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Effect of β-Casomorphin-7 on Dyslipidemia and Oxidative Stress in Aged Mice

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ABSTRACT

The impact of β-Casomorphin-7 (β-CM-7) on lipid metabolism was investigated in aged mice. Mice were treated without or with different doses of β-CM-7 for 30 days. At the end of the experiment, all mice were collected for serum and tissue samples. Histopathological studies showed the tissue protective role of β-CM-7 in aged mice. The treatment of β-CM-7 in aged mice significantly increased the triglyceride (TG) level with decreasing high density lipoprotein (HDL) level in serum. The superoxide dismutase (SOD), glutathione peroxidase (GPx) activities and malondialdehyde (MDA) level were significantly increased in liver tissues with β-CM-7 treated. β-CM-7 decreased the fatty acid synthase (FAS) level significantly. The results suggest that β-CM-7 can change the dyslipidemia which is induced by aging. The mechanisms of the regulating effects likely tend to pass through controlling the oxidative stress and balancing the level between FAS and acetyl-CoA carboxylase (ACC).

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Authors' Contribution
YH conceived and designed the experiments. YD, LJ, DJ and CD performed the data analysis. YD and HY wrote the manuscript drafts.

Key words
β-casomorphin-7, Aging, Dyslipidemia, Lipid metabolism, Oxidative stress.

INTRODUCTION

Our population is aging, but longevity does not always represent good health. There has been a considerable rise in the prevalence of age-related chronic, non-communicable diseases (WHO, 2017). An increasing number of our elderly population suffers from metabolic syndrome (MSyn), the characterizations of which include dyslipidemia (Iwaniak *et al*., 2018). The metabolic disorders of glycolipid metabolism, such as type 2 diabetes, hyperlipidemia, and osteoporosis, are the key factors for the aging of the body.

The versatile properties of bioactive milk peptides are increasingly recognized (Mohantya *et al*., 2016). It could show positive effects on human physiology and metabolism either, directly or through enzymatic hydrolysis *in vivo* or *in vitro* (Kitts, 2003). β-Casomorphins (βCM) belong to a family of opioid peptides derived from food protein. β-CM-7 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) was first separated from the enzymatic digest of bovine β-casein (Brantl *et al*., 1979). Current researches show that β-CM-7 can regulate glucose, antioxidative, immunological, gastrointestinal, hormonal and neurological responses (Kaminski *et al*., 2007; Yin *et al*., 2010, 2012; Zhang *et al*., 2013; Zoghbi *et al*., 2006). There is however no report about the effect of β-CM-7 on dyslipidemia in aged mice. Examples of peptide MSyn-biological activities involve antihypertensive, anti-oxidative, anti-obesity, anti-diabetic, and cholesterol level reducing effects (Iwaniak *et al*., 2018). Based on these which related to metabolism, we conjecture that β-CM-7 might have some regulatory (dyslipidemia) effects on aged mice.

The purpose of this study was to investigate the effect of β-CM-7 on dyslipidemia in aged mice.

MATERIALS AND METHODS

Chemicals and reagents

β-CM-7 was purchased from Nanjing Peptide Biotech Co., Ltd. (Nanjing, China). The commercial assay kits for measurement of monoamine oxidase (MAO), superoxide dismutase (SOD), malondialdehyde (MDA), fatty acid synthase (FAS), glutathione peroxidase (GPx) and acetyl-CoA carboxylase (ACC) were obtained from Jiancheng Biologic Project Company (Nanjing, China). All the other chemicals and drugs were of reagent analytical grade.

Animals

Forty elderly male KM mice (weighing 52-72g, 10 months old) and ten young KM mice (weighing 40-50g, 2 months old) were purchased from Nanjing Qinglongshan Animal Center (Nanjing, China). They were placed under controlled ambient conditions of temperature (22±2°C), 12 h light/12 h dark cycle, and maintained on standard food particles and tap water (unless otherwise specified). All animal care and procedures were conducted in accordance with animal health and welfare policies of the nation and institutions. All mice specimens collection and field study

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were guided by using precedure of Animal Management Committee of Jiangsu Province and the principles of laboratory animal care of Yangzhou University. The animals were acclimatized for 1 week before the study.

