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Effects of Nicotinamide Riboside and Beta-hydroxybutyrate on C. elegans Lifespan

Jeffery Peters
East Tennessee State University

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By

Jeffery Dylan Peters

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J. Dylan Peters 4/27/20

Jeffery D. Peters Date

Patrick C Bradshaw 4/29/2020

Dr. Patrick C. Bradshaw, Thesis Mentor Date

Joseph R Bidwell 4/28/20

Dr. Joseph R. Bidwell, Reader Date

Hugh A Miller III 4/28/20

Dr. Hugh A. Miller, Reader Date

Abstract

The nicotinamide riboside (NR) form of vitamin B_3 and the ketone body ß-hydroxybutyrate (BHB) are two of the most promising natural compounds yet identified for the treatment of aging and aging-related diseases. Forms of vitamin B_3 are precursors for the synthesis of the coenzymes nicotinamide adenine dinucleotide (NAD(H)) and nicotinamide adenine dinucleotide phosphate (NADP(H)). In aged cells levels of NAD^+ decline, decreasing metabolism and decreasing activity of protective sirtuin protein deacetylases. In aged cells NR, but not more common forms of vitamin B3, boost NAD^+ levels. BHB is naturally produced by the body when individuals fast or consume a ketogenic (KD) or calorically restricted (CR) diet. These diets have been shown to extend lifespan in mice, while they are also protective in many disease models. *Caenorhabditis elegans*, a roundworm with a short mean lifespan of roughly 2 to 3 weeks depending upon the temperature, is used as a model system to study aging. BHB has been previously shown to increase lifespan by roughly 20% when administered to *C. elegans.* We administered NR and BHB individually and together to *C. elegans* starting at two different developmental stages (larval stages 1 and 4) and measured lifespan. We found that administration of 20 mM DL-BHB decreased lifespan when first given at the L1 stage, while it robustly increased lifespan when first given at the L4 stage. Administration of 0.5 mM NR increased lifespan when first given at L1, with only a very slight increase when first given at L4. When initiating administration at L1, NR greatly mitigated the BHB-mediated decline in longevity, however, NR did not increase BHB-mediated lifespan extension when first administered at L4.

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Introduction

Nicotinamide adenine dinucleotide (NAD^+) is an important coenzyme that is used in hundreds of enzymatic reactions and almost all major metabolic pathways [1]. Levels of NAD^+ decline with human aging, and this appears to contribute to mitochondrial dysfunction and other metabolic decline in age-related diseases [2-6]. It has also been shown that NAD^+ levels decrease with age in mice and the nematode *Caenorhabditis elegans*. Genetically decreasing NAD^+ levels also decreases lifespan [7]. Therefore, increasing cellular levels of NAD^+ has been hypothesized to increase lifespan in mammals. This hypothesis was tested in *C. elegans* and as expected, the increased NAD^+ levels upregulated sirtuin protein activity, decreased metabolic decline, and increased lifespan [7].

Nicotinamide riboside (NR) is a form of vitamin B_3 that was first shown in yeast to be a precursor to NAD^+ [8]. Not long after, administration of NR to yeast cells was shown to increase lifespan [9]. More recently, a large number of NAD^+ precursor feeding studies were performed in rodents that showed that increasing NAD^+ levels had "a favorable outcome" on a number of age-related disorders characterized by "chronic oxidative stress, inflammation, and impaired mitochondrial function" [10]. It is likely that NR treatment increases nucleo-cytoplasmic and mitochondrial NAD^+ levels and the activity of sirtuin proteins, which leads to increased lifespan. In summary, these data show that NR has great potential for the treatment of age-related diseases in humans.

The oldest known method of lifespan extension is the calorie restriction diet (CR). CR was first shown to increase lifespan in rats over 80 years ago [11]. It is hypothesized that CR extends lifespan by slowing and/or delaying the process of aging. It has been shown in mice that CR causes a shift from glucose to fatty acid and ketone body metabolism, because on this diet

carbohydrates are present at too low of levels to supply all of the energy the body needs [12]. However, neurons are unable to readily oxidize fatty acids, but can instead oxidize ketone bodies when glucose levels are low. Recently, scientists became interested in the effects on longevity of a ketogenic diet (KD), where the main source of energy is obtained through a high fat, low carbohydrate diet. During ketosis, the body, especially neurons, does not have enough carbohydrates to supply energy needs, and so fatty acids are broken down into the ketone bodies acetone, acetoacetate, and ß-hydroxybutyrate (BHB) in the liver and then distributed to peripheral tissues in the circulation, which fill this energetic need. BHB has both D- and L-isomers, with the D- form being the one produced in the body under carbohydrate depleted conditions.

