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Rosmarinic acid regulates lipid accumulation through lipid metabolism and anti-inflammatory

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ABSTRACT: Obesity caused by poor eating habits has become a global public health issue and harms human health. Rosmarinic acid (RA) is a natural active substance with many biological functions like antioxidant. However, the function of RA's lipid-lowering effect and mechanism are not entirely clear. This study aimed to explore the effect of RA on obesity and its action mechanism in *Caenorhabditis elegans*. RA not only alleviated intestinal inflammation and change the composition of unsaturated fatty acid, but also decreased fat storage and adjusted the size and quantity of fat droplets without threatening development and reproduction or affecting energy intake. In addition, RA improved the integrity of intestinal barrier function in *C. elegans*. RNA sequencing and qRT-PCR comprehensively revealed that the lipid-lowering mechanism mediated by RA might involve lipid metabolism and anti-inflammatory pathways. Among them, RA regulates lipid metabolism by reducing fat synthesis through SBP-1and increasing fatty acid β-oxidation via NHR-49. Furthermore, the anti-inflammatory pathway was associated with RA's enhancement of the ratio of unsaturated fatty acid and alleviation of inflammatory reactions caused by PAO1. Therefore, RA regulated lipid metabolism and relieved inflammation, which had the potential for dietary supplements or functional foods.

Keywords: Rosmarinic acid; *Caenorhabditis elegans*; Lipid metabolism; Inflammation; RNA sequencing

1. Introduction

In recent years, obesity has posed a serious threat to human health. As a global epidemic, obesity is associated with an increased risk of multiple health complications, including hyperlipidemia, type II diabetes, cardiovascular diseases and some cancers [1]. Epidemiology clearly indicates that the formation of obesity is positively correlated with high-fat diet [2]. Long-term high-fat diet can also damage intestinal villi, affect intestinal absorption and digestion of nutrients, cause intestinal inflammation [3], and lead to a decrease of intestinal barrier function [4].

Chronic low-grade inflammation plays a pivotal role in obesity development [5]. Inflammation is a coordinated body response when subjected to external stimulus [6]. Obesity is associated with low-grade

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chronic inflammation, promoting insulin resistance and diabetes [7]. Inflammation in the internal environment of obese patients is mediated by multiple factors and physiological processes related to fat metabolism can be negatively affected. Several key inflammatory markers have been consistently associated with obesity and the risk of adverse outcomes in obesity-associated diseases. Meanwhile, the changes in intestinal permeability are potential triggers of inflammation in obesity [8]. Currently, the main treatment for obesity is through medication. The side effects of general treatment methods such as taking drugs to treat obesity is obvious [9]. Therefore, it is necessary to prevent and treat obesity through the natural active substances in our diet [10].

Rosemary extract actives have high biological activity and are therefore widely used in the food industry [11, 12]. Rosmarinic acid (RA) is an ester of caffeic acid and 3,4-dihydroxyphenyllactic acid, which is widely found in the plant kingdom including rosemary [13, 14]. Most research have focused on the antioxidative and antiviral effect of RA whereas the effect on weight loss attracts few attention [15, 16]. In recent years, the lipid-lowering effect and intestinal protection of RA has attracted more and more attention [14, 17, 18], but the underlying mechanism has not been elucidated comprehensively. For example, does RA regulate fat accumulation or lipid metabolism? Is there a correlation between its regulated fatty acid synthesis and innate immunity? Therefore, it is necessary to fully clarify the anti-fat action and mechanisms of RA.

The nematode *Caenorhabditis elegans*, a free-living coelomic organism, is a genetic model widely used in the study of modern biological analysis [19]. The superiority of the short life cycle, a large number of offspring, simple structure, and easy maintenance make *C. elegans* a desirable model for *in vivo* evaluation [20, 21]. Most importantly, the advantages of the full sequence genome, well-described genetic backgrounds and availability of gene mutations make it easy to identify the underlying molecular basis of various drugs to inhibit the occurrence and development of diseases [22]. Therefore, *C. elegans* has been highly recognized in the study of fat metabolism. The intestinal toxicity is reflected as inflammation and edema in *C. elegans* and evaluated by healthspan after pathogenic bacteria infection [23-25]. Treatment of *C. elegans* with pathogenic bacteria *Pseudomonas aeruginosa* (PAO1) can simulate intestinal inflammation of *C. elegans* [26]. Therefore, in this current research, the model organism *C. elegans* was used to study the lipid-lowering effect and mechanism of RA, and nematodes infected with PAO1 were used as intestinal barrier damage models to explore the ability of RA to improve intestinal immune barrier function. The results of this study not only provide references for the application of RA in obesity and inflammatory diseases caused by obesity, but also provide a basis for the mechanism of RA in lipid metabolism.

2. Materials and Methods

2.1 C. elegans Maintenance and Sample Treatment

C. elegans strains were used including wild-type N_2, ZXW618 hkdIs618[*dhs-3*p::*dhs-3*::GFP], CE541[*sbp-1(ep79)*], CE548[*sbp-1*::GFP::SBP-1 + *rol-6(su1006)*], GG43[*fasn-1(g43)* I], DG4153[*pod-2(tn1691)* II], BX14[*elo-1(wa7)* IV], BC4849[*let-767(s2819)* III], BX24[*fat-1(wa9)* IV], BX26[*fat-2(wa17)* IV], BX30[*fat-3(wa22)* IV], BX52[*fat-4(wa14) fat-1(wa9)* IV], BX107[*fat-5(tm420)* V], BX106[*fat-6* (tm331) IV], BX153[*fat-7* (wa36) IV], VS20[*atgl-1*p::*atgl-1*::GFP + *mec-7*::RFP],

RB1716[*nhr-49 (ok2165)*], WBM170[*acs-2*p::GFP + *rol-6*(su1006)], VC4060[*ech-1.1(gk5134)* V], VS24[*kat-1(tm1037)* II], DG2179[*tub-1 (nr2044)* II], WBM170[*acs-2*p::GFP + *rol-6(su1006)*], ZXW618[*hkdIs618* (*dhs-3p::dhs-3*::GFP)]. All strains were obtained from *Caenorhabditis Genetics Center* (CGC, University of Minnesota, Minneapolis, MN, USA). Age-synchronized nematodes were obtained and cultured according to our previous protocol [14]. As previously described [14], RA was produced by Wuling Yangguang Biotechnology Co., Ltd (Hunan, China) and its purity was above 95%. RA treatment of *C. elegans* started from the egg stage. The RA stock solution (90 mmol/L in pure water) was diluted to the appropriate concentration and mixed with *E. coli* OP_{50} bacterial fluid.

2.2 Detection of the Size of Anterior Intestine

Nematodes of the same size were anesthetized with 1% sodium azide and fixed on a glass slide. The length of anterior intestine were measured by microscope (CX-41, Olympus Co., Tokyo, Japan). More than 30 worms were measured per assay, which was repeated three times [23]. The experimental treatment is divided into general experiment (Treatment was conducted with RA and PAO1 for 3 days), preventive experiment (Treatment was conducted with RA for 3 days and then fed with PAO1 for 2 days) and therapeutic experiment (Treatment was carried out with PAO1 for 2 days firstly, and then with RA for 3 days).

2.3 Lifespan Assay of Pathogenic Bacteria Infection

Fifty worms treated with or without RA for three or six days were transferred to a new nematode growth medium (NGM) covered with PAO1. According to our previous protocol, the survival assay was performed on three independently biological replicates [14, 23]. The experimental treatment is divided into general experiment, preventive experiment and therapeutic experiment.

2.4 Oil Red O (ORO) Staining Assay

ORO staining was performed as the previous method with a minor modification [27]. Firstly, worms were collected and washed with M9 buffer. Next, worms were anesthetized with 0.5% sodium azide in PBS and then fixed in 4% paraformaldehyde. After three cycles of freeze and thaw, the samples were suspended in 60% isopropanol for 20 min to dehydrate. Finally, worms were stained with freshly diluted ORO stain for 12 h in the dark. Stained nematodes were photographed under a optical microscope (CX-41, Olympus Co., Tokyo, Japan). The dye intensity was measured and quantified by Image J. More than 15 worms were measured and experiments were repeated at least three times.