Experimental design

The young mice (n = 10) were as the matched group (group I) while the elderly mice (n = 40) were assigned into 4 groups randomly (group II to V, 10 per group): Group I (Con) is the normal control mice in whcih each animal was given free access to basal diet and intragastric administration of physiological saline solution for 30 days. Group II (M Con) is the model control mice in which each animal was given free access to a basal diet and intragastric administration of physiological saline solution for 30 days. Group III (L) is the β-CM-7 treated mice in which each animal was put on a basal diet and intragastric administration of the low dose of β-CM-7 (2×10^{-7} mol·d^{-1}, 30 d). Group IV (M) is the β-CM-7 treated mice in whcih each animal was put on a normal diet and intragastric administration of the moderate dose β-CM-7 (1×10^{-6}mol·d^{-1}, 30 d). Group V (H) is the β-CM-7 treated mice in which each animal was put on a normal diet and intragastric administration of the high dose β-CM-7 (5×10^{-6}mol·d^{-1}, 30 d).

Collection of serum and organ tissues

On the last day of the experiment, mice were fasted for 12 h, anaesthetized with ether and collected blood from the orbital plexus into clean centrifuge tubes. Serum samples were extracted from blood samples by centrifugation (3000 rpm, 4°C) for 10min and stored at −20°C until assayed. Sacrifice all mice and perform the postmortem examination immediately. Liver and spleen were dissected out, cleaned with phosphate buffer saline, blotted with filter paper and weighted. A part of the liver samples was fixed in 4% neutral-buffered polyoxymethylene while the rest was placed in liquid nitrogen rapidly and then stored at −70°C until analysis.

Serum parameters measurements

Biochemical analysis was performed to determine the levels of triglyceride (TG), glucose (GLU), high density lipoprotein (HDL) and low density lipoprotein (LDL) utilizing automated biochemical analyzer in accordance with the manufacturers' instructions.

Histopathological observation

Liver tissue fixed in 4% neutral-buffered polyoxymethylene were embedded in paraffin. Five-micrometer thick sections were cut and stained with hematoxylin and eosin (H&E) for histopathological observation. The histological changes were observed by light microscopic examination at a magnification of 20 ×.

Assays of SOD, GPx, MAO, MDA, FAS and ACC in liver tissues

Liver samples (500 mg) were homogenized with nine times the volume of ice-cold physiological saline. The homogenates were centrifuged at 3000 rpm for 15 min at 4°C to obtain the supermatant, and then determined their protein concentration by Coomassie light blue method. The level of MDA and the activities of SOD, GPx, MAO, FAS and ACC in liver tissues were estimated with commercial assay kits on the basis of the instructions.

Statistical analysis

Data were expressed as the mean ± SD of 10 mice per group though analyzed statistically using the SPSS 16.0 software. Experimental results were evaluated with one-way ANOVA followed by least significant difference (LSD) post-hoc tests. $P < 0.05$ or $P < 0.01$ were considered statistically significant.

Table I.- Effect of β-casomorphin-7 on organ index in mice (mean ± SD, n = 10)[a].

	Liver index/%	Slpeen index/%
Control	4.06±0.41	0.23±0.04
Model control	3.78±0.83	0.19±0.04
Low dose	3.62±0.46	0.17±0.03
Moderate dose	3.69±0.44	0.16±0.03
High dose	3.83±0.35	0.18±0.05

[a] Organ index (%) = Organ weight/Body weight × 100.

RESULTS

Liver and spleen index in different groups

The organ index can reflect the effect of intragastric administration on the organs of mice to a certain extent. As shown in Table I, the organ index of the model control group has a decreasing trend when compared with the normal control group. However, there was no significant difference in the organ index among these groups.