Studies were performed in order to determine if KD or other diets that increase ketone body levels could induce anti-aging effects similar to those induced by the CR diet. Studies using either *C. elegans* or mice showed that increasing BHB levels increased lifespan in a similar way as that occurs when signaling through the insulin/insulin-like growth factor receptor is decreased [13]. Another study found that KD further helped to preserve "motor function, memory, and muscle mass in aged mice", further showing that KD can be a successful way to increase lifespan and improve health over the lifespan of animals undergoing this diet [14]. Another study showed that a cyclic KD (where high carbohydrate, high fat, and ketogenic diet cycles were alternated each week from 12-24 months of age) in mice showed a reduction in mid-life mortality rates and preserved memory function [15].

With KD being shown to increase lifespan, the effects on lifespan of individual ketone bodies that are increased by the KD were also studied. The most abundant and most stable ketone body is BHB, and as such most research has focused on it. In fact, one study showed that

treatment of *C. elegans* with D-BHB increased lifespan by an average of 20% [16]. One pathway by which BHB was shown to extend lifespan was by inhibiting histone deacetylases (HDACs). BHB was shown to increase histone acetylation leading to upregulation of the expression of the FOXO3a transcriptional regulator in mammals and likely the homologous *daf-16* gene in *C. elegans*. Another proposed protective pathway is the stimulation of mitochondrial metabolism, where increased BHB levels increase mitochondrial metabolism of BHB, causing increased citric acid cycle (CAC) intermediates (which lead to activation of DAF-16/FOXO). BHB-mediated lifespan extension was also shown to require activation of the Nrf2 (SKN-1 in *C. elegans*) transcriptional regulator, which can be activated by increased reactive oxygen species (ROS) produced from increased mitochondrial metabolism. SKN-1/Nrf-2 increases antioxidant gene expression [17]. These proposed pathways overlap and are shown in Figure 1. The study using *C. elegans* indicated that D-BHB does indeed extend lifespan through the HDAC inhibition pathway, as well as through "conserved stress response pathways" such as the DAF-16/FOXO and SKN-1/Nrf-2 antioxidant pathways described above [16].

The mechanisms through which increased activities of the DAF-16/FOXO and SKN-1/Nrf2 transcriptional regulators extend lifespan are not completely known, as each regulates the expression of hundreds of genes. But since these factors increase the resistance to multiple stresses (oxidative stress, thermal stress, unfolded protein stress, starvation, etc.) they are master regulators capable of fine tuning many cellular functions to improve metabolism, antioxidant defense, and protein synthesis, stability, and turnover. The genes and proteins they encode likely evolved these functions for organisms to delay reproduction during times of stress as egg-laying is frequently delayed when these transcriptional regulators are activated. The lifespan extension

that occurs may therefore be a byproduct of the increased stress resistance mechanisms established to be able to delay reproduction.

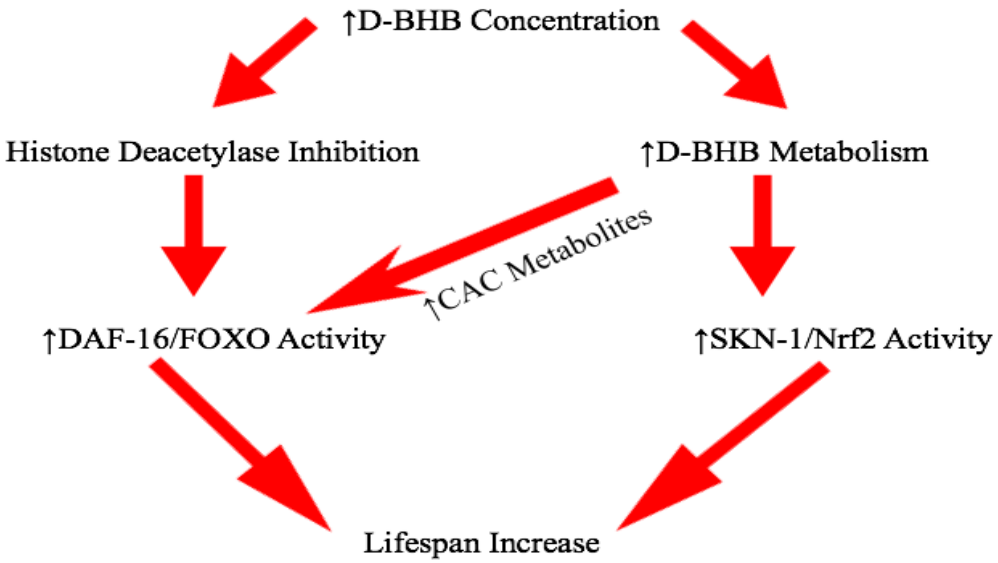

Figure 1: Possible Pathways Mediating Lifespan Extension via BHB Treatment in *C. elegans.*

Part of the rationale behind performing this research was to determine if NR and BHB have an additive effect on lifespan, which would occur if they function through independent pathways, or alternatively if they activate a shared metabolic pathway that would prevent an additive effect. *C. elegans* serves as a good model for such an experiment. These roundworms are small, roughly about 1 mm in size, and have four larval stages. If kept at room temperature for three days, they reach their gravid stage of development, being able to lay eggs at that time [18]. They are also useful in the study of human aging and aging-related disease, as 40% of human disease genes have a homolog present in the *C. elegans* genome [19]. They also have an average lifespan of 2-3 weeks, making their use more time-efficient than using longer-lived mammalian organisms [20].

Because of these benefits, *C. elegans* serves as an efficient model to quickly perform lifespan assays and to gather data in a relatively small amount of time. While NR and BHB are both compounds of interest in the fields of longevity research, their effects on lifespan when added together remain unknown. It is hypothesized that NR and BHB will confer an additive effect on lifespan in *C. elegans*, working on independent metabolic pathways to increase lifespan further than either compound would when added individually. There is also interest regarding whether the lifespan-extending effects of these compounds occur during the developmental stages as this is the case for some long-lived mitochondrial mutant strains or strictly during adulthood. Therefore, treatment was first initiated at either the first larval stage (L1) or the final larval stage (L4).

Materials and Methods

Cultures of mixed age wild-type N2 strain *C. elegans* nematodes were grown on nematode growth media (NGM) agar plates and washed from the agar into 50 mL conical tubes using a 0.1 M NaCl solution. Worms were then suspended in a 1% bleach and 0.5 M NaOH solution to kill the larvae and adult *C. elegans*, leaving only eggs that were protected by their thick eggshell. The eggs obtained are age-synchronized to within 9 hours, in order to have a same-age population for lifespan assays. Eggs were then placed in M9 minimal growth media overnight, when worms will hatch into larval L1 stage. *C. elegans* will stay overnight in this stage, as they will not develop further until fed [18]. Worms in the larval L1 stage were then transferred into 8-micron cell culture inserts placed in 12-well microplates with roughly 25-60 eggs per insert. The inserts provide a semi-permeable membrane that allow food (bacteria) and waste products to move through the membrane while keeping *C. elegans* from escaping. 1.35 mL of liquid S-media was added to the outer portion of each of the 12 well plates, followed by 0.15 mL 9 x10^9 (10 mg protein/mL) HT115(DE3) empty vector *E. coli* cells/mL which served as food. *C. elegans* were given live bacteria up until day 3 of each experiment, to allow for proper nutrition during their developmental stages, after which worms were exclusively fed only heat-killed bacteria (of the same concentration and strain) to prevent the bacteria from metabolizing the treatment compounds. In total, this caused the final volume of each well to equal roughly 1.5 mL. Microplates were incubated at 20˚C and shaken at 60 rpm to provide aeration throughout the duration of the experiment. After 3 days, worms reached adulthood and 0.4 mM fluorodeoxyuridine (FUdR), a DNA synthesis inhibitor, was added to each of the 12 well plates. This prevented *C. elegans* from laying eggs, which maintained the synchronous population. The

worms were counted under a microscope and the culture media, FUdR, and bacteria were replaced every Monday, Wednesday, and Friday for the entirety of each assay.

On these same days, some cohorts of worms (excluding the control groups) were also treated with either a 20 mM 50:50 mixture of D- and L-BHB, 0.5 mM nicotinamide riboside, or both. The only difference between the two experiments performed was whether *C. elegans* were first treated with NR, BHB, or both on day 1 of the experiment (when *C. elegans* were at the L1 stage of development) or day 3 of the experiment (when *C. elegans* were at the L4 stage). Upon death of the entire population of *C. elegans* for each well of the 12-well plate, the percent survival over time for each trial was averaged and the mean lifespans over time were calculated using Kaplan-Meier survival curves and Log-rank statistical analysis. In total, 3 independent trials were performed for both conditions, initiating treatment at either the L1 or L4 stage of development in *C. elegans.* The data collected is displayed in the Results and Discussion section below.

Results and Discussion

The effects of BHB or NR on *C. elegans* mean lifespan when added alone or together starting at either the L1 (Table 1) or L4 (Table 2) larval stages is summarized in Table 3. Overall, the data show that 0.5 mM NR increases mean lifespan greatly when first given at L1, but only very slightly when initiated at L4, while 20 mM DL-BHB greatly decreased mean lifespan when first given at L1, but greatly increased mean lifespan when first given at L4. When NR was added to BHB starting at L1, it blocked the detrimental effects of BHB on lifespan by 45%, but when NR was added with BHB starting at L4, it had no statistically significant effect on the large BHB-mediated mean lifespan extension.

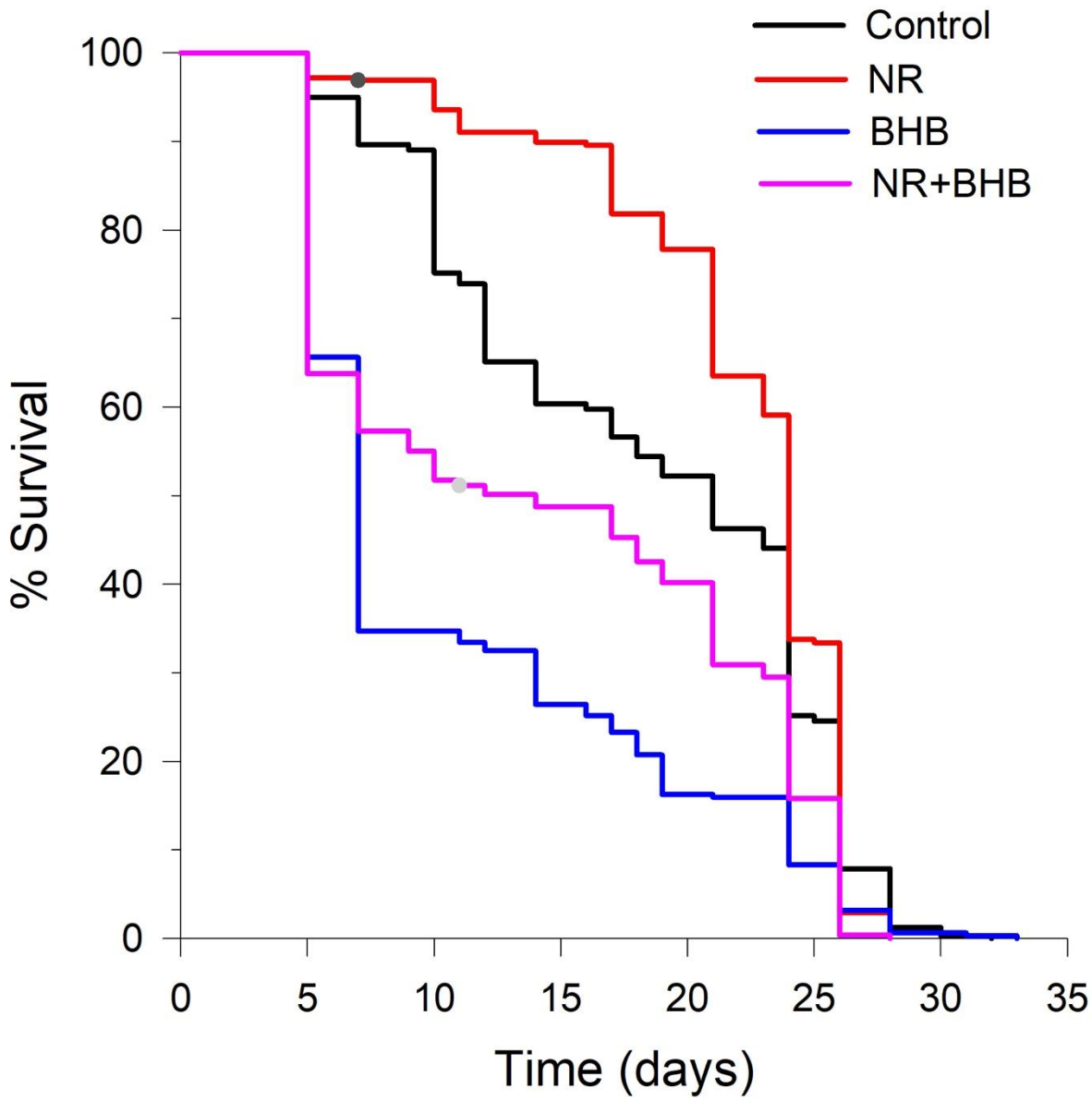

Figure 2: Survival analysis when NR, BHB, or both were first administered at L1