2.5 Nile Red Staining of C. elegans

Nile red staining assay was performed according to the previous method with a minor modification [27]. Worms were collected and fixed following the same method as that of ORO staining. The fixed worms were rotated in the dark at 200 r/min at 26ºC for 6 h before being imaged. The fluorescent images were acquired by using fluorescence microscopy (Zeiss, Germany). More than 30 worms were measured per assay, and the assay was repeated three times.

2.6 Triglyceride (TG) Content Assay and Fatty Acid Composition Analysis

The worms in both the normal and high-fat diet were prepared. The homogenate of worms was prepared as before [27]. TG quantification in *C. elegans* was conducted with a commercial TG assay kit (Nanjing Jiancheng, China), and the protein concentration was quantified with the BCA protein assay kit (Nanjing Jiancheng, China). Fatty acid composition analysis was determined by gas chromatography-mass spectrometry (GC-MS) as previously described [27]. Three independent biological experiments were carried out.

2.7 Label-Free Quantitative Analysis of Lipid Droplets in ZXW618

The label-free quantification of a lipid droplet in ZXW618 worms was conducted as previously described [27]. The ZXW618 strain incubated with or without RA for three days from eggs was anesthetized with 100 mmol/L sodium azide prior to imaging. Fluorescent images were performed using confocal laser scanning microscopy (LSM800, Carl Zeiss, Jena, Germany). More than 20 worms were measured for each condition, and the images were analyzed by Image J. Experiments were independently repeated three times.

2.8 Intestinal Permeability Assay

The intestinal barrier assay was performed based on the method previously described with moderate modification [28]. Briefly, after exposed to RA, 15 nematodes were collected and fed for 3 h in the OP_{50} bacterial liquid mixed with 5% blue food dye (Sigma, USA). Subsequently, nematodes were anesthetized with 1% sodium azide. The presence or absence of the blue food dye in the intestine of nematode by microscope (CX-41, Olympus Co., Tokyo, Japan). The experimental treatment is divided into general experiment, preventive experiment and therapeutic experiment.

2.9 Defecation Duration Assay and Body Size Assay

These measurements were performed as described previously [29]. To assay defecation duration, after exposure to RA, an individual animal was examined for a fixed number of cycles, and a cycle period was defined as the interval between the initiations of two successive posterior body-wall muscle contractions. Thirty nematodes were examined in each treatment and experiments were independently repeated three times. The experimental treatment is divided into general experiment, preventive experiment and therapeutic experiment. Body size assay was performed as described, including body length, width and area [30].

2.10 Gonadal Staining Analysis and E. coli OP_{50} Bacterial Growth Assay

The gonad staining analysis was performed according to the previous studies [31]. After 96 h treatment of RA, the worms were stained in DAPI solution (200 ng/mL) and incubated in darkness for 20 min. At least 60 individuals were photographed by using fluorescence microscopy (Axio Imager. Z2, Zeiss). The bacterial growth assay of *E.coli* OP_{50} was carried out as described before [30]. The determination was carried out for 6 h (OD_{600}) and three times independently.

2.11 Attraction Assay and Food Clearance Assay

It was implemented according to the previous scheme [32]. Approximately 100 untreated worms were placed in the center of the NGM plate (6 cm × 6 cm) containing four alternating bacterial spots containing RA

or solvent water. The number of worms on each spot was determined after 2 h. The attraction index was as follows. At least six independent experiments were carried out.

$$\text{Attraction Index}=\frac{\text{N(RA/CK)}}{(\text{N(RA)}+\text{N(CK)})}\times 100\%$$

Food clearance assay was carried out essentially as describe[33]. After 60 h of incubation with or without RA in medium, the sample was shaken for 25 min and measured at 600 nm using Multifunctional Microplate Reader (PerkinElmer, Waltham, MA, USA). The food intake was calculated by dividing the bacterial clearance rate by the number of worms per well (approximately 12 wells). Food intake was expressed as a percentage relative to control animals.

2.12 RNA sequencing analysis and Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Total RNA was extracted from 3 days old adult worms using total RNA extraction kit according to the manufacturer's protocols. Nematodes were divided into control groups (CK) and RA groups respectively. RNA samples that met standards were submitted to the Shanghai Majorbio Biopharm Technology Co., Ltd. for strand specific cDNA library construction and Illumina paired-end sequencing (HiSeq, 2000, Illumina Inc., San Diego, CA). Gene expression analysis was carried out in accordance with the previously published protocol [34, 35]. The qRT-PCR experiment was repeated in triplicate with an independent RNA preparation (The primers were listed in Table S1).

2.13 SBP-1::GFP and ACS-2::GFP Fluorescence Visualization Assay

The green fluorescent protein (GFP)-labeled SBP-1 and ACS-2 were determined according to previously published protocols [36]. CE548 and WBM170 mutants were visualized using a fluorescence microscope (Axio Imager. Z2, Zeiss, Jena, Germany). More than 20 worms were measured for each condition, and the images were analyzed by Image J. Experiments were independently repeated three times.

2.14 Statistical analysis

Results were presented as the mean of triplicate ± SD. The statistical analyses were performed using one-way analysis of variance followed by post hoc comparison (LSD and Duncan tests, SPSS software, version 25). The different letters above bars indicated a significant difference at $P < 0.05$. Lifespan was analyzed Prism (version 9.0, GraphPad Software, Inc., La Jolla, CA, USA) by log-rank (Mantel-Cox) test. 3. Results and Discussion

3.1 RA Alleviated Intestinal Inflammation in C. elegans.

According to previous research reports, after feeding the nematodes with PAO1, the pathogenic bacteria colonizes the intestine of nematodes slowly, producing virulence-related membrane vesicles, and causing accumulation of electron-dense biofilm-like material on intestinal cells and abnormalities in them [23]. Therefore, PAO1 is a pathogenic bacterium that can cause intestinal inflammation in *C. elegans*. In order to investigate whether RA can alleviate the inflammatory response in *C. elegans*, we tested the size of anterior intestine and lifespan of *C. elegans* infected with PAO1. When the nematodes are fed with pathogenic bacteria

such as PAO1, the anterior intestine of the nematodes will expand abnormally [23, 37]. The normal morphology of anterior intestine and the abnormal swelling morphology of anterior intestine after inflammatory infection were shown in Figure 1A. First, we conducted the general experiment and detected the changes in the size of the anterior intestine of PAO1 infected nematode. Under this feeding of RA, a significant recovery of the abnormal swelling of anterior intestine was observed in nematode (Figure 1B). In order to further demonstrate the preventive and therapeutic effects of RA on intestinal damage, preventive experiment and therapeutic experiment were carried out. In the preventive experiment, the average size of the anterior intestine of the CK group and the RA treatment group was 35 μm and 25.2 μm, respectively (Figure 1C), so RA significantly reduced the anterior intestine size of nematode. At last, in order to explore the therapeutic effect of RA on anterior intestine, we conducted therapeutic experiments. However, RA treatment had no significant effect (Figure 1D, $P > 0.05$).

We further explored the effect of RA on the lifespan in inflammation-induced *C. elegans*. First, we conducted the general experiment. As shown in the Figure 1E, the survival curve of RA treatment groups moved right significantly compared with the CK groups ($P < 0.001$). The statistical results showed that RA treatment could significantly improve the maximum lifespan and median lifespan. Furthermore, the average lifespan of nematode increased by 71.3% (Table 1). In the preventive experiment, the survival curves of RA treatment groups were significantly shifted to the right (Figure 1F, $P < 0.001$). RA significantly increased the median lifespan and maximum lifespan of nematode (Table 1). And compared with the CK group, RA treatment prolonged the mean lifespan of nematode by 36.7% (Table 1). In the therapeutic experiment, the survival curves of nematode treated with RA were significantly shifted to the right (Figure 1G, $P < 0.01$). After RA treatment, the average lifespan of nematode increased by 19.5%. And the median lifespan and maximum lifespan were significantly prolonged (Table 1). The above results comprehensively demonstrated that RA treatment had both preventive and therapeutic intestinal inflammation induced by PAO1.