Histological analysis

The liver in the normal control group appeared almost normal upon histologic examination (Fig. 1A). H&E staining of liver sections found significant inflammation, infiltration, hepatocellular degeneration and the cell lining of the hepatic cords appeared abnormal in the model control group (Fig. 1B). Compared with the model control group,

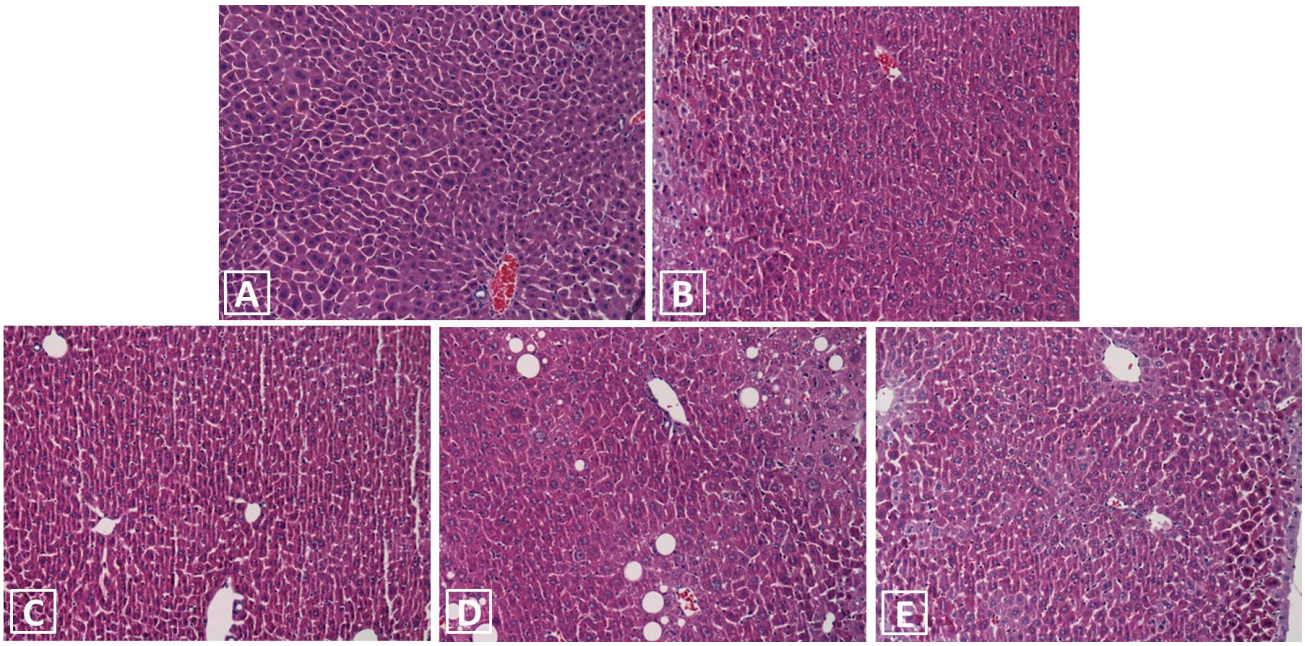

Fig. 1. Effect of β-casomorphin-7 on Histologic structure of liver tissues in aged mice. A, control; B, model control; C, low dose of β-casomorphin-7 treated; D, moderate dose of β-casomorphin-7 treated; E, high dose of β-casomorphin-7 treated.

significantly less cell degeneration and tissue damage were observed in mice treated with the low dose of β-CM-7, however, with the formation of lipid droplets (Fig. 1C). These changes were more obvious in the moderate dose group (Fig. 1D). There was no sign damage of liver tissues in the high dose group (Fig. 1E).

Table II.- Change of serum parameters (mean±SD, n=10).

