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## Article

# Nicotinamide Riboside and Phycocyanin Oligopeptides Affect Stress Susceptibility in Chronic Corticosterone-Exposed Rats

Cemal Orhan <sup>1</sup>, Emre Sahin <sup>2</sup>, Mehmet Tuzcu <sup>3</sup>, Nurhan Sahin <sup>1</sup>, Abdullah Celik <sup>1</sup>, Sara Perez Ojalvo <sup>4</sup>, Sarah Sylla <sup>4</sup>, James R. Komorowski <sup>4</sup> and Kazim Sahin <sup>1,\*</sup>

<sup>1</sup> Department of Animal Nutrition, Faculty of Veterinary Medicine, Firat University, Elazig, Turkey; nsahinkm@yahoo.com (K.S.); nsahin@firat.edu.tr (N.S.); corhan@firat.edu.tr (C.O.); acelik@beu.edu.tr (A.C.)

<sup>2</sup> Department of Animal Nutrition, Faculty of Veterinary Medicine, Bingol University, Bingol, Turkey; esahin@bingol.edu.tr

<sup>3</sup> Department of Biology, Faculty of Science, Firat University, Elazig, Turkey; mtuzcu@firat.edu.tr

<sup>4</sup> Research and Development, Nutrition 21, Harrison, NY 10577, USA; sperezoyalvo@nutrition21.com (S.P.O.); ssylla@nutrition21.com (S.S.); jkomorowski@nutrition21.com (J. R. K.)

\* Correspondence: nsahinkm@yahoo.com; Tel.: +904242370000/3938

**Abstract:** Nicotinamide riboside (NR) is an NAD<sup>+</sup> precursor capable of regulating mammalian cellular metabolism. Phycocyanin oligopeptide (PC), a phytonutrient found in blue-green algae, has antioxidant and anti-inflammatory properties. This study explored the effects of NR, PC, and their combination on telomere length, inflammatory and antioxidant status in rats under chronic stressed conditions (CS). Forty-nine rats were allocated into seven groups: Control, chronic stress (CS), CS with NR (26.44 mg/kg), a low dose of 2.64 mg/kg of PC (PC-LD), or a high dose of 26.44 mg/kg PC (PC-HD), NR+PC-LD and NR+PC-HF. Rats were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days. NR and PC supplementation, particularly NR plus PC, increased the serum antioxidant enzyme activities, hepatic nicotinamide adenine (NAD<sup>+</sup>) content, and telomere length ( $P < 0.001$  for all) compared with the CS group. The levels of serum malondialdehyde (MDA), liver interleukin-6 (IL-6), tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ), IL-1 $\beta$ , and IL-8 were reduced under CS condition ( $P < 0.001$ ). In addition, CS decreased the levels of hepatic telomere-related proteins and sirtuin1 (SIRT1-3), while supplementation of NR and PC alone or in combination increased the levels of telomere-related proteins (e.g., POT1b, TRF1, and TRF2), and SIRT3, NAMPT  $P < 0.05$ . In conclusion, NR and PC, especially their combination, can alleviate metabolic abnormalities by enhancing hepatic cytokines, SIRT3, NAMPT, and NAD<sup>+</sup> levels in CS-exposed rats. More research is needed to elucidate further the potential health effects of the combination of NR and PC in humans.

**Keywords:** corticosterone; phycocyanin; nicotinamide riboside; NAD; hepatic metabolism

## 1. Introduction

Stress is a rapid and coordinated response by an organism to environmental challenges. These responses require the organism to divert energy resources from one tissue to serve the needs of another to counteract the adverse effects of stress. Glucocorticoids (GCs), a class of adrenal steroids released during and after stress, mediate daily physiological changes and coordinate stress. GCs regulate various physiological progressions, mainly by releasing energy stores through gluconeogenesis and suppressing glucose uptake and activity in specific tissues such as adipose and immune tissue [1,2]. Chronic corticosterone (CORT) exposure increases total body fat mass and decreased insulin sensitivity [3]. The formation of reactive oxygen species (ROS), such as hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), hydroxyl radicals (HO), and superoxide anion radicals (O<sub>2</sub><sup>-</sup>), which lead to lipid peroxidation, is a metabolic consequence of the stress-induced increase in energy production [4].

Additionally, the elevated CORT levels induce redox imbalance and ROS generation in several tissues [5]. Oxidative stress mediates antioxidant defense system changes, which is also associated with chronic stress [6]. Therefore, the body primarily uses enzymatic and non-enzymatic antioxidant defense systems to neutralize ROS [7]. Cellular oxidative stress may reduce telomere length by attenuating telomerase activity, which controls the de novo synthesis of telomeres [8]. Various age-related diseases have been shown to cause cellular damage [9], and cellular senescence increases due to ROS-induced oxidative stress [10].

Nicotinamide adenine dinucleotide (NAD<sup>+</sup>) plays a crucial role in energy metabolism and genome integrity by acting as a substrate for different enzyme families, such as sirtuins (SIRT6) and poly(ADP-ribose) polymerases (PARPs) [11]. NAD<sup>+</sup> levels decrease in cellular oxidative reactions [12] that may occur during metabolic and age-related diseases and neurodegeneration [11]. SIRT6 regulate DNA damage and improve mitochondrial biogenesis [13]. The increased NAD<sup>+</sup> levels may improve SIRT6 activity [12] and prevent telomere shortening under stress conditions [14]. Natural NAD<sup>+</sup> precursors such as nicotinamide riboside (NR) can alleviate telomere damage by inhibiting ROS production and mitochondrial impairment [12]. NR supplementation can prevent NAD<sup>+</sup> decline in mitochondria and improves mitochondrial activity and biogenesis in hepatocytes, thus ameliorating oxidative stress-related pathologies and extending the health span [15]. Similarly, phycocyanin (PC), a biliprotein derived from spirulina, has potent antioxidant and anti-inflammatory effects [16,17]. PC could activate nuclear transcription factor erythroid-2-like factor 2 (Nrf2)/heme oxygenase-1 (HO-1) signaling, which attenuates oxidative stress-induced DNA damage in the liver [18]. A recent study by Komorowski et al. [19] indicated that the novel patented PC oligopeptide might be superior to standard PC owing to its high cellular antioxidant capacity. However, the protective effects of PC oligopeptide and its combination with NR are still unclear. Therefore, this study was conducted to test the effects of PC oligopeptide and NR supplementation on the hepatic abnormalities, NAD<sup>+</sup> levels, and underlying mechanisms in rats reared under chronic stress conditions.

## 2. Materials and Methods

### 2.1. Animals

A total of 49 male Wistar albino rats (8 weeks old, 180±20g) were used, and animals were reared in polypropylene cages conditioned at a temperature of 22±2°C, 55±5% humidity, and a 12/12h light/dark cycle. A regular chow diet and water were provided ad libitum. The experiment was conducted under the protocol approved by the Firat University Animal Experiments Ethics Committee (16/03/2020-384972) according to The European Parliament and the Council directive (2010/63/EU, 2010) and European Economic Community guidelines (Directive 86/609/EEC, 1986). The animal experiments were carried out in the Experimental Research Center of Firat University (FUDAM). All experimental procedures were reported according to the Animal Research: Reporting of In Vivo Experiments (ARRIVE) guidelines.

### 2.2. Experimental Design

After a one-week adaptation period, 49 rats were randomly separated into seven groups (n=7) as follows: 1) Control; rats were treated with saline containing tween 80 and DMSO in normal conditions; 2) Chronic stress (CS); rats were injected subcutaneously with corticosterone (CORT, 40 mg/kg) daily for 21 consecutive days, 3) NR; rats were injected with CORT and administered NR (26.44 mg/kg), 4) PC-LD; rats were injected with CORT and administered a low dose of 2.64 mg/kg of PC, 5) PC-HD; rats were injected with CORT and administered a high dose of 26.44 mg/kg PC, 6) NR+PC-LD; rats were injected with CORT and administered NR (26.4 mg/kg) and a low dose of 2.64 mg/kg PC, 7) NR+PC-HD; rats were injected with CORT and administered NR (26.44 mg/kg) and a high dose 26.44 mg/kg PC. NR and PC were administered by oral gavage daily for 21 days.

Doses of PC oligopeptide and NB were determined based on the Human Equivalent Dose for Drug Development, according to [20]. Human doses (30 or 300 mg HED) were converted to animal

doses based on body surface area. A conversion factor of 6.17 was used to convert human doses to rat doses. It was determined that low-dose PC equals 2.64 mg/kg, high-dose PC, and NR equals 26.44 mg/kg for rats. CORT (Sigma–Aldrich Co., St. Louis, MO, USA) was suspended in saline with 0.1% Tween 80% and 0.2% DMSO.

### 2.3. Biochemical Analysis

Serum levels of glucose, cholesterol, blood urea nitrogen (BUN), creatinine, and activities of aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were analyzed with a portable automated chemistry analyzer (Samsung LABGEO PT10, Samsung Electronics Co., Suwon, Korea). Total serum corticosterone (LifeSpan Biosciences, Seattle, WA, USA) levels and antioxidant enzyme activities [superoxide dismutase (SOD), glutathione peroxidase (GSH-Px), and catalase (CAT)] were measured using the relevant commercial kits (MyBioSource, Inc. San Diego, CA, USA) by enzyme-linked immunosorbent assay (ELISA, Elx-800, Bio-Tek Instruments Inc, Vermont, USA). Also, liver NAD<sup>+</sup>, nicotinamide (NAM), nicotinic acid (NA), nicotinamide adenine dinucleotide phosphate (NADPH), and nicotinamide mononucleotide (NMN) GSH (MyBioSource, Inc. San Diego, CA, USA) levels were measured using a commercial kit following the manufacturer's instructions by ELISA. The serum MDA levels were analyzed by HPLC with an SPD-20A UV detector (Shimadzu). Tissue samples (300 mg) were homogenized and centrifuged, and supernatants were injected (injection volume, 20  $\mu$ L) into an HPLC system. The mobile phase was 30 mM KH<sub>2</sub>PO<sub>4</sub>-methanol (82.5 + 17.5, v/v %, pH 3.6), the flow rate was 1.2 mL/minute, and detection was at 250 nm.

### 2.4. Relative Telomere Length Measurement

Telomere length in the liver was determined by quantitative real-time PCR (qPCR). Genomic DNA was obtained from the liver tissues of rats using a commercial kit (DNA mini kit, Qiagen, Germany), and the DNA concentration was determined for each sample by a microvolume spectrophotometer (MaestroNano, Maestrogen Inc., USA). For PCR, 20 ng of DNA was diluted in an SYBR Green PCR Kit (QuantiFast, Qiagen, Germany) with AT1 (F: ACGTGTTCAGCATCGACCGCTACC, R: AGAATGATAAGGAAAGGGAACAAGAAGCCC) and telomere (tel 1 F: GGTTTTTGAGGGTGAGGGTGAGGGTGAGGGTGAGGG; tel 2 R: TCCCGACTATCCCTA TCCCTATCCCTATCCCTATCCCTA) primers. For telomere primers, PCR was conducted at 95 °C for 3 min, 95 °C for 15 s (40 cycles), and 54 °C for 1 min on a Rotor-Gene Q machine (Qiagen, Germany). Telomeric DNA normalized to AT1 quantity. Relative telomere length expressed as normalized telomere/single gene copy AT 1 receptor. The relative telomere length of the CORT groups was demonstrated as the relative fold change of the control group.

### 2.5. Western Blotting Analysis

The levels of inflammatory cytokines [interleukin-6 (IL-6), tumor necrosis factor-alpha (TNF- $\alpha$ ), IL-1 $\beta$ , and IL-8], telomere shelterin proteins [protection of telomeres protein 1a (POT-1a), Pot-1b, telomeric repeat-binding factor 1 (TRF1), TRF2, and TRF1-interacting nuclear protein 2 (Tin2)], sirtuin-1 (SIRT1), SIRT3, and nicotinamide phosphoribosyltransferase (NAMPT) in the liver were detected by Western blotting. Liver tissues were mechanically homogenized and pooled at 4 °C in tissue lysis buffer and centrifuged at 15,000  $\times$  g for 20 min at 4 °C. Protein quantification was performed by a microvolume spectrophotometer (MaestroNano, Maestrogen Inc.). Then, liver homogenates were mixed with 2x Laemmli buffer (1:1 ratio) and boiled for 5 minutes in microtubes. Protein samples were separated using 10% sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose (NC) membranes. NC membranes were blocked with 5% bovine serum albumin for 2 hours. The membranes were incubated with rat-specific primary antibodies diluted to 1:1000 [IL-6, TNF- $\alpha$ , IL-1 $\beta$ , IL-8, POT-1a, Pot-1b, TRF2, Tin2, SIRT1, SIRT3, NAMPT (Santa Cruz Biotechnology, Heidelberg, Germany), and TRF1 (Thermo Fisher Scientific, Waltham, MA, USA)] overnight at 4°C. After washing, the membranes were incubated with appropriate secondary antibodies diluted to 1:5000 (Santa Cruz Biotechnology) for 2h. The

bands were visualized using a diaminobenzidine substrate solution. Finally, the membranes were scanned, and protein levels were measured densitometrically. Protein loading was checked by  $\beta$ -actin protein (Santa Cruz Biotechnology, Heidelberg, Germany). The blots were repeated at least three times.

2.6. Statistical Analysis

Sample size (N=49) was calculated based on a power of 85%, an effect size of 0.65, and a P value of 0.05 using the G\*power program (Version 3.1.9.3) [21]. Given that assumption, a sample size of seven per group was calculated. The data were analyzed using Statistical Analysis Software (IBM SPSS version 22.0). The Shapiro–Wilk and Levene tests were used to determine the normality of the data and homogeneity of variance, respectively. The groups were compared using ANOVA and Tukey posthoc test.  $P<0.05$  was considered to be statistically significant. Data are shown as the mean  $\pm$  standard deviation.

3. Results

3.1. The Effects of PC and NR on Body Weight and Biochemical Parameters

CORT administration significantly reduced the final body weight compared to the control group ( $P<0.001$ , Table 1). Serum glucose levels were not affected by CORT administration in the CS, NR, PC-LD, and NR+PC-HD groups compared to the control group ( $P>0.05$ ). However, a dose of 26.44 mg/kg NR plus 2.64 mg/kg PC administration decreased the serum glucose level compared to the control ( $P<0.05$ ), CS ( $P<0.001$ ), and NR groups ( $P<0.05$ , Table 1). The serum creatine, BUN, ALT, and AST levels did not differ among the groups ( $P>0.05$ ).

**Table 1.** Effects of nicotinamide riboside and phycocyanin oligopeptides on serum biochemical parameter in rats subject to chronic corticosterone.

	Groups						
	Control	CS	NR	PC-LD	PC-HD	NR+PC-LD	NR+PC-HD
Final BW, g	276.57 $\pm$ 9.85 <sup>a</sup>	210.43 $\pm$ 8.28 <sup>b</sup>	222.86 $\pm$ 23.11 <sup>b</sup>	221.29 $\pm$ 11.25 <sup>b</sup>	221.86 $\pm$ 9.92 <sup>b</sup>	225.14 $\pm$ 13.23 <sup>b</sup>	217.86 $\pm$ 19.42 <sup>b</sup>
Glucose, mg/dL	119.14 $\pm$ 8.43 <sup>ab</sup>	127.86 $\pm$ 5.05 <sup>a</sup>	119.86 $\pm$ 7.10 <sup>ab</sup>	118.57 $\pm$ 2.64 <sup>abc</sup>	114.14 $\pm$ 5.24 <sup>bc</sup>	109.29 $\pm$ 5.47 <sup>c</sup>	118.29 $\pm$ 4.68 <sup>bc</sup>
Creatine, mg/dL	0.40 $\pm$ 0.06	0.38 $\pm$ 0.07	0.37 $\pm$ 0.08	0.40 $\pm$ 0.08	0.39 $\pm$ 0.06	0.36 $\pm$ 0.09	0.45 $\pm$ 0.05
BUN, mg/dL	21.46 $\pm$ 3.57	23.01 $\pm$ 2.32	22.90 $\pm$ 1.87	22.13 $\pm$ 2.52	21.59 $\pm$ 3.00	22.20 $\pm$ 4.11	23.17 $\pm$ 3.65
ALT, U/L	95.57 $\pm$ 8.42	98.14 $\pm$ 12.81	98.00 $\pm$ 5.55	98.71 $\pm$ 6.82	98.03 $\pm$ 7.36	96.10 $\pm$ 5.78	98.72 $\pm$ 4.79
AST, U/L	110.69 $\pm$ 11.79	117.46 $\pm$ 7.53	114.71 $\pm$ 10.92	112.60 $\pm$ 13.13	117.43 $\pm$ 15.25	114.06 $\pm$ 12.59	116.20 $\pm$ 19.06

Data are presented as the mean and standard deviation. a-c: Means in the same line without a common superscript differ significantly ( $P<0.05$ , ANOVA and Tukey post hoc test). CS: chronic stress induced with corticosterone; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days. BUN: Blood urea nitrogen; ALT: Alanine transaminase; AST: Aspartate aminotransferase.

Changes in the concentrations of serum corticosterone, MDA, SOD, GSH-Px, and CAT to determine the level of stress and antioxidant capacity were shown in Table 2. As expected, the serum corticosterone level was markedly increased in the CORT-treated groups compared to the control group ( $P<0.001$ ). Similarly, the serum MDA levels were elevated after CORT administration compared to the control group ( $P<0.001$ ). The serum MDA levels were reduced by NR and PC supplementation ( $P<0.001$ ). An apparent decline of the MDA levels was observed in the NR+PC-LD group compared to the NR ( $P<0.001$ ), PC-LD ( $P<0.001$ ), and PC-HD ( $P<0.05$ ). Conversely, serum SOD, GSH-Px, and CAT levels notably decreased after CORT injection ( $P<0.001$ , Table 2). NR supplementation increased the GSH-Px ( $P<0.05$ ) and CAT levels ( $P<0.01$ ), while it did not change the serum SOD levels ( $P>0.05$ ) compared to the CS group. The PC- PC-LD and PC-HD groups had higher serum SOD and CAT levels than the NR group ( $P<0.05$ ). Furthermore, the combination of NR with

each dose of PC prominently boosted serum SOD and GSH-Px levels compared to other groups ( $P<0.05$ ), except the control group ( $P>0.05$ ).

**Table 2.** Effects of nicotinamide riboside and phycocyanin oligopeptides on serum biochemical parameter in rats subject to chronic corticosterone.

	Groups						
	Control	CS	NR	PC-LD	PC-HD	NR+PC-LD	NR+PC-HD
Corticosterone, ng/ml	46.33±5.82 <sup>b</sup>	110.22±6.15 <sup>a</sup>	102.16±8.68 <sup>a</sup>	104.48±3.67 <sup>a</sup>	105.91±6.39 <sup>a</sup>	105.46±8.13 <sup>a</sup>	104.99±5.34 <sup>a</sup>
MDA, nmol/ml	0.55±0.07 <sup>f</sup>	1.97±0.13 <sup>a</sup>	1.74±0.07 <sup>b</sup>	1.53±0.07 <sup>c</sup>	1.41±0.07 <sup>cd</sup>	1.26±0.07 <sup>e</sup>	1.28±0.09 <sup>de</sup>
SOD, U/ml	128.00±5.48 <sup>a</sup>	54.54±6.39 <sup>e</sup>	61.36±4.5 <sup>e</sup>	72.70±3.05 <sup>d</sup>	86.41±6.34 <sup>c</sup>	96.84±6.77 <sup>b</sup>	98.27±4.65 <sup>b</sup>
GSH-Px, U/ml	64.87±3.08 <sup>a</sup>	19.62±1.37 <sup>e</sup>	26.28±1.98 <sup>d</sup>	31.76±4.96 <sup>cd</sup>	37.10±2.80 <sup>c</sup>	44.68±5.58 <sup>b</sup>	45.98±4.10 <sup>b</sup>
CAT, U/ml	164.98±6.13 <sup>a</sup>	102.03±4.80 <sup>e</sup>	114.69±4.82 <sup>d</sup>	124.55±5.48 <sup>c</sup>	131.43±3.76 <sup>bc</sup>	140.55±6.76 <sup>b</sup>	139.05±7.81 <sup>b</sup>

Data are presented as the mean and standard deviation. a-f: Means in the same line without a common superscript differ significantly ( $P<0.05$ , ANOVA and Tukey post hoc test). CS: chronic stress induced with corticosterone; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed condition or NR and PC were orally administered for 21 days. MDA: Malondialdehyde; SOD: Superoxide dismutase; GSH-Px: Glutathione peroxidase; CAT: Catalase.

### 3.2. The Effects of PC and NR on Liver Nicotinamide Metabolites and GSH

We tested the liver NAD<sup>+</sup>, NAM, NA, NMN, and NADPH levels to assess hepatic NAD<sup>+</sup> metabolism. The level of liver NAD<sup>+</sup> and its metabolites decreased in the CS group compared to other groups ( $P<0.05$ , Table 3). We found that PC supplementation elevated NAD<sup>+</sup> and NAM levels in rat liver compared to NR supplementation ( $P<0.05$ ). The NR+PC-LD dose group had markedly higher NAD<sup>+</sup> and NADPH levels than the NR ( $P<0.05$ ) and NR+PC-HD groups ( $P<0.01$ ). Interestingly, liver NAM concentrations were similar between the NR and NR+PC-HD groups ( $P>0.05$ ), while this metabolite was remarkably elevated in the NR+PC-LD group compared to the NR group ( $P<0.05$ ). CORT injection effectively diminished liver GSH status compared to the control group ( $P<0.001$ ). The NR- and PC-supplemented groups significantly reversed the GSH reduction following CORT injection ( $P<0.001$ ; Table 3). The NR group had a similar GSH activity to the NR+PC-LD and NR+PC-HD groups ( $P>0.05$ ). The NR+PC-LD group had a higher GSH activity than the NR+PC-HD group ( $P<0.05$ ).

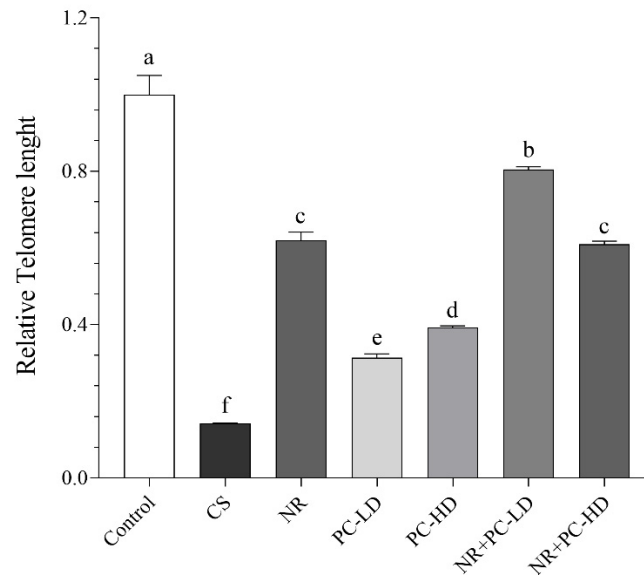
**Table 3.** Effects of nicotinamide riboside and phycocyanin oligopeptides on serum biochemical parameter in rats subject to chronic corticosterone.

	Groups						
	Control	CS	NR	PC-LD	PC-HD	NR+PC-LD	NR+PC-HD
NAD <sup>+</sup> , μmol/g	0.76±0.05 <sup>a</sup>	0.33±0.04 <sup>f</sup>	0.59±0.04 <sup>c</sup>	0.42±0.05 <sup>e</sup>	0.49±0.06 <sup>de</sup>	0.67±0.05 <sup>b</sup>	0.55±0.06 <sup>cd</sup>
NAM, μmol/g	2.36±0.15 <sup>a</sup>	1.01±0.08 <sup>e</sup>	1.60±0.06 <sup>c</sup>	1.31±0.09 <sup>d</sup>	1.43±0.13 <sup>d</sup>	1.78±0.10 <sup>b</sup>	1.66±0.10 <sup>cb</sup>
NA, μmol/g	1.79±0.09 <sup>a</sup>	1.22±0.12 <sup>d</sup>	1.63±0.09 <sup>abc</sup>	1.46±0.10 <sup>c</sup>	1.57±0.17 <sup>bc</sup>	1.71±0.13 <sup>ab</sup>	1.73±0.12 <sup>ab</sup>
NMN, μmol/g	0.45±0.05 <sup>a</sup>	0.15±0.02 <sup>e</sup>	0.30±0.02 <sup>bc</sup>	0.20±0.02 <sup>de</sup>	0.24±0.03 <sup>cd</sup>	0.36±0.05 <sup>b</sup>	0.32±0.07 <sup>b</sup>
NADPH, nmol/g	62.65±4.13 <sup>a</sup>	30.84±3.52 <sup>e</sup>	44.42±2.31 <sup>c</sup>	39.88±3.04 <sup>cd</sup>	41.38±3.57 <sup>cd</sup>	52.52±5.39 <sup>b</sup>	37.89±2.23 <sup>d</sup>

Data are presented as the mean and standard deviation. a-f: Means in the same line without a common superscript differ significantly ( $P<0.05$ , ANOVA and Tukey post hoc test). CS: chronic stress induced with corticosterone; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed condition or NR and PC were orally administered for 21 days. Nicotinamide adenine dinucleotide; NAD<sup>+</sup>: Nicotinamide adenine dinucleotide; NAM: Nicotinamide; NA: Nicotinic acid; NMN: Nicotinamide mononucleotide; NADPH: nicotinamide adenine dinucleotide phosphate; GSH: glutathione.

### 3.3. The Effects of PC and NR on Telomere Length

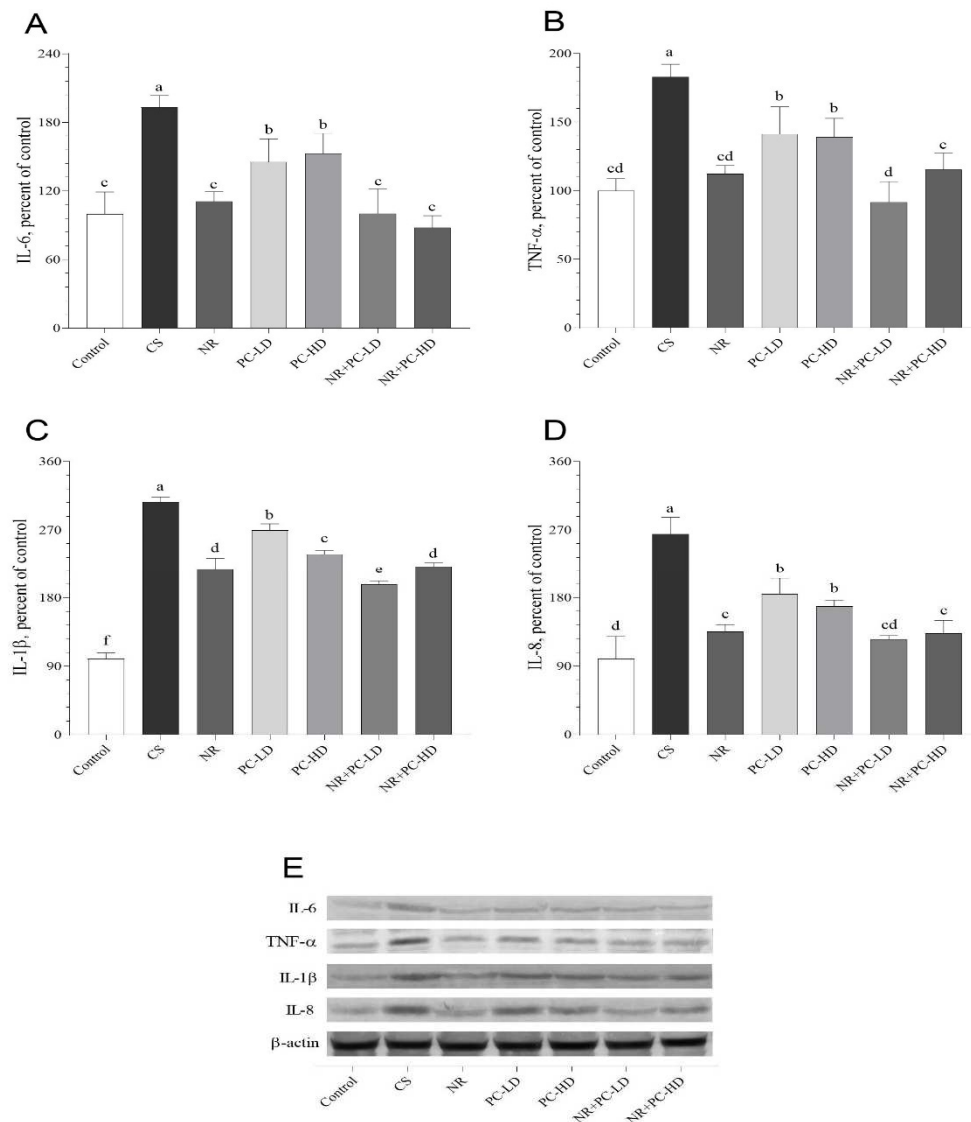
As seen in Figure 1, CORT injection noticeably decreased the telomere length, and the NR+PC-LD group had the most protective activity against telomere shortening among the CORT-injected groups ( $P<0.001$ ). The relative telomere length was similar between the NR+PC-HD and NR groups ( $P>0.05$ ).



**Figure 1.** Effects of NR and PC oligopeptides on liver relative telomere length in rats subject to chronic corticosterone (CORT). Data are expressed as a relative fold change compared to the control. The error bars above the lines indicate the standard deviation of the mean. Different symbols (a-f) indicate significant differences among the groups (ANOVA and Tukey post hoc test;  $P<0.05$ ). CS: chronic stress; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days.

### 3.4. The Effects of PC and NR on Inflammatory Mediators

As expected, inflammatory cytokine levels were significantly increased in CORT-injected rats compared to other groups (Figure 2A-E;  $P<0.01$ ). The hepatic IL-6 (Figure 2A) levels were similar between the control, NR, NR+PC-LD, and NR+PC-HD groups ( $P>0.05$ ). NR plus low-dose PC-supplemented rats had an inhibitory effect on TNF- $\alpha$  (Figure 2B). It was observed that the NR+PC-LD group had the most inhibitory effect on IL-1 $\beta$  activity compared to the other CORT-injected groups ( $P<0.001$ , Figure 2C). The NR ( $P<0.01$ ) and NR+PC-HD ( $P<0.05$ ) groups had higher IL-8 levels than the control group (Figure 2D), while NR plus low-dose PC supplementation reduced the hepatic IL-8 level compared to the control group level ( $P>0.05$ ).

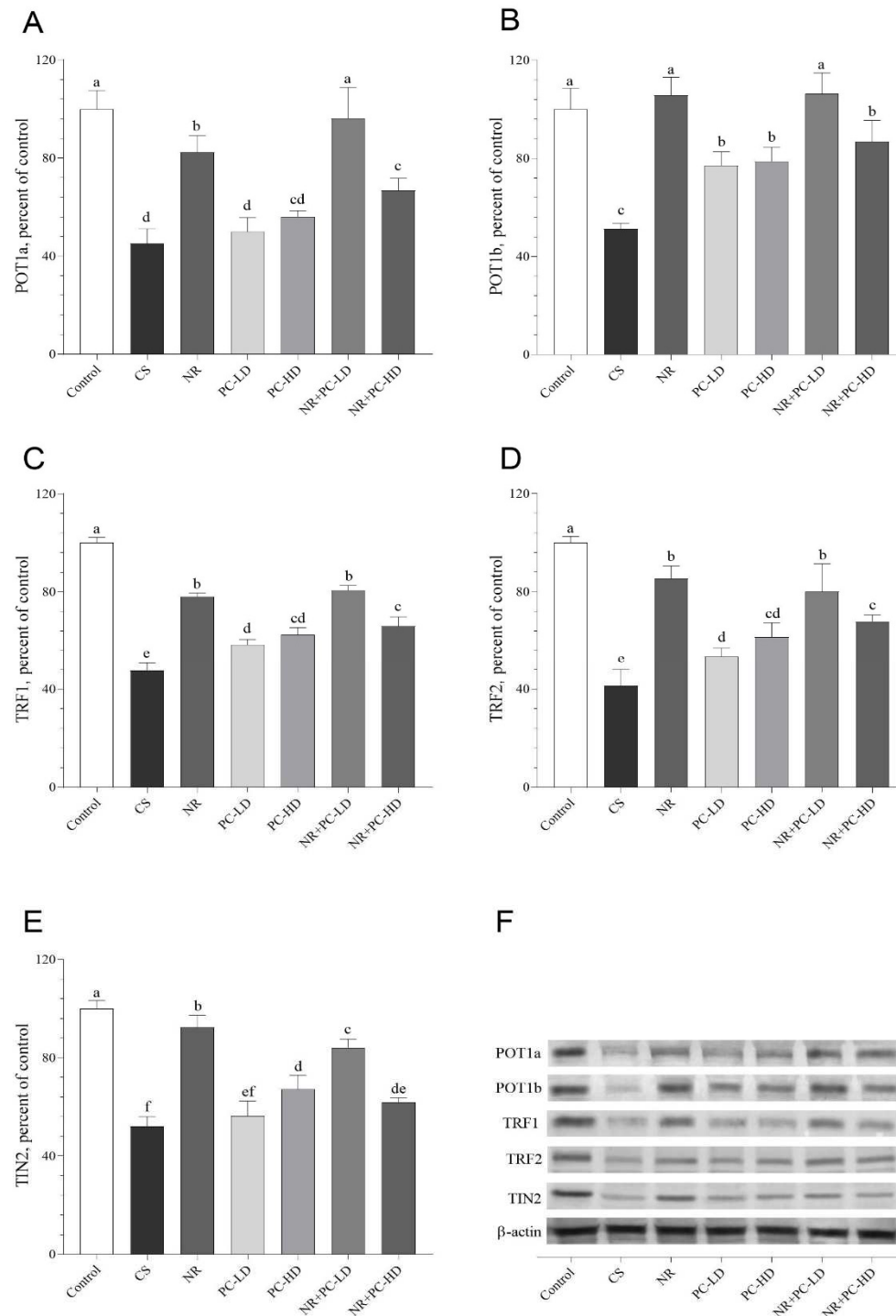


**Figure 2.** Effects of NR and PC oligopeptides on liver IL-6 (A), TNF- $\alpha$  (B), IL-1 $\beta$  (C) and IL-8 (D) levels in rats subject to chronic corticosterone (CORT). The densitometric analysis of the relative intensity according to the control group of the western blotting bands was performed with  $\beta$ -actin normalization to ensure equal protein loading (E). Blots were repeated at least three times ( $n = 3$ ), and a representative blot was shown. Data are expressed as a percent of the control set at 100%. The error bars above the lines indicate the standard deviation of the mean. Different symbols (a-f) indicate significant differences among the groups (ANOVA and Tukey post hoc test;  $P < 0.05$ ). IL-6, interleukin-6; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IL-1 $\beta$ , interleukin-1 $\beta$ ; IL-8, interleukin-8. CS: chronic stress; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days.

### 3.5. The Effects of PC and NR on Telomere Sheltering, SIRT $\alpha$ s, and NAMPT

We next analyzed protein levels of telomere shelterin complexes by western blotting. As shown in Figure 3 and Figure 4. NR or NR+PC supplementation increased the telomere shelterin complex, SIRT1, SIRT3, and NAMPT protein levels, which were reduced after CORT administration ( $P < 0.05$ ). The control and NR+PC-LD groups had similar POT1a protein levels ( $P > 0.05$ , Figure 3A). Additionally, we detected that the POT1b levels did not differ between the control, NR, and NR+PC-LD groups ( $P > 0.05$ , Figure 3B). Although the NR and NR+PC-LD groups had significantly higher

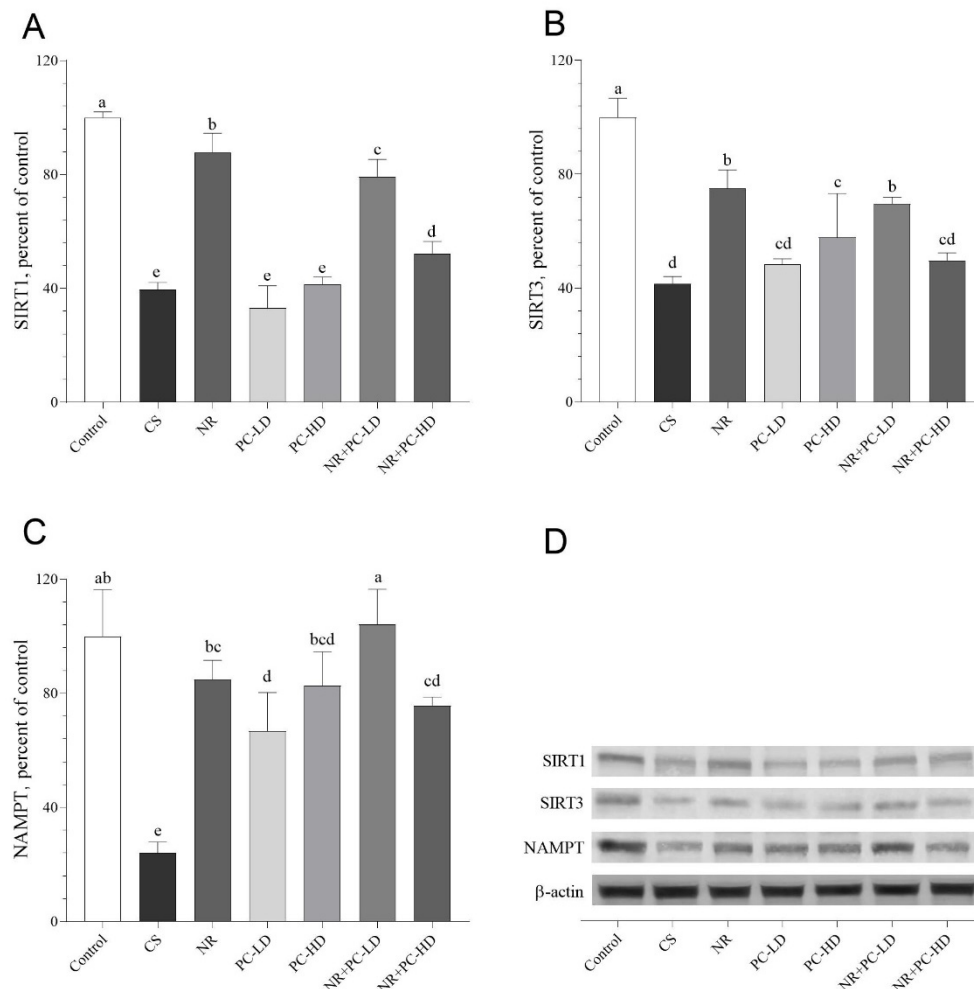
TRF1 (Figure 3C) and TRF2 (Figure 3D) levels than the other CORT-administered groups ( $P < 0.01$  for all), these groups could not reach the level of TRF1 and TRF2 of the control group. Moreover, we found that Tin2 levels in the NR+PC-LD group noticeably increased compared to those in the CS group ( $P < 0.05$ , Figure 3E). Unfortunately, none of the CORT-injected groups had significantly elevated Tin2, TRF1, or TRF2 levels compared to the control ( $P < 0.05$ ).



**Figure 3.** Effects of NR and PC oligopeptides on liver POT1a (A), POT1b (B), TRF1 (C), TRF2 (D) and Tin2 (E) levels in rats subject to chronic corticosterone (CORT). The densitometric analysis of the relative intensity according to the control group of the western blotting bands was performed with β-actin normalization to ensure equal protein loading (F). Blots were repeated at least three times ( $n = 3$ ), and a representative blot was shown. Data are expressed as a percent of the control set at 100%. The error bars above the lines indicate the standard deviation of the mean. Different symbols (a-f) indicate

significant differences among the groups (ANOVA and Tukey post hoc test;  $P < 0.05$ ). POT1a, protection of telomeres protein 1a; POT1b, protection of telomeres protein 1b; TRF1, telomeric repeat-binding factor 1; TRF2, telomeric repeat-binding factor 2; TIN2, TRF1-interacting protein 2. CS: chronic stress; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days.

In order to explore the effect of NR and PC oligopeptides on telomere lengthening, we measured the levels of SIRT1, SIRT3, and NAMPT, which are NAD<sup>+</sup>-related enzymes. The NR group had the highest SIRT1 levels among the CORT-injected groups (Figure 4A). Any doses of PC without NR did not alter the SIRT1 level compared to the CS group ( $P > 0.05$ , Figure 4A). Similarly, the PC-LD and NR+PC-HD did not significantly increase the SIRT3 levels compared to the CS group ( $P > 0.05$ , Figure 4B). However, the NR and NR+PC-LD groups had the highest SIRT3 levels among the CORT-injected groups ( $P < 0.05$ , Figure 4B). On the other hand, the NR+PC-LD group remarkably boosted the NAMPT levels compared to the other groups, except the control ( $P < 0.05$ , Figure 4C). Additionally, the NR+PC-LD group had a higher NAMPT level than the control group, but the difference was not statistically significant ( $P > 0.05$ , Figure 4C).



**Figure 4.** Effects of NR and PC oligopeptides on liver SIRT1 (A), SIRT3 (B) and NAMPT (C) levels in rats subject to chronic corticosterone (CORT). The densitometric analysis of the relative intensity according to the control group of the western blotting bands was performed with  $\beta$ -actin normalization to ensure equal protein loading (D). Blots were repeated at least three times ( $n = 3$ ), and a representative blot was shown. Data are expressed as a percent of the control set at 100%. The error bars above the lines indicate the standard deviation of the mean. Different symbols (a-e) indicate

significant differences among the groups (ANOVA and Tukey post hoc test;  $P < 0.05$ ). SIRT1, sirtuin 1; SIRT3, sirtuin 3; NAMPT, nicotinamide phosphoribosyltransferase. CS: chronic stress; NR: Nicotinamide riboside (26.44 mg/kg); PC-LD: Phycocyanin oligopeptide low dose (2.64 mg/kg); PC-HD: Phycocyanin oligopeptide high dose (26.44 mg/kg). Rats except the control group were given daily corticosterone injections (40 mg/kg) to induce stressed conditions, or NR and PC were orally administered for 21 days.

#### 4. Discussion

This study showed that chronic CORT exposure increased oxidative stress by increasing MDA content and decreasing the antioxidant activity of SOD, CAT, and GSH-Px. At the same time, it also increased serum corticosterone concentrations and liver anti-inflammatory (TGF- $\beta$ ) and pro-inflammatory (TNF- $\alpha$ , IL-1 $\beta$ , IL-6) cytokines levels. Similar to our results, it has been well documented in previous studies that chronic CORT exposure increases MDA levels [22], stimulates inflammatory cytokines [22,23], suppresses antioxidant enzyme activity [23], and accelerates telomere shortening [23,24]. As in our findings, it has been reported that NR and PC may exert inhibitory effects on oxidative stress-induced tissue damage in animals [16,25] and humans [12,25] by attenuating short telomere-induced DNA damage [12].

CORT-induced oxidative and inflammatory status probably increases PARP1 and cyclic ADP ribose hydrolase (CD38) activation, which are the primary consumers of NAD<sup>+</sup>, and restricts NAD<sup>+</sup> bioavailability to SIRT1 and SIRT3 [26] that prevent liver damage, fat accumulation, and fibrosis [27]. Therefore, in the present study, the limited NAD<sup>+</sup> bioavailability caused by CORT-induced liver inflammation may have reduced SIRT1 and SIRT3 levels in the liver in CORT rats. NR can enter the NAD<sup>+</sup> salvage pathway via phosphorylation to NMN by nicotinamide riboside kinases (NRKs), and NMN is converted into NAD<sup>+</sup> to feed the NAD<sup>+</sup> biosynthesis cycle [28,29]. Oral NR supplementation achieves the replenishment of cellular NAD<sup>+</sup> reduced by inflammation and may diminish circulating inflammatory cytokine activities [30]. Thus, NR can attenuate oxidative inflammatory tissue damage by inhibiting mitochondrial ROS production and reducing MDA levels while elevating SOD levels [31,32]. Analogously, PC supplementation boosts antioxidant enzyme levels in the liver and alleviates hepatic oxidative stress owing to its radical scavenging, lipid peroxidation prevention [33], and mitochondrial respiratory complex protective activity [34]. The anti-inflammatory ability of PC may stem from its inhibitory action on activating programmed cell death five protein (PCDP5)/nuclear factor  $\kappa$ -B or toll-like receptor 2/NF- $\kappa$ -B pathways [35,36].

In the present study, NR plus PC supplementation effectively increased liver NAD<sup>+</sup>, NAM, and NADPH levels. Similar to our results, It has been shown that PC has inhibitory activity on NADPH oxidase (NOX) [37], producing ROS by utilizing NADPH [26]. Fan et al. [38] showed that NOX inhibition augments NADPH and NAD<sup>+</sup> levels. Based on Fan et al. [38]'s report, we assumed that NADPH levels increased following NOX inhibition by PC, and NAD<sup>+</sup> and NAM levels were elevated in parallel depending on the reduced NADPH requirement. The elevated NAM level might have stimulated NAMPT upregulation in the NR plus low-dose PC-supplemented group to promote the NAD<sup>+</sup> salvage pathway. NAMPT can mediate the activity of NAD<sup>+</sup>-dependent enzymes and alleviates hepatic steatosis in a SIRT-dependent manner in mice [39]. NAMPT overexpression can independently influence liver regeneration, and this regeneration process may also be restored by NR treatment in NAMPT knockout mice through SIRT1-dependent pathways [40]. In contrast to other spirulina-derived PCs [41], we found that novel PC oligopeptides could not have effectively stimulated SIRT1 while increasing the SIRT3 levels in the liver. Also, NR+PC supplementation was not as effective as NR supplementation in elevating SIRT3 levels. Under inflammatory conditions, increased NAM levels by phytochemical administration may negatively affect SIRT1 activity due to the activation of the PARP1-dependent anti-inflammatory response pathway [42]. Although we could not demonstrate why SIRT1 was not stimulated by PC, our results suggested that the PC oligopeptide exerts SIRT1-independent anti-inflammatory effects in the liver of CORT-treated rats.

We also explored the effects of NR and PC on telomere shelterin complex proteins. Recently, Shen et al. [43] showed that CORT exposure impaired the telomere shelterin protein complex and

reduced telomere length in the liver of rats. TRF1 has both lengthening and shortening effects on telomeres to avoid DNA damage and protect telomere stability [44]. Similar to our results, Badmus et al. [45] reported that liver TRF1 levels decreased after four weeks of CORT feeding in broiler chickens. TRF2 positively regulates telomere function and continually interacts with TRF1 and POT1 to control telomere length [46]. An in vitro study showed that oxidative damage could interfere with telomere length by suppressing TRF2 activity [47]. Xie et al. [24] indicated that CORT exposure reduced liver TRF2 activity in rats. In parallel with these results, we found that POT1a and POT1b levels were reduced after CORT injection because POT1 binding to the TRF1 and TRF2 complex is mediated by TIN2-interacting protein (TPP1) and TIN2 [48]. The interaction between POT1, TPP1, and TIN2 orchestrates the functions of telomere shelterin complex proteins and telomere integrity [49]. Shen et al. [43] demonstrated that liver TIN2 and POT1a levels decreased after CORT exposure in rats, and this report supports our findings. Promoting NAD<sup>+</sup> metabolism after NR supplementation may prevent ROS-induced DNA damage and mitochondrial impairment in DC fibroblasts [12]. Because NR activates the NAD<sup>+</sup>/SIRT1 pathway, it increases mitochondrial biogenesis and decreases ROS generation; thus, NR may prevent telomere shortening [12]. On the other hand, PC supplementation presumably regulates telomere shelterin proteins, owing to its potent antioxidant activity [19,50] and protective effect on the mitochondrial respiratory complex [34] rather than the SIRT1-dependent pathway. Although we did not measure the liver mTOR levels, PC might have increased the telomere length by diminishing mTOR complex activation, as reported before by Joly et al. [51]. Furthermore, we elucidate that PC partly stimulated SIRT3 activity, which improved mitochondrial biogenesis and antioxidant defense [52]. Collectively, NR plus PC administration may have concurrently prevented hepatic telomere shortening in CORT rats.

Interestingly, this formulation was less effective than the low-dose PC formulation when combined with the NR high-dose PC oligopeptide. This is because antioxidants exert hormetic effects on the redox state and their mechanisms of action may vary depending on the dose [53]. Thus, the protective effect of NR on hepatic telomere length, inflammation, and NAD<sup>+</sup> metabolism may have been attenuated due to the cumulative activity of NR plus high-dose PC supplementation.

## 5. Conclusions

In conclusion, the present study showed that NR and PC oligopeptide supplementation in CORT-treated rats might be relevant in improving hepatic telomere shortening, energy metabolism by regulating the response of the liver through a metabolic increase in both cellular NAD<sup>+</sup> availability and hepatic cytokines, SIRT-3, and NAMPT pathways. However, more preclinical and clinical studies are needed to reveal the molecular mechanisms of action of these combinations.

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