Tc) and suppressor T cells (Ts)²². We found that compared with the Ctrl group, the number of the two spleen T lymphocytes in the LPG group was increased significantly and there was no significant improvement in the HPG group, suggesting that the ability of the mice to produce specific immune cells during the aging process was decreased. Some scholars called this age-related decline in antigen-specific response capacity as immune aging²³ which can increase the body's susceptibility to infection and cancer². The results showed that Kupffer cells increased, non-specific inflammation was enhanced, and generation of specific immune cells was reduced during the aging process in mice. Long-term low-protein diets has a protective

effect on age-related changes in the immune system of mice, while long-term high-protein diets have the opposite effect. The mTOR kinase is highly conserved in evolution. It promotes growth and development during the development of organisms, and further expression of the mTOR promotes some age-related changes in the body after maturation²⁴. Modern studies generally believe that this signal pathway can regulate longevity. When mTOR signal is reduced or inactivated, the lifespan of filariasis and fruit flies is prolonged, and organisms exposed to low-dose rapamycin can produce similar changes²⁵. Rapamycin, a specific inhibitor of mTOR, was isolated from the soil samples of the East Islands in the 1970s and is a macrolide compound with anti-fungal effects produced by *Streptomyces hygroscopicus*²⁶. In the 1990s, Heitman et al²⁷ discovered that mTOR was its target *in vivo*. The mechanisms by which mTOR lengthens life span are varied, and one important aspect is its effect on the immune system. Many immunological studies have found that mTOR plays an important role in regulating T cell proliferation, differentiation and formation of effector cells²⁸. In our work, immunohistochemistry and Western blot analysis of mTOR protein results showed that: long-term low-protein diet can reduce the expression of mTOR in liver of middle-aged and old mice and the results of the indicators in HPRG group and LPG group showed the same trend, indicating that Rapamycin inhibits the adverse effects of long-term high-protein diets on middle-aged and old mice. It is suggested that diet control can reprogram the metabolic process by reducing activity of mTOR, an important nutrient receptor in the body, thereby partially to extend the lifespan of the organism²⁹.

Conclusion

To sum up, we found that liver cell exhibited balloon-like changes during the aging process in mice, and the number of non-specific inflammatory cells in the liver and intestines increased, along with the decrease of the number of T lymphocytes in the spleen. Long-term administration of a low-protein diet, by downregulating the mTOR protein in the tissue, delayed the related changes in the aging process in mice. In the future, by examining the content of mTOR in other tissues and the changes of downstream

signal molecules of mTOR, as well as cell experiments, the research group will continue to further explore this issue and the role of mTOR in the immune system during the gradual aging of the body.

Acknowledgements

This study was supported by the Shanxi Natural Science Foundation (Grant number: 2013011054-2).

Conflict of Interest

The Authors declare that they have no conflict of interests.

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