	GLU (mmol/l)	TG (mmol/l)	HDL-c (mmol/l)
Con	5.59±1.35	2.82±0.75	1.88±0.28
M Con	6.03±1.31	1.67±0.46**	2.16±0.29 *
L	5.87±1.74	2.14±0.31 #	1.85±0.19 #
M	5.72±1.07	1.70±0.60	1.51±0.20 ##
H	6.14±1.28	1.70±0.38	1.78±0.44 ##

*$P < 0.05$ vs the control group; **$P < 0.01$ vs the control group; #$P < 0.05$ vs the model control group; ##$P < 0.01$ vs the model control group. L, low dose (2×10^{-7} mol·d^{-1}) of β-casomorphin-7 treated; M, moderate dose (1×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated; H, high dose (5×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated.

Serum parameters in different groups

We assayed the levels of serum TG, GLU, HDL-c and LDL-c with automated biochemistry analyzer so as to study the effect of β-CM-7 on abnormalities of lipid metabolism in the elderly mice, but the level of LDL-c could not be measured. As shown in Table II, the GLU level of the model control group was higher than the normal control group, but it was not significant.

Compared with the normal control group, the levels of TG ($P < 0.01$) and HDL-c ($P < 0.05$) were significantly decreased in the model control group. The treatments with the low dose of β-CM-7 significantly raised the TG level ($P < 0.05$) and reduced the HDL-c level ($P < 0.05$), and the treatments with the moderate and high doses of β-CM-7 extremely significantly reduced the HDL-c level ($P < 0.01$) when compared with the model control group.

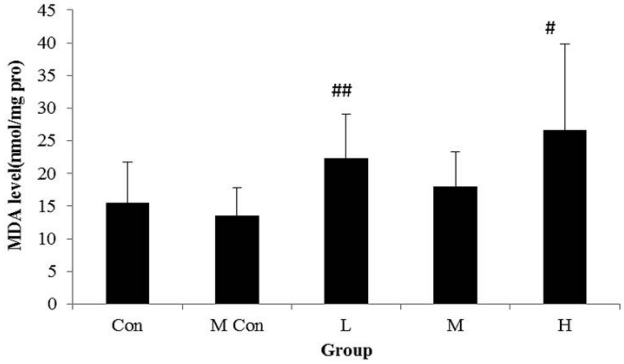

Fig. 2. Effect of β-casomorphin-7 on MDA level of liver tissues (mean ± SD, n = 10) of aged mice. #$P < 0.05$ vs the model control group; ##$P < 0.01$ vs the model control group; L, low dose (2×10^{-7} mol·d^{-1}) of β-casomorphin-7 treated; M, moderate dose (1×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated; H, high dose (5×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated.

Change of antioxidant capacities in liver tissues

As shown in Figure 2, the MDA level in liver tissues of the model control mice slightly decreased when compared with the normal control mice. Compared with the model control group, the treatments with the low ($P < 0.01$) and high ($P < 0.05$) doses of β-CM-7 significantly raised the MDA level.

As shown in Table III, the high dose of β-CM-7 increased the activity of SOD in liver tissues significantly($P < 0.05$), compared to the model control group. There was decrease in the GPx activity of the model control mice when compared with the normal control mice, though it was not significant. Compared with the model control group, the activity of GPx significantly increased in the low dose of β-CM-7 ($P < 0.05$).