Table 1 Statistical analysis of the lifespan of nematodes under stress of PAO1 by RA

Condition		sMean lifespan	Median lifespan	Maximum Lifespan	Mean fold increase	*P*-value
PAO1 and RA 3 day	OP_{50}	6.82 ± 0.34^{a}	6.00 ± 0.00^{a}	10.00 ± 0.00^{a}	-	-
	RA	11.68 ± 0.10^{b}	10.33 ± 0.27^{b}	15.33 ± 0.27^{b}	71.3	< 0.0001
RA 3 day + PAO1 2 day	OP_{50}	12.30 ± 0.08^{a}	12.00 ± 0.00^{a}	18.67 ± 0.19^{a}	-	-
	RA	16.82 ± 0.18^{b}	16.00 ± 0.00^{b}	21.67 ± 0.27^{b}	36.7	< 0.0001
PAO1 2 day + RA 3 day	OP_{50}	9.89 ± 0.05^{a}	9.67 ± 0.13^{a}	15.00 ± 0.00^{a}	-	-
	RA	11.82 ± 0.02^{b}	10.33 ± 0.27^{b}	16.67 ± 0.27^{b}	19.5	< 0.0001

The data were analyzed by one-way ANOVA (SPSS 25), and different letters in a column denote values that are significantly different ($P < 0.05$). The mean fold increase was calculated by $(T - C)/C \times 100$, where T is the mean survival time of *C. elegans* treated with CA and C is the mean survive time of CK groups (OP_{50}).

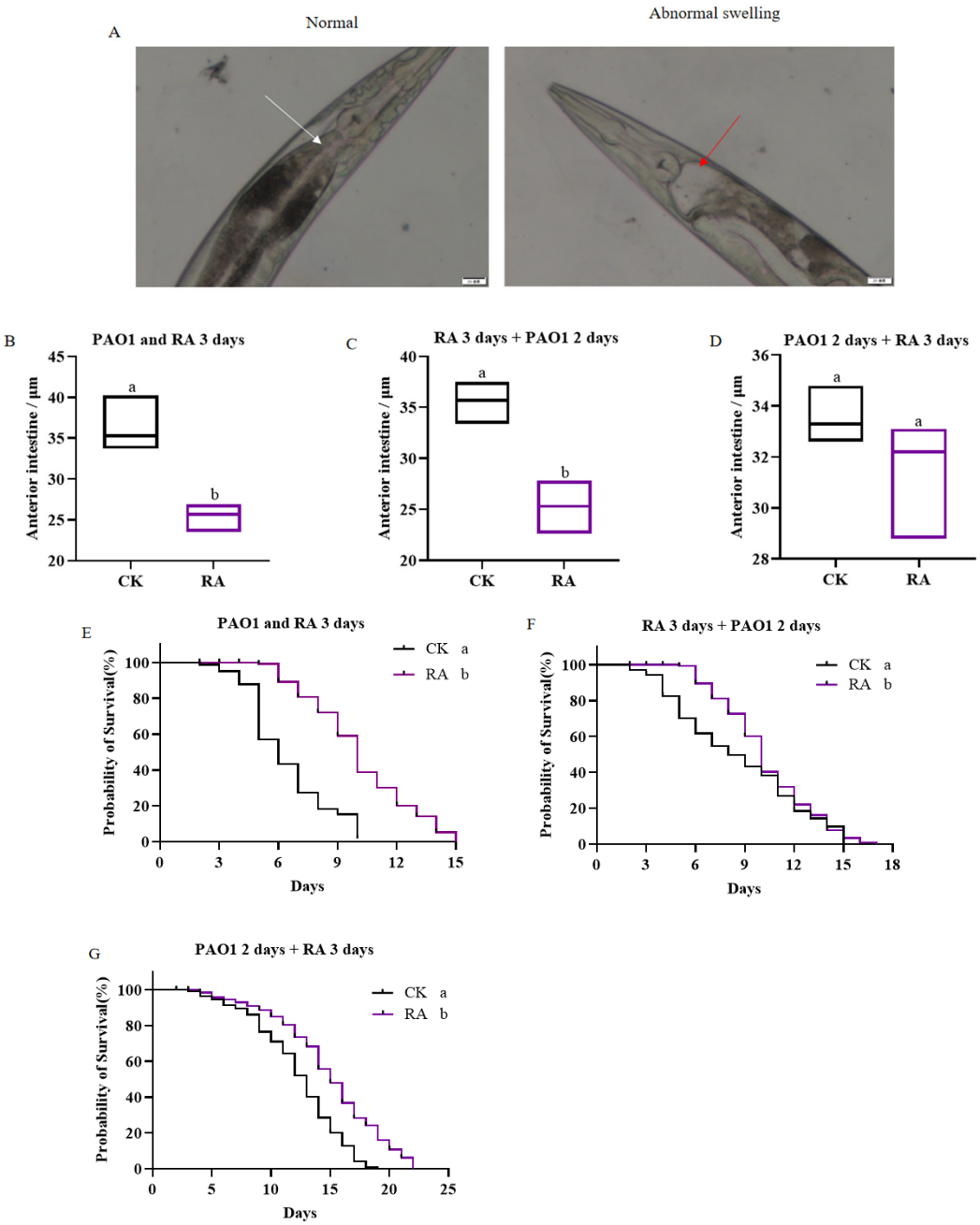


Figure 1. Effect of RA on the abnormal swelling of anterior intestine and lifespan under PAO1 infection in *C. elegans*. (A) Representative images of normal anterior intestine and abnormal swelling of anterior intestine in *C. elegans*. (B) Treatment with RA significantly repair the abnormal swelling of anterior intestine in nematodes in general experiments. (C) Treatment with RA significantly repair the abnormal swelling of anterior intestine in nematodes in preventive experiment. (D) Treatment with RA significantly repair the abnormal swelling of anterior intestine in nematodes in therapeutic experiment. Different letters were significantly different among these groups ($P < 0.05$). (E) Treatment with RA significantly extended the lifespan of nematodes in general experiments. (F) Treatment with RA significantly extended the lifespan of nematodes in preventive experiment. (G) Treatment with RA significantly extended the lifespan of nematodes in therapeutic experiment. At least three independent experiments were performed. Different letters were significantly different among these groups ($P < 0.05$).

3.2 RA Promoted the Decrease of Fat Accumulation in C. elegans.

It was reported that chronic lowgrade inflammation played a pivotal role in obesity development [5]. By detecting cytokines in obese mice induced by high-fat diet, it was found that a high-fat diet can lead to chronic inflammation. Based on the excellent performance of RA in alleviating nematode inflammation, we further investigated whether RA holds the potential to alleviate fat deposition in high-fat nematodes, N2 wild-type

worms fed with high glucose diets with RA was tested. To comprehensively evaluate the body fat accumulation, lipid droplet visualization was performed using ORO staining. Results from the ORO staining methods exhibited that RA induced a similar effect in reducing body fat of high-fat worms as the CK groups (Figure 2A-B). Although RA's inhibitory effect was weaker than that of positive control orlistat, RA significantly altered the fat content of nematode, 9% decrease compared with the CK groups in ORO staining. However, this result was opposite to the previous results reported by Pietsch that RA does not reduce fat content in nematodes [32]. We speculated that the possible reason for the difference in results was the difference in staining methods, as the lipid dye Nile red did not clearly indicate the main fat storage in nematode. Therefore, for further verification, the effect of RA on the fat content of nematode was determined by immobilized Nile red staining. The results were consistent with the results of ORO staining, RA significantly alleviated fat deposition in nematode, and the effect was similar to that of orlistat group (Figure 2C-D). Concurrently, the content of TG in worms was significantly reduced by 22% after RA treatment (Figure 2E). The above results demonstrate that RA can significantly promote the reduction of fat accumulation in *C. elegans*.

