



Potential of dietary restriction-induced lifespan extension by polyphenols

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ABSTRACT

Dietary restriction (DR) extends lifespan across multiple species including mouse. Antioxidant plant extracts rich in polyphenols have also been shown to increase lifespan. We hypothesized that polyphenols might potentiate DR-induced lifespan extension. Twenty week old C57BL/6 mice were placed on one of three diets: continuous feeding (control), alternate day chow (Intermittent fed, IF), or IF supplemented with polyphenol antioxidants (PAO) from blueberry, pomegranate, and green tea extracts (IF + PAO). Both IF and IF + PAO groups outlived the control group and the IF + PAO group outlived the IF group (all $p < 0.001$). In the brain, IF induced the expression of inflammatory genes and p38 MAPK phosphorylation, while the addition of PAO reduced brain inflammatory gene expression and p38 MAPK phosphorylation. Our data indicate that while IF overall promotes longevity, some aspects of IF-induced stress may paradoxically lessen this effect. Polyphenol compounds, in turn, may potentiate IF-induced longevity by minimizing specific components of IF-induced cell stress.

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1. Introduction

Dietary restriction (DR) extends lifespan in multiple species including flies, yeast, worms, mice, and rats [1]. Different DR regimens, including alternate day or intermittent fasting (IF), have been extensively studied in rodents [2] and shown to modify expression of genes involved in oxidative stress, tumorigenesis, cell bioenergetics, inflammation, and mRNA splicing [3]. DR influences the energy sensing AMP kinase system [4] and the sirtuin family of NAD-dependent deacetylases [5]. It affects mitochondrial maintenance [4], and alters chromatin architecture [6]. These changes are demonstrable in a variety of tissues and can be tissue-specific. It has been hypothesized that DR slows the basic aging process through effects that involve a number of interactive gene networks. While DR has not yet been shown to

increase human longevity, it does appear to mitigate age-associated increases in insulin resistance, cholesterol, and blood pressure [7].

Several plant polyphenols have also been shown to increase longevity. In particular, crude blueberry extracts and green tea polyphenols prolong lifespans of worms and mice, respectively [8,9]. These effects have been attributed both to antioxidant effects and to the direct activation of SIRT1 by compounds such as resveratrol that are present in the polyphenol mixtures. While pomegranate has not been reported to extend lifespan, polyphenol-rich pomegranate fruit extract inhibited oxidative stress in human keratinocytes [10]. Although it has been proposed that polyphenols mediate life prolongation primarily via their anti-oxidant properties, some studies suggest that some antioxidant chemicals not only fail to provide a life prolonging effect, but also unexpectedly reduce the life-extension capacity of DR. In mice, an antioxidant mixture containing 2-mercaptoethylamine plus ethoxyquin countered DR-induced lifespan extension [11]. N-acetylcysteine, vitamin C, and the vitamin E derivative trolox abolished a DR mimetic-induced lifespan increase in *Caenorhabditis elegans* [12]. Similarly, in humans, vitamins C and E reduced the benefits of endurance training [13]. In this study, we examined the impact of

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polyphenol-plant derived antioxidants (PAO) on DR's longevity-promoting effects.

2. Material and methods

2.1. Survival experiment

Survival studies were performed using 148 (12 control, 68 IF and 68 IF + PAO) male C57BL/6 mice that were obtained from Jackson Laboratories at 4–6 weeks of age. Individual mice were housed in separate clear plastic cages on a 12:12 h light:dark schedule. All mice were maintained on an AL diet until 20 weeks of age. At 20 weeks of age the mice were randomized to 3 different dietary groups. AL-fed mice (the control group) had uninterrupted food access. IF mice had 24 h of no food access followed by 24 h of food access (fasted 3 days per week and fed high fat chow ad libitum 4 days per week). IF + PAO mice had 24 h of no food access followed by 24 h of food access (fasted 3 days per week and fed high fat chow supplemented with blueberry, green tea, and pomegranate extracts ad libitum 4 days per week). The control and IF groups consumed TD.94045 AIN-93G chow (Harlan-Teklad). The IF + PAO mice consumed TD.07226 (Harlan Teklad), which is TD.94045 modified to contain 2% blueberry extract, 115 ppm Sunphenon (epigallocatechin gallate; EGCG), and 0.3% pomegranate powder at the expense of corn starch. All mice had uninterrupted access to drinking water.