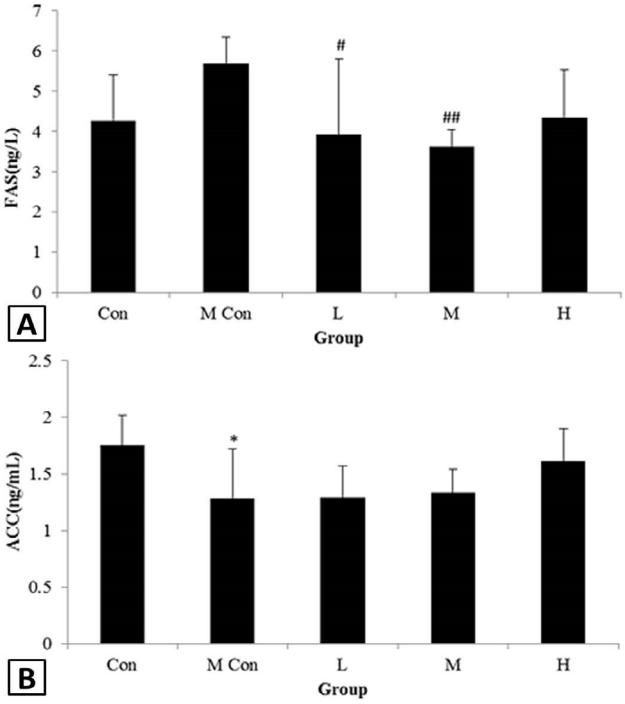

Fig. 3. Effect of β-casomorphin-7 on activities of FAS (A) and ACC (B) in liver tissues (mean ± SD, n = 10) of aged mice. $^{*}P < 0.05$ vs the control group; $^{\#}P < 0.05$ vs the model control group; $^{\#\#}P < 0.01$ vs the model control group; L, low dose (2×10^{-7} mol·d^{-1}) of β-casomorphin-7 treated; M, moderate dose (1×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated; H, high dose (5×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated.

Change of the enzymatic activities related with lipid metabolism in liver tissues

Enzymatic activities related with lipid metabolism in liver tissues are shown in Figure 3. The approximately significant increase was observed in the FAS level of the model control group when compared with the normal control group ($P = 0.06$). Compare to the model group, the treatments with the low ($P < 0.05$) and moderate ($P < 0.01$) doses of β-CM-7 significantly reduced the FAS level (Fig. 3A) and the high dose approximate significantly ($P = 0.07$). As shown in Figure 3B, the ACC level of the model control mice significantly decreased when compared with the normal control mice ($P < 0.05$). A uptrend of the ACC level were able to be observed with the dose of β-CM-7 treated adding, though it was not significant, while the treatments with the high dose of β-CM-7 raised the ACC level approximately significant ($P = 0.08$), compared with the model control mice.

Table III.- Change of antioxidant activity of liver tissues (mean ± SD, n = 10).

	SOD (U/mg pro)	GPx (U/mg pro)	MAO (U/mg pro)
Con	41.32±10.58	109.18±70.16	52.61±19.68
M Con	56.88±19.58	68.79±54.08	73.02±34.42
L	76.69±22.26	156.47±80.97 $^{\#}$	87.23±25.45
M	39.72±15.59	99.92±21.21	66.41±25.92
H	79.38±24.04 $^{\#}$	134.83±78.02	73.90±32.41

$^{*}P < 0.05$ vs the control group; $^{\#}P < 0.05$ vs the model control group; L, low dose (2×10^{-7} mol·d^{-1}) of β-casomorphin-7 treated; M, moderate dose (1×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated; H, high dose (5×10^{-6}mol·d^{-1}) of β-casomorphin-7 treated.

DISCUSSION

Aging is a major risk factor for many chronic, debilitating diseases or imbalances in human metabolism, it affects the life quality of the elderly and aggravates the burden of health care. There will be more and more cost paying for age-related diseases with the life extension of the world's population. Kaminski *et al*. (2007) noticed, 'β-CM-7 may play a role in the aetiology of human diseases'. Thus it would be advantageous to consider whether β-CM-7 has effects on and how to regulating dyslipidemia which is caused by aging (Aditi *et al*., 2015).

In the present study, animals tolerated well the experimental conditions and the weight was not statistically different between groups. The difference of organ index in each group is not significant which suggests that β-CM-7 does not have adverse effects on mice. Compared with the normal control group, the organ index of the model control group decreased but not significant, it points out that the aged mice in the experimental group did not enter the aging state completely (Ephraim *et al*., 1993) or they were reasonably healthy. The serum GLU level-no visible increase, the histological observation of the

liver tissues-no severe inflammation and necrosis, and the activity of MAO-no significant difference in the aged mice also demonstrated this point. However, the experiment discovered the serum TG level of an abnormality. It is interesting to see the decrease of the TG level which represents the aging of male mice (Liu and Li, 2015).