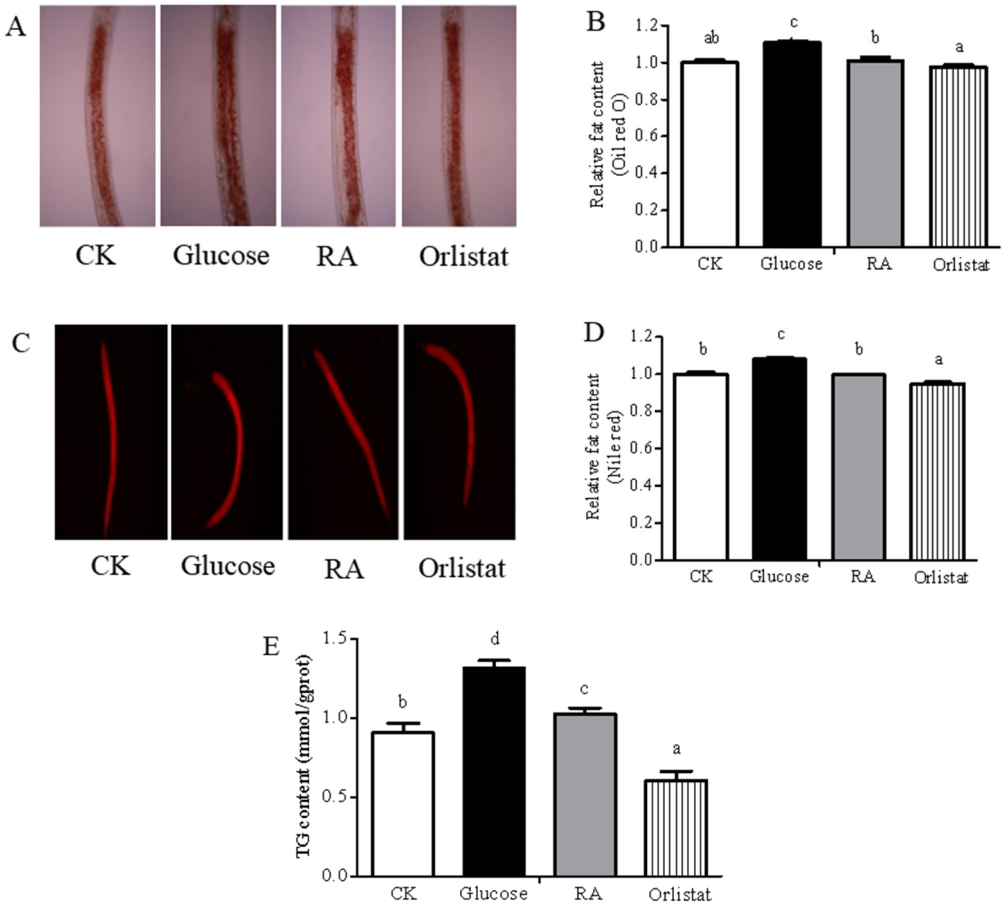
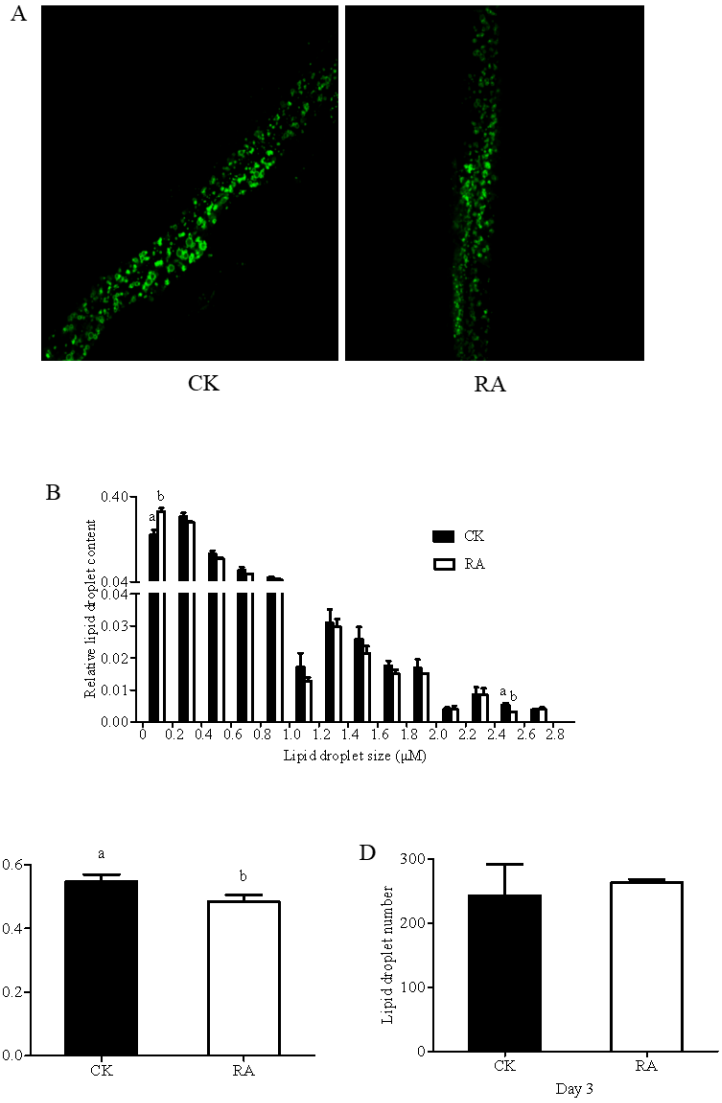


Figure 2. Effect of RA on fat accumulation in high fat N2 *C. elegans*. (A) Representative images of ORO staining were presented (the anterior was on the top and the posterior was on the bottom). (B) The ORO lipid staining intensity was quantitatively analyzed. (C) Representative fluorescent images of Nile Red lipid staining were presented (the anterior was on the top and the posterior was on the bottom). (D) The fluorescence intensity of Nile Red lipid staining was quantitatively analyzed. (E) The relative TG content in the total lipid was determined by normalization with total protein concentration. The experiment was conducted at least three biological replicates. Different letters were significantly different among these groups ($P < 0.05$).

3.3 RA Improved Lipid Metabolism and Polyunsaturated Fatty Acid Composition in C. elegans.

The size and number of lipid droplets can also reflect the status of fat metabolism in the body of nematodes. To investigate the details of fat deposition alleviation of RA, ZXW618 transgenic worms were used to explore the state (size and number) of fat droplets. As an intestinal lipid droplet marker, the green fluorescence protein (GFP)-fused DHS-3 (DHS-3::GFP) is located on the surface of the lipid droplets and forms a ring structure in the ZXW618 worm, so the change of lipid droplets can be directly observed (Figure 3A) [27]. Interestingly, RA led to a change in the distribution of lipid droplets with the small-sized lipid droplets (0-0.2 μm in diameter) portion increasing while the large-sized lipid droplets (2.4-2.6 μm in diameter) portion declining (Figure 3B). Although no significant differences were detected in the average number of lipid droplets ($P > 0.05$, Figure 3D), a significant decrease in the average droplet diameter was observed (Figure 3C). Thus, RA displayed a marked effect on reduction of fat storage, which was accompanied by a decrease in the size of lipid droplets.