2.2. Microarray experiments

In addition to the mice used in the survival experiment, another 8 male mice and 16 female mice were maintained on the above specified diets in order to generate biospecimens for microarray experiments. Brain tissue was collected from these mice at 2 years of age, flash frozen in liquid nitrogen, and stored at -80°C until use. For male mice, brains were collected at the end of a feeding day for comparisons of the control ($n=2$), IF ($n=3$) and IF + PAO ($n=3$) groups. For female mice, brains were collected at the end of a feeding day for comparisons of IF ($n=4$) and female IF + PAO mice ($n=4$), as well as at the end of a fasting day (IF group $n=4$; IF + PAO group $n=4$). For each specimen total RNA was isolated from 50 to 100 mg of freshly ground tissue using TRIzol reagent with phase lock gel, and subjected to quality assessment with Agilent RNA kits before reverse transcription. Total cDNAs were amplified once and hybridized to Affymetrix Genechip Array Mouse 430 2.0. Data were analyzed by Affymetrix Expression Console RMA-PARTEK (2 data points) and by GeneSpring for gene annotation. To evaluate the expression levels of gene sets, Gene Set Enrichment Analysis (GSEA, <http://www.broadinstitute.org/gsea/index.jsp>) software was used. The enrichment score (ES) and normalized enrichment score (NES) were calculated to reflect the over-represented degree [14]. GSEA gene ontology collection v3.0 was used (<http://www.broadinstitute.org/gsea/msigdb/index.jsp>) for pathway expression analysis. False Discovery Rate (FDR) q values and nominal p values were calculated by GSEA for each gene set.

2.3. Immunoblotting

Another 15 male C57BL/6 mice were placed onto the different diets and used to prepare brain protein lysates. For this part of the analysis, the control, IF, and IF + PAO groups each contained five mice. After one month on their respective diets these mice were sacrificed by decapitation. The control group had access to food up to the time of sacrifice, while the IF and IF + PAO groups had no food access for 24 h prior to sacrifice. Brains were rapidly removed and placed in liquid nitrogen within 1 min of decapitation. The frozen brains were later thawed, on ice, to the point that homogenates could be generated and protein lysates were subsequently prepared using a NE-PER

kit (Pierce) according to the manufacturer's instructions. A primary antibody to the phospho-p38 (Cell Signaling Technology) mitogen-activated protein kinase (MAPK) was used to measure p38 activation