The disorder of lipid metabolism refers to the abnormality of lipids and their metabolites in blood or other tissues and organs produced by congenital or acquired factors. Aging can cause the lipid metabolism which arouses changes in the body's environment and excessive oxygen free radicals, that leads to oxidative stress. SOD and GPx are the key antioxidant enzymes existed in liver.

Reactive oxygen species such as free radicals and peroxides may cause some damage of important cellular components, and the two enzymes can prevent it to a certain extent (Edwin *et al*., 2016). MDA is the product of lipid peroxidation (Zhu *et al*., 2017). MAO is a positive enzyme related to aging, which is also one of the indexes of hepatocirrhosis. Its raised activity leads to the increased decomposition of monoamine transmitters and the metabolic disorder of cells, which results in cell aging. It has shown that β-CM-7 in A1 beta-casein play a physiological role in the oxidation of peroxidation of lipid components (Stanisław *et al*., 2007).

In this study, the MDA level and SOD, GPx and MAO activities in liver homogenates were measured. The mice treated with β-CM-7 were shown with significant increase in SOD and GPx activities. It is basically consistent with the previous reports (Yin *et al*., 2012). However, unlike the previous findings, an increase in MDA level was observed with β-CM-7 in our results. The histological observation showed that the formation of lipid droplets in treated mice with low and moderate dose of β-CM-7, but significant less cell degeneration and tissue damage. Most interestingly, the study found the liver tissues treated with high dose of β-CM-7 show almost no lipid droplets and relatively healthy. These were given expression on the increase TG level which regulated the dyslipidemia. This may prompt the antioxidant effect of β-CM-7 is not the important mechanism. It is just to balance the oxidative stress more possibly. It seemed to be more confirmed this point by the investigation of lipid metabolism markers of liver tissues like FAS and ACC in mice.

The liver secrets TGs in the form of very-low density lipoproteins (VLDL), which assemble with Apoprotein B (Zhenwei *et al*., 2017).The cholesterol and fatty acids (FAs) are synthesized from their precursor acetyl-CoA, which is converted to cholesterol in a pathway involving at least 23 enzymes and forming FAs in pathways involving up to 12 enzymatic steps (Ramasamy, 2016). FAS catalyzes the synthesis of fatty acids from acetyl-CoA and malonyl-CoA, which is the catalyzing last step in FA synthesis. ACC is an important regulator of these metabolic transitions (Chow *et al*., 2014; Liu *et al*., 2018), which is an enzyme involved in synthesizing and oxidating FAs. It has been a target to treat the metabolic diseases such as type 2 diabetes and dyslipidemia, as a potential intervention point (Bourbeau *et al*., 2015). The current study showed that β-CM-7 significantly reduced the FAS activity--similar to the results of Shituleni *et al*. (2016), and increased the ACC activity approximately significantly. Unlike to our study, most experiments about ACCs are reported to improve liver fat metabolism through inhibiting them. However, it seems to confirm the role of ACC from the opposite side in these experimental mice. Therefore, the treatment of β-CM-7 in aged mice may regulate the dyslipidemia though balancing the FAS and ACC activities. Yet the effects of the β-CM-7 on regulating dyslipidemia in aging is little known, its mechanism is worthy of further study, which may enhance our comprehension with regard to dyslipidemia with aging and be beneficial for the effective treatment.

On the basis of our findings, we come to a conclusion that the β-CM-7 treatment has potential regulating effects on dyslipidemia caused by aging, via controlling the oxidative stress and balancing the level between FAS and ACC.

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Statement of conflict of interest

The authors have declared no conflict of interests.

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