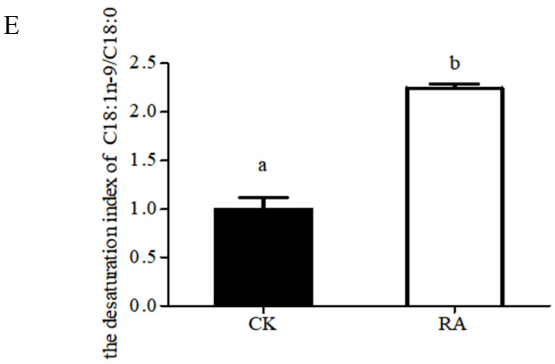


Figure 3. Effect of RA on lipid metabolism in *C. elegans*. (A) Representative DHS-3::GFP-labeled lipid droplet fluorescent imaging of the ZXW618 worms with or without RA treatment. (B) The distribution of lipid droplets in strain ZXW618 with or without RA culture was quantitatively analyzed. (C) The average size of lipid droplets with or without RA administration in ZXW618 was quantitatively analyzed. (D) The average number of lipid droplets in the ZXW618 strain with or without RA pretreatment was quantified. (E) The desaturation index of oleic and stearic acids was determined using GC-MS. The experiment was conducted at least three biological replicates. Different letters were significantly different among these groups ($P < 0.05$).

Unsaturated fatty acids can reduce the inflammatory reaction of the body. Previous studies have shown that RA can alleviate inflammation, and it is curious whether the administration of RA will affect the fatty acid desaturation index, thereby improving lipid metabolism. GC-MS was used to determine the desaturation index of oleic vs. stearic acids, the ratio of C18:1n-9/C18:0 was increased dramatically by RA administration compared to the CK (Figure 3E). The result strengthened that the weight loss effect of RA was closely related to the promotion of oleic acid, which in a kind of unsaturated fatty acid.

3.4 RA Improved the Integrity of Intestinal Barrier Function in C. elegans.

Obesity caused by a high-fat diet leads to changes in gut microbiota and increases intestinal permeability [38]. We further investigated whether RA can improve intestinal permeability of nematodes through blue food dye experiment and defecation experiment. The blue food dye was used to measure the integrity of intestinal barrier. If the intestinal integrity of the nematode is damaged, the blue food dye will leak into the body cavity of the nematode, making the surrounding intestine and even the whole nematode blue (Figure 4A, CK). However, nematode with complete intestinal barrier only have blue food dye in the intestine (Figure 4A, RA) [39-41]. To evaluate the effects of RA on the integrity of nematode intestinal barrier, first, we conducted a general experiment to evaluate intestinal integrity of nematode. As shown in Figure 4A and B, compared with the CK group, the proportion of blue food dye leakage of RA-treated nematode due to intestinal damage was significantly reduced ($P < 0.05$). The results indicated that RA had the effect of repairing the intestinal leakage of nematode infected by PAO1. In order to further demonstrate the preventive and therapeutic effects of RA on intestinal damage, preventive experiment and therapeutic experiment were designed respectively. In the preventive experiment, it was found that about 68.2%~80.0% of the nematode in the CK group had intestinal leakage. However, the proportion of nematode with intestinal leakage was significantly lower in the RA treatment groups compared with that in the CK groups, with only 48.5% of nematodes leaking blue food dye into the body cavity (Figure 4C and D, $P < 0.05$). The results indicated that RA could prevent intestinal barrier damage caused by PAO1 infection. In the therapeutic experiment, the results showed that only 49.7% of

nematode had leaky intestine in the RA treatment groups, and the proportion of leaky intestine nematodes were significantly lower than that in the CK group (Figure 4E and F, $P < 0.05$).

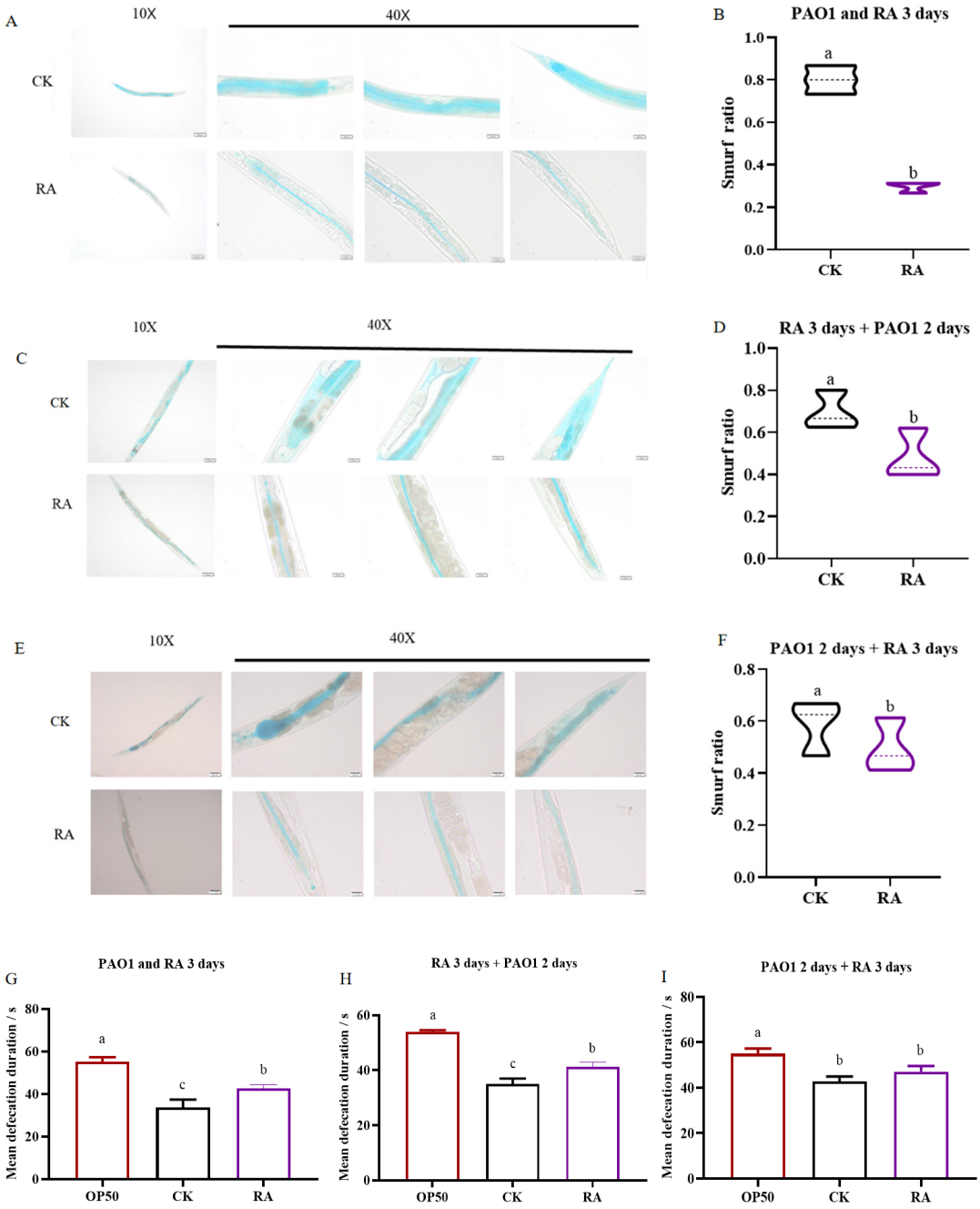


Figure 4. Intestinal barrier permeability of *C. elegans* treated with RA. (A) Representative images of intestinal barrier integrity of *C. elegans* in general experiments. (B) and comparison of the smurf ratio. (C) Representative images of intestinal barrier integrity of *C. elegans* in preventive experiment. (D) and comparison of the smurf ratio. (E) Representative images of intestinal barrier integrity of *C. elegans* in therapeutic experiment. (F) and comparison of the smurf ratio. (G) Treatment with RA significantly improved the defecation behavior of nematodes in general experiments. (H) Treatment with RA significantly improved the defecation behavior of nematodes in preventive experiment. (I) Treatment with RA did not improved the defecation behavior of nematodes in therapeutic experiment. Different letters were significantly different among these groups ($P < 0.05$).