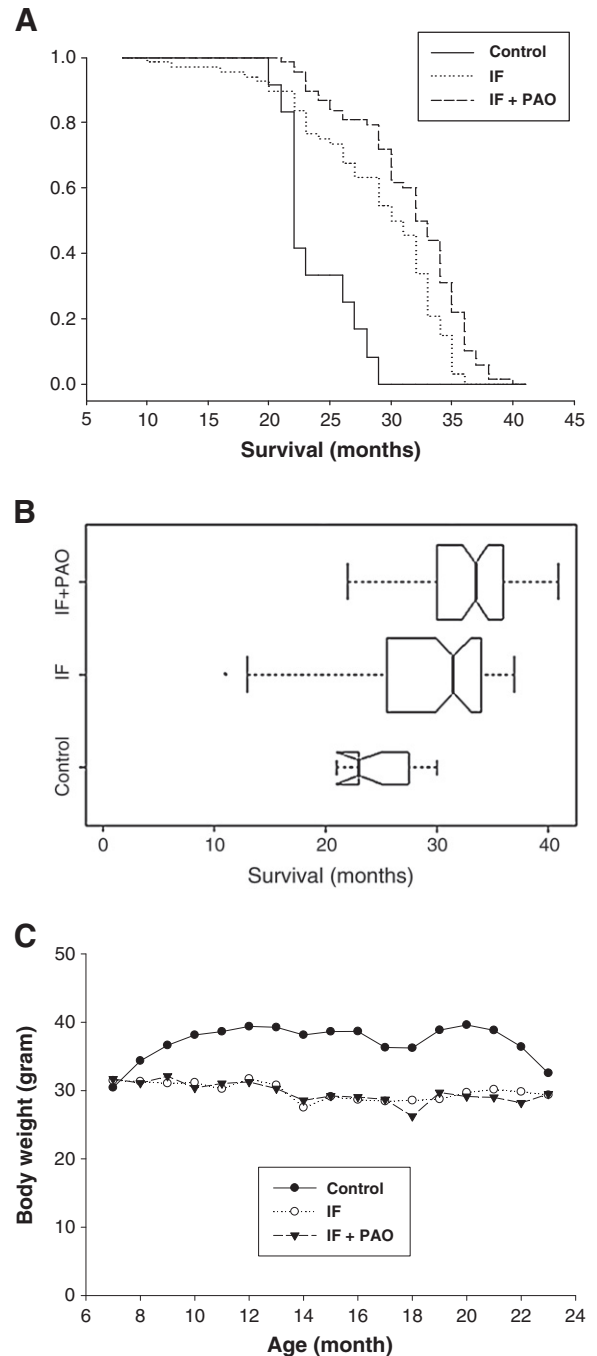


Fig. 1. Comparison of survival (A, B) and body weights (C) for male C57BL/6 mice. Mice were fed a high fat chow ad libitum (control, $n=12$), high fat chow on alternate days four days a week (intermittent fed group, IF, $n=68$), or alternate day high fat chow supplemented with blueberry, pomegranate, and green tea extracts (IF + PAO, $n=68$). (A) Kaplan-Meier survival curves are used to represent the distribution of survival time in months. (B) Box plots represent the distribution of survival time in months for the three groups. The median survival time, first and third quartile, and outlying times are represented by the center line, left and right bounds of the box, and plotted points, respectively. Boxes are drawn with widths proportional to the number of observations per group. Whiskers extend to the most extreme observation within a distance of 1.5 times the interquartile range ($IQR=Q_3-Q_1$). The notches in the side of each box extend to $\pm 1.58(IQR/\sqrt{n})$ and represent an asymptotic but robust 95% confidence interval estimate for median survival time. Strong evidence of a difference in the median survival time between two groups is indicated by non-overlapping notches of the plots of those groups. (C) Mean body weights across the duration of the study. Control mice stabilized at 37 g. IF and IF + PAO groups stabilized at 29 g.

Table 1
Survival analysis and Kruskal–Wallis rank sum test ($\chi^2 = 24.47$, $p < 0.001$) results.

A. Kaplan–Meier estimates of median survival		
Group	N	Median survival (months)
Control	12	23.0
IF	68	31.5
IF + PAO	68	33.5
B. Kruskal–Wallis rank sum tests of significance		
χ^2 , p	Control	IF
Control	–	–
IF	17.9 (<0.001)	–
IF + PAO	43.9 (<0.001)	12 (<0.001)

in the cytosolic lysate fraction. Primary antibody binding was revealed using horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology) and Supersignal West Femto Maximum Sensitivity Substrate (Thermo Scientific). Densitometry was performed using a ChemiDoc XRS with Quantity One software. To ensure equivalent protein loading, the blot was subsequently stained with a primary antibody to actin (Santa Cruz Biotechnology). The phospho-p38 band densities were divided by their corresponding actin densities to yield a relative density value. Relative density values were summarized by mean and standard error. Means were compared by one-way ANOVA followed by a Least Squares Deviation test, with p values less than 0.05 considered significant.

3. Results

To examine the effects of PAO on DR lifespan extension, we placed five month old C57BL/6 mice on three different diets. The control diet consisted of a high-fat chow, which was selected over standard chow in order to better reflect diet patterns typical of Western countries.

Table 2
Deceased mice necropsy results.

	IF	IF + PAO
Died naturally and underwent necropsy	32	30
Severely enlarged liver, likely liver tumor	12 (34%)	12 (32%)
Visibly enlarged liver, possibly due to liver tumor	6 (17%)	7 (18%)
Short or no top teeth	14 (40%)	11 (30%)

Table 3
20 gene sets were significantly down-regulated by addition of PAO to IF (FDR $q < 0.0001$, NOM $p < 0.001$).

Pathway	Description	ES	NES	NOM p-val	FDR q-val
WIELAND_HEPATITIS_B_INDUCED	Immune response	–0.69	–2.63	0.00	0.00
ICHIBA_GVHD	Immune response	–0.52	–2.44	0.00	0.00
SANA_IFNG_ENDOTHELIAL_UP	Immune response	–0.63	–2.26	0.00	0.00
FLECHNER_KIDNEY_TRANSPLANT_REJECTION_UP	Immune response	–0.56	–2.17	0.00	0.00
NEMETH_TNF_UP	Immune response	–0.55	–2.16	0.00	0.00
DER_IFNG_UP	Immune response	–0.59	–2.15	0.00	0.00
SANA_TNFA_ENDOTHELIAL_UP	Immune response	–0.56	–2.13	0.00	0.00
IGLESIAS_E2FMINUS_UP	Immune response	–0.52	–2.20	0.00	0.00
LAL_KO_6MO_UP	Inflammation	–0.61	–2.27	0.00	0.00
LAL_KO_3MO_UP	Inflammation	–0.61	–2.15	0.00	0.00
CARIES_PULP_UP	Inflammation, DNA repair, cell differentiation	–0.53	–2.35	0.00	0.00
LIAN_MYELOID_DIFF_GRANULE	Cell differentiation	–0.80	–2.54	0.00	0.00
TGFBETA_EARLY_UP	Cell differentiation	–0.66	–2.29	0.00	0.00
TAKEDA_NUP98_HOXA9_8D_DN	Cell differentiation	–0.49	–2.13	0.00	0.00
YU_CMYC_DN	Tumorigenesis	–0.58	–2.15	0.00	0.00
LEE_MYC_E2F1_UP	Tumorigenesis	–0.61	–2.14	0.00	0.00
EMT_UP	metastasis/tumorigenesis	–0.59	–2.15	0.00	0.00
GILDEA_BLADDER_UP	Metastasis/tumorigenesis	–0.70	–2.15	0.00	0.00
NADLER_OBESITY_UP	Adipogenesis	–0.63	–2.30	0.00	0.00
AGEING_BRAIN_UP	Antioxidant and DNA repair	–0.55	–2.43	0.00	0.00

We established two treatment groups: an IF group that underwent repeated fasting and refeeding (in 24 h cycles) with the high fat chow, and an IF group whose high fat chow was supplemented with blueberry, pomegranate, and green tea extracts (the IF + PAO group).

As shown in Fig. 1A, compared to the control group the IF and IF + PAO groups had significant lifespan prolongation ($p < 0.001$ and $p < 0.001$, respectively) (see also Table 1). IF + PAO mice also significantly outlived IF mice with a median survival of 33.5 months compared to 31.5 months for the IF group ($p < 0.001$) (Fig. 1A and B, Table 1). Major postmortem pathological findings did not differ between IF and IF + PAO mice (Table 2). Relative to the control group, body weight was significantly reduced in both the IF and IF + PAO groups but IF and IF + PAO body weights were themselves comparable (Fig. 1C).

GSEA was used to identify gene ontology pathways that were differentially expressed in IF + PAO versus IF mouse brain tissue. These studies were performed using both male and female aged C57BL/6 mice. For 2 year-old male and female mice sacrificed at the end of a feeding day, no pathways were significantly altered. Although we did not analyze gene expression in aged male mice at the end of a fasting day, in female mice sacrificed at the end of a fasting day GSEA identified 20 gene sets that were down-regulated by addition of PAO to IF (FDR $q < 0.0001$, NOM $p < 0.001$). Table 3 lists these 20 sets by biological function. Of the 20, 11 were related to immune response or inflammation, 4 to tumorigenesis/metastasis, 3 to cell differentiation, 1 to adipogenesis, and 1 to oxidative stress.

These gene array findings suggest that addition of PAO to the IF protocol prevents the activation of pro-inflammatory pathways. In support of this, we analyzed the activation status of brain p38 MAPK protein. The p38 MAPK acts as a pro-inflammatory signaling pathway intermediate that is activated by oxidative stress and oxidative stress-related conditions. We found that relative to the control diet the un-supplemented IF diet actually induced p38 phosphorylation, while the addition of PAO to the IF diet blocked this effect (Fig. 2).

4. Discussion

This study investigated the impact of PAO on DR-induced lifespan extension. While our data show both IF and IF + PAO significantly prolong lifespan, adding PAO to IF further extends lifespan significantly beyond IF alone.

We previously reported that the addition of this PAO combination to this IF DR protocol did not alter insulin sensitivity or glucose tolerance in male C57BL/6 mice [19]. We now report that addition of PAO

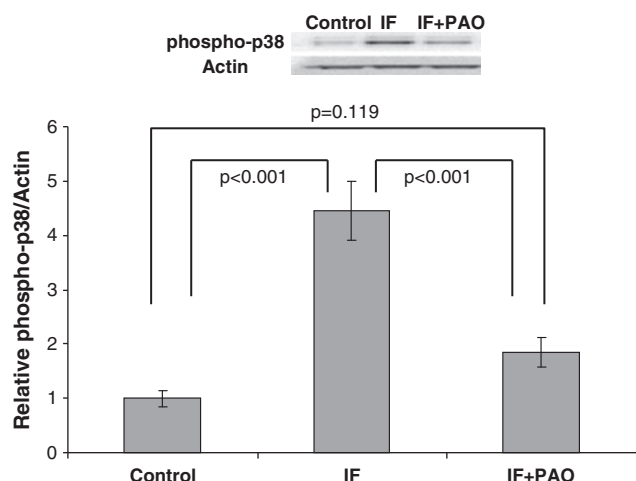


Fig. 2. p38 phosphorylation in brain protein lysates from control, IF, and IF + PAO mice. A Western blot approach was used to determine phospho-p38 and actin band densities. The density value for each phospho-p38 band was divided by its corresponding actin band density value to correct for differences in protein loading. Each group contained brain lysates from five different mice. Data are presented as mean values \pm SEM. The groups were analyzed by one-way ANOVA. Phospho-p38 in the IF group was higher than it was in the control group, which suggests 24 h of fasting activated p38 MAPK signaling. PAO supplementation prevented the apparent fast-induced increase in p38 phosphorylation.

to the IF protocol did not affect the weight of the mice. Together, results from our previous and current studies suggest that changes in weight, insulin sensitivity, and glucose tolerance cannot explain the beneficial effect of PAO supplementation on mouse longevity. Rather, our data suggest that this benefit may be explained by changes in immune response, inflammation, cell differentiation, oxidative stress, DNA repair, and adipogenesis-related gene expression. In particular, eleven of twenty PAO-induced GSEA changes involved inflammation/immunity gene sets. Our data, therefore, further suggest that although IF extends longevity in caged mice, it also simultaneously induces stress that may paradoxically counteract its overall beneficial effects.

Epidemiological data suggest that in humans DR may prolong lifespan and reduce mortality from cancer and coronary heart disease [20]. Uncontrolled data from people practicing self-imposed DR suggests that it protects against diabetes, left ventricular diastolic dysfunction, and inflammation [21]. Examining whether polyphenols potentiate DR benefits in humans will likely require logistically complex randomized clinical studies. Our data on polyphenol–DR combinations can potentially inform the design of such studies.

We selected a high-fat chow for these experiments because we wanted to mimic the effects of a Western diet. Our use of a high-fat diet does, however, raise several interpretive considerations. One caveat arises from the possibility that even though the mice were on an alternate day feeding protocol, our high-fat baseline diet may have rendered the mice obese. To assess this, we reviewed prior CR studies of male C57BL/6 mice [15–18]. Body weights in both our control and IF groups were comparable to those from other studies (Table 4).

Control group longevity in our study was reduced relative to what was reported in these from other studies (Table 4), which could be due to the high fat chow these ad libitum-fed mice were maintained on. However, compared to the other studies the high fat chow did not appear to negatively impact the life expectancy of our IF group. Since our study did not focus on differences between IF and control, but rather on differences between IF and IF + PAO, the fact that our IF group lived as long as non-high-fat IF groups from other studies indicates IF-induced lifespan extension in this study was not simply an artifact of reduced life expectancy in our control group.

Our study has several procedural limitations. The fasting GSEA data were obtained only in female mice and the survival data were

Table 4

Comparison of approximate body weights and median life spans for C57BL/6 mice from different DR studies.

	Control	DR
A. Comparison of approximate body weights among various studies (in grams)		
Non-obese		
Turturro	41	23
Aires	37	29
Pearson	34	30
Pugh	33	25
Weindruch	32	25
Obese		
Pearson's obese mice	47	
B. Comparison of approximate median life span among various studies (in months)		
Non-obese		
Turturro	26.8	32.1
Aires	23	31.5
Pearson	29.5	31.3
Pugh	29.8	34.6
Weindruch	30	31.2
Obese		
Pearson's obese mice	27	

obtained only from male mice. This occurred because the male mice were sacrificed before the need to sacrifice mice on a fasting day was appreciated, and thus no gene array data were obtained from the post-fasted male mice. The fact that these pathway changes were only observed in mice on the fasting day raises the possibility that PAO benefits require the presence of DR, and that PAO without DR might not prolong longevity. Finally, the p38 MAPK biochemical analysis was performed on relatively young male mice, as opposed to aged mice. Repeating these measurements in appropriately aged mice would be required to determine whether or not the ability of PAO to reduce IF-induced oxidative stress is age-dependent or age-independent.

5. Conclusion

Our study is the first to demonstrate that polyphenols, when used in conjunction with an intermittent feeding DR protocol, can enhance the longevity-promoting effects of that DR protocol (at least in mice). The underlying mechanism appears to involve reduction of cell stress and inflammation-related activity, some of which seems to be a byproduct of DR itself. In the absence of definitive clinical trials that address the health benefits of DR–polyphenol combinations, our data may have practical relevance for humans seeking to engage in health-promoting behaviors.

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