To further explore the regulatory effect of RA on intestinal function of gastrointestinal injured nematodes, the changes in nematode defecation duration under PAO1 infection were examined. Normally, the defecation of nematode is a rhythmic behavior, and the interval of each defecation cycle is about 50 seconds [25]. As shown in Figure 4G-I, compared with nematode treated with *E. coli* OP_{50}, the defecation

duration of nematode treated with PAO1 decreased significantly, which was due to faster muscle contractions when intestine of nematode is stressed. First, the general experiment was conducted. As shown in the Figure 4G, RA could improve the defecation behavior of nematode ($P < 0.05$). Secondly, we conducted preventive experiments. It can be seen from the experimental results that RA could improve the defecation behavior of nematode and increase the defecation duration (Figure 4H, $P < 0.05$). The result also showed that RA could treat PAO1-mediated intestinal dysfunctions. At last, we conducted therapeutic experiments. Under this treatment, there was no significant change in the defecation duration of nematode (Figure 4I, $p > 0.05$). The above results indicate that the treatment of RA could improve the intestinal permeability and intestinal regulatory function of PAO1-infected nematode.

3.5 RA has no Effect on the Development Situation and Energy Intake in C. elegans.

To determine if the reduction of fat content of RA was acted by inhibiting food intake, several parameters, including the growth of *E. coli* OP_{50}, food attraction and food clearance rate were examined. The results revealed that except for the significant inhibitory effect in the third hour, there was no significant change of *E. coli* OP_{50} in the subsequent time (Figure S1A). Thus, speculation about the anti-obesity mechanism caused by the reduction in food supply due to antibacterial action was rejected. Second, attractiveness experiment verifies whether RA has an indirect dietary restriction effect. The attractiveness analysis manifested that administering RA did not result in significant chemical rejection of worms in general ($P > 0.05$, Figure S1B). Therefore, RA did not affect the feeding behavior of worms and indirectly caused changes in food intake. Third, food clearance was assessed by further measuring food clearance. Consistently, no significant differences were found ($P > 0.05$, Figure S1C). In general, these results suggested that RA did not alter the energy consumption involved in nematode development and reproduction, nor did it inhibit the energy intake involved in dietary restrictions.

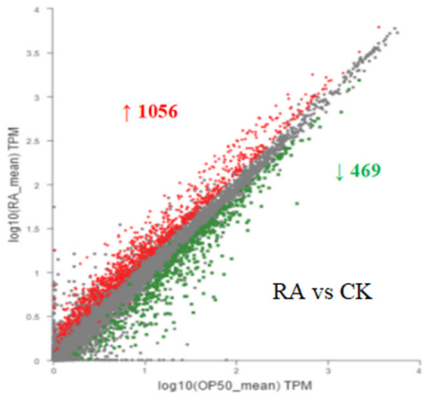
To determine whether lipid-lowering effects of RA affected basic physiological functions, the development and reproduction of normal nematode treated for 60 h were tested. First, several growth rate parameters, including body length, width and area, were measured. RA did not have an obvious change in the morphology of *C. elegans* (Figure S1D). Consistently, quantitative analysis showed that the body area of the worms treated with RA was not significantly different from that of the CK groups ($P > 0.05$, Figure S1E). Meanwhile, there was no significant difference in body length and width (Figure S1F and S1G). The gonads of the nematode supplemented with RA were intact, and the morphology was not different from CK groups (Figure S1H, $p > 0.05$)). Interestingly, developmental embryos were also found in the uterus. Therefore, it was inferred that RA did not change the number of offspring of nematodes [14].

3.6 RA Improved Lipid Metabolisms and Inflammatory Reaction by Activating Four Signaling Pathways.

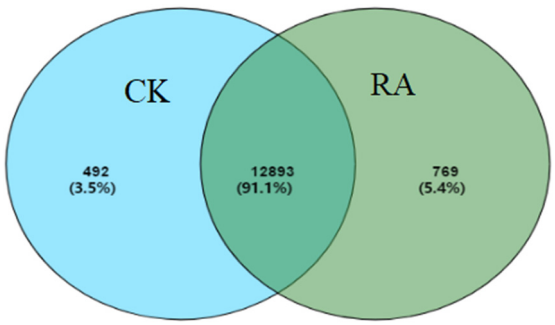
In order to further clarify the mechanism of RA in relieving inflammation and lipid metabolism of nematode, we used RNA sequencing to investigate the gene transcription and expression level of N_2 nematode. In the experiment, the common points and differences in gene expression levels of each treatment group were detected respectively. As shown in Figure 5A, compared to the OP_{50} group, there were a total of

1525 differentially expressed genes (DEGs) (1056 up and 469 down regulated genes) in the group under RA treatment. Difference Venn Diagram (Figure 5B) demonstrated it appeared 12893 overlapping genes between CK and RA group. We further annotated DEGs with GO terms to analyze the functional process, including biological process, cellular component and molecular function. Compared to the CK group, the enriched GO terms of RA group were 24 in biological process, 14 in cellular component and 13 in molecular function (Figure 5C). The enriched biological process mainly included cellular process, single-organism process and metabolic process. Cellular component mainly included cell, membrane and membrane part. Molecular function changes occurred slightly different, and were binding and catalytic activity. To further understand genes biological functions of *C. elegans* response to RA, KEGG pathway related database to identify enriched pathways in DEGs. The detailed pathways were shown in Figure 5D and Table S2. There were 155 pathways related to RA and there were four most significant pathways including Glycerolipid metabolism, PPAR signaling pathway Biosynthesis of unsaturated fatty acids, and Antigen processing and presentation. The first two signaling pathways were related with lipid metabolism, and the last two signaling pathway were associated with anti-inflammatory. Among them, Glycerolipid metabolism signaling pathway is connected with lipogenisis and lipolysis, PPAR signaling pathway is correlated with fatty acid β-oxidation, Biosynthesis of unsaturated fatty acids signaling pathway is relevant to the changes of unsaturated fatty acid composition and reducing inflammatory response and Antigen processing and presentation signaling pathway is correlated with alleviating inflammatory reactions (Including reducing the anterior intestine size and extending infection lifespan of nematode). The RNA sequencing demonstrated that RA played a significant regulatory potential in lipid metabolism and anti-inflammatory of nematode. Based on the above research results, the expression levels of key genes involved in the relevant pathways were detected by qRT-PCR.

A



B



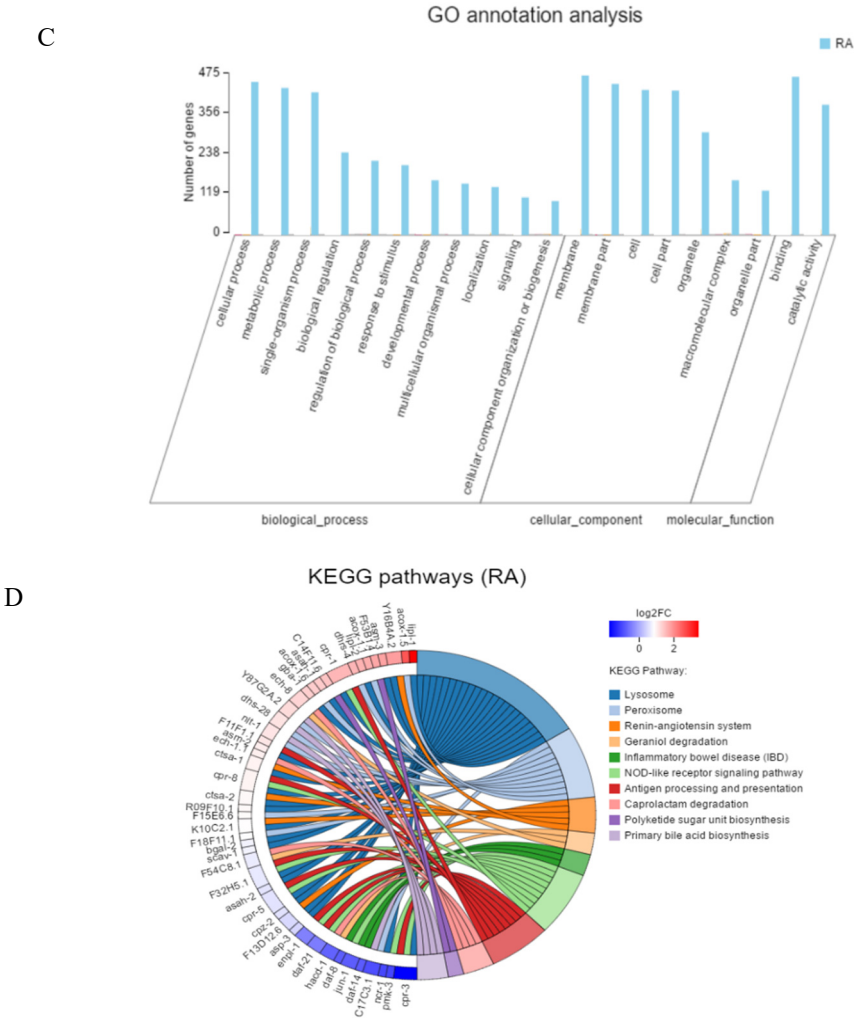


Figure 5. Results of transcriptomics and reverse transcription-polymerase chain reaction(qRT-PCR) in *C. elegans*. (A-B) Differentially expressed genes in *C. elegans* of RA treatment. (A) Scatter plot of DEGs. (B) Overlapping genes. (C-D) GO and KEGG pathway enrichment analysis. (C) Major GO terms for *C. elegans* of RA exposure. (D) KEGG pathway enrichment for *C. elegans* of RA exposure. Effect of RA on lipid metabolism-related genes.

Firstly, we investigated the genes related to lipogenesis and lipolysis involved in the Glycerolipid metabolism signaling pathway. Fat synthesis is regulated by transcription factors and enzymes related to lipid metabolism [42, 43]. In terms of transcriptase, the expression of these genes encoding lipogenesis, lipolysis and fatty acid β-oxidation was explored. First, the gene expression levels were studied involved in fat synthesis, including an acetyl CoA carboxylase (ACC) (*pod-2*), fatty acid synthase (FAS) (*fasn-1*), fatty acid desaturases (*fat-1* through *fat-7*) and fatty acid elongases (*elo-1* and *let-767*) [42, 43]. As shown in Figure 6A, RA did not change the expression of genes: *pod-2* and *fasn-1*, suggesting that the observed RA effect on reducing fat storage might be independent on the first and second steps in the de novo fatty acid biosynthesis. In addition, no significant changes were detected in genes related to fatty acid elongases (*elo-1* and *let-767*) in RA treatment group when compared to that of CK ($P > 0.05$, Figure 6A). Strikingly, the expression of *fat-4* and *fat-5* associated with desaturase was significantly down-regulated, although no variation was observed on the expression of *fat-1*, *fat-2* and *fat-3* (Figure 6A), demonstrating that RA affected the expression of fatty acid desaturases genes. Interestingly, RA upregulated the expressions of *fat-6* and *fat-7*, which may be related to the synthesis of unsaturated fatty acids (Figure 6A). Next, the role of

RA in lipolysis was investigated. There are homologs of lipases that hydrolyze triglycerides in *C. elegans*: ATGL-1 (a homolog of fatty triglyceride lipase) and HOSL-1 (a homolog of a hormone-sensitive lipase) [42, 43]. The expression of *atgl-1* or *hosl-1* was not regulated by RA in *C. elegans* ($P > 0.05$, Figure 6A), so the fat-lowering effect of RA might be independent of the effect on lipolysis.

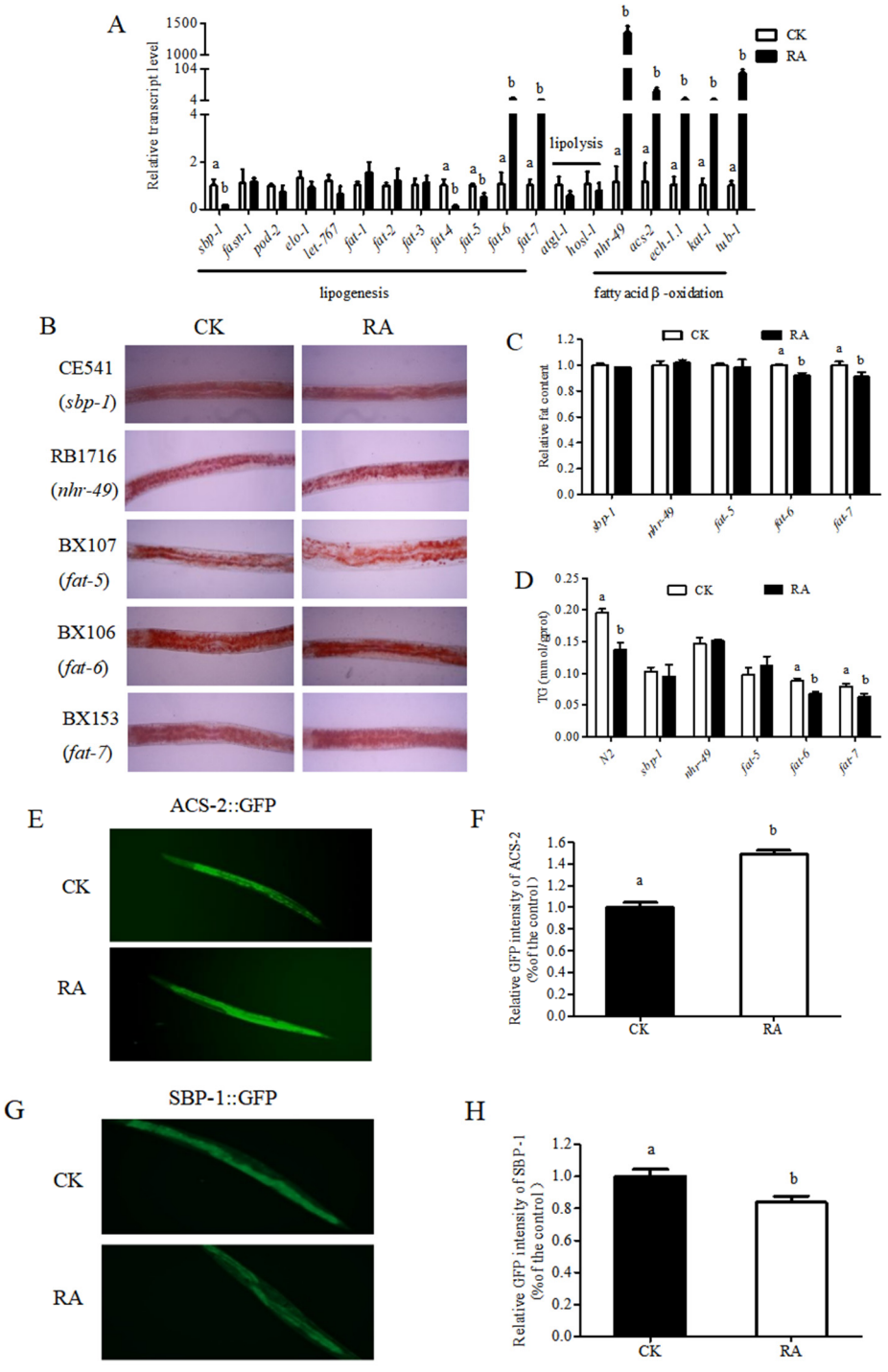


Figure 6 Effect of RA on lipid metabolism-related genes in *C. elegans*.

(A) Effect of RA on the mRNA expressions of lipid metabolism related genes measured by qRT-PCR (encoding lipogenesis, lipolysis and fatty acid β-oxidation). (B) Representative pictures of ORO staining of mutant strains (anterior was on the left). (C) Quantitative determination of ORO staining of each mutant. (D) The relative TG content was determined in each mutant. (E) Representative image of ACS-2::GFP fluorescence of WBM170 worms with or without exposure to RA. (F) The fluorescence intensity of ACS-2::GFP was quantitatively analyzed in the presence or absence of RA. (G) Representative image of SBP-1::GFP fluorescence of CE548 worms with or without exposure to RA. (H) The fluorescence intensity of SBP-1::GFP was quantitatively analyzed in the presence or absence of RA. Different letters were significantly different among these groups ($P < 0.05$).

Second, key genes associated with fatty acid β-oxidation were further explored, such as *acs-2* (encodes an acyl-CoA synthetase), *ech-1.1* (encodes a trifunctional β-oxidation), and *kat-1* (encodes a 3-ketoacyl-coA thiolase) [44]. The study found that the transcription level of all genes was dramatically up-regulated (Figure 6A), suggesting the possible involvement of fatty acid β-oxidation in RA's fat reduction effect. The role of *acs-2* in the function of RA was further traced by the transgenic strain WBM170 (ACS-2::GFP). As shown in Figure 6E, the fluorescence intensity of ACS-2::GFP dramatically increased after RA treatment (Figure 6F), which was consistent with the up-regulation of *acs-2* expression at transcriptional level. Furthermore, the expression level of *tub-1* (an ortholog of mammalian TUBBY), which co-acted with *kat-1* in the regulation of fatty acid β-oxidation in *C. elegans*, was also significantly increased (Figure 6A). This result was consistent with the enrichment of PPAR signaling pathways closely related to fatty acid β-oxidation in RNA sequencing.

Third, the upstream of RA-mediated lipid-lowering mechanism may involve SBP-1 and NHR-49 dependent pathways. In *C. elegans*, SBP-1 plays a crucial role in regulating stearoyl-CoA desaturase (SCD) and other fat-synthesis genes [42, 43]. NHR-49 encodes a functional homolog of the mammalian PPAR in mammals [42, 43] and targets fatty oxidation signal genes to regulate fatty acid β-oxidation [36, 45]. To determine whether the down-regulation of *fat-4* and *fat-5* expression depends on *sbp-1*, the transcriptional level of *sbp-1* was detected. Consistently, the mRNA level of *sbp-1* was significantly down-regulated by RA (Figure 6A). In addition, the protein levels of *sbp-1* detected by the fluorescence intensity of CE548 transgenic worms (SBP-1::GFP) were reduced accordingly (Figure 6G and 6H). Fat-lowering effects of RA were abolished in the *sbp-1* mutant CE541 (Figure 6B-6D). Collectively, these results suggested that the lipid-lowering effect of RA was achieved by down-regulating *fat-4* and *fat-5* via the SBP-1-dependent pathway. Furthermore, the transcriptional level of *nhr-49*, the upstream genes of *acs-2*, *ech-1.1*, *kat-1* and *tub-1*, was markedly upregulated compared with the CK (Figure 6A), which was consistent with the trend of changes in genes *acs-2*, *ech-1.1*, *kat-1* and *tub-1*. Therefore, the results indicated that the lipid-lowering effect of RA might depend on NHR-49 pathway with upregulation of fatty acid β-oxidation via *acs-2*, *ech-1.1*, *kat-1* and *tub-1*. There have also been many researches on the role of SBP-1 and NHR-49 in the regulation of lipid lowering at the cell and animal experimental level. The sterol regulatory element-binding protein (SREBP) transcription factor family is a critical regulator of lipid and sterol homeostasis in eukaryotes. In mammals, SREBPs are highly active in the high fat feeding state to promote the expression of lipogenic and cholesterogenic genes and facilitate fat storage. In cell experiments, studies have shown that the lipid-lowering regulatory mechanism of active substances on 3T3-L1 adipocytes is closely related to the activation of SREBP-1c[46]. In *C. elegans* model, it possesses a SREBP ortholog, *sbp-1*, which is expressed in all metabolic tissues. Mutation of *sbp-1* in *C. elegans* exhibits reduction of fat levels and changes in the expression of lipogenesis genes. These observations suggest that SBP-1 shows the same function in *C. elegans* as SREBP-1c does in mammals. In addition, in relevant studies, hazardous substances increased the expression of CCAAT/enhancer binding protein (C/EBPα) and peroxisome proliferator-activated receptor-γ

(PPARγ), which is the homolog of NHR-49, in 3T3-L1 adipocytes[47]. The above reports indicate that the mechanism found in *C. elegans* model involve SBP-1 and NHR-49 dependent pathways also apply to mammalian cells.

At last, we studied the genes involved in Biosynthesis of unsaturated fatty acids signaling pathway. Given that *fat-5*, *fat-6* and *fat-7* are three crucial SCD genes in worms, the effect of RA on SCD genes was accordingly investigated with mutant strains so as to confirm the expression of the *fat-5*, *fat-6* and *fat-7* gene. By ORO staining and TG content analysis, *fat-5* mutations could abrogate the effect of RA on reducing fat storage, whereas the fat reduction effect of RA remained in the mutants of *fat-6* and *fat-7* (Figure 6B-D). It was indicated that the observed effect of RA on reducing fat storage might depend on SCD via *fat-5* instead of *fat-6* and *fat-7*. In view of the increased transcription levels of *fat-6* and *fat-7*, as well as literature indicated that administering RA significantly upregulated the expression of *fat-6* and *fat-7* genes with activation potential for anti-inflammatory in nematode [48]. Due to significant upregulation of *fat-6* and *fat-7* also observed in qPCR experiment (Figure 6A), we inferred that the anti-inflammatory effect of RA was closely related to Biosynthesis of unsaturated fatty acids signaling pathway, which might involve the unsaturated fatty acid regulation by *fat-6* and *fat-7* and their upstream gene *nhr-49*. Combined with the improvement effect of RA on intestinal inflammation and RNA sequencing enrichment to Antigen processing and presentation signaling pathway, the up regulation of Biosynthesis of unsaturated fatty acids signaling pathway can improve the anti-inflammatory capacity of nematodes, which is consistent with the results reported in the literature [48].

Taken collectively, these data add to the known health-promoting effects of RA, and suggest the mechanism of RA-mediated health improvement is closely related to lipid metabolism and anti-inflammatory. Among them, lipid metabolism-related pathways involve the reduction of fat synthesis via SBP-1 and the increase in fatty acid β-oxidation via NHR-49. And the anti-inflammatory pathway involves NHR-49 mediated unsaturated fat acid synthesis.

4. Conclusion

RA is among the most important active constituents of rosemary polyphenolic compounds. In this study, we found that RA provided strong alleviated intestinal inflammation function and significantly reduced fat accumulation and improved the fatty acid desaturation index. Experimental results of ZXW618 transgenic worms and TG levels also verified the lipid-lowering effect of RA. In addition, RA improved the integrity of intestinal barrier function in nematodes. RNA sequencing and qRT-PCR comprehensively revealed the lipid-lowering mechanism mediated by RA might involve lipid metabolism and anti-inflammatory. Among them, lipid metabolism got involved with RA's reduction in fat synthesis through the SBP-1 and increase in fatty acid β-oxidation via NHR-49. The anti-inflammatory pathway was associated with RA's enhancement of the ratio of unsaturated fatty acid and alleviation inflammatory reactions caused by PAO1. These findings afforded a mechanistic insight into alleviating fat accumulation by RA, consequently providing a basis for the high-value utilization of RA.

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Supporting Information

RA has no Effect on the Development Situation and Energy Intake in *C. elegans*. (**Figure S1**), Primer sequences for qRT-PCR analysis (**Table S1**). Analysis of KEGG Pathway of RA Treated Nematodes (**Table S2**).

Declaration of Competing Interest

The authors have no declaration of interest statement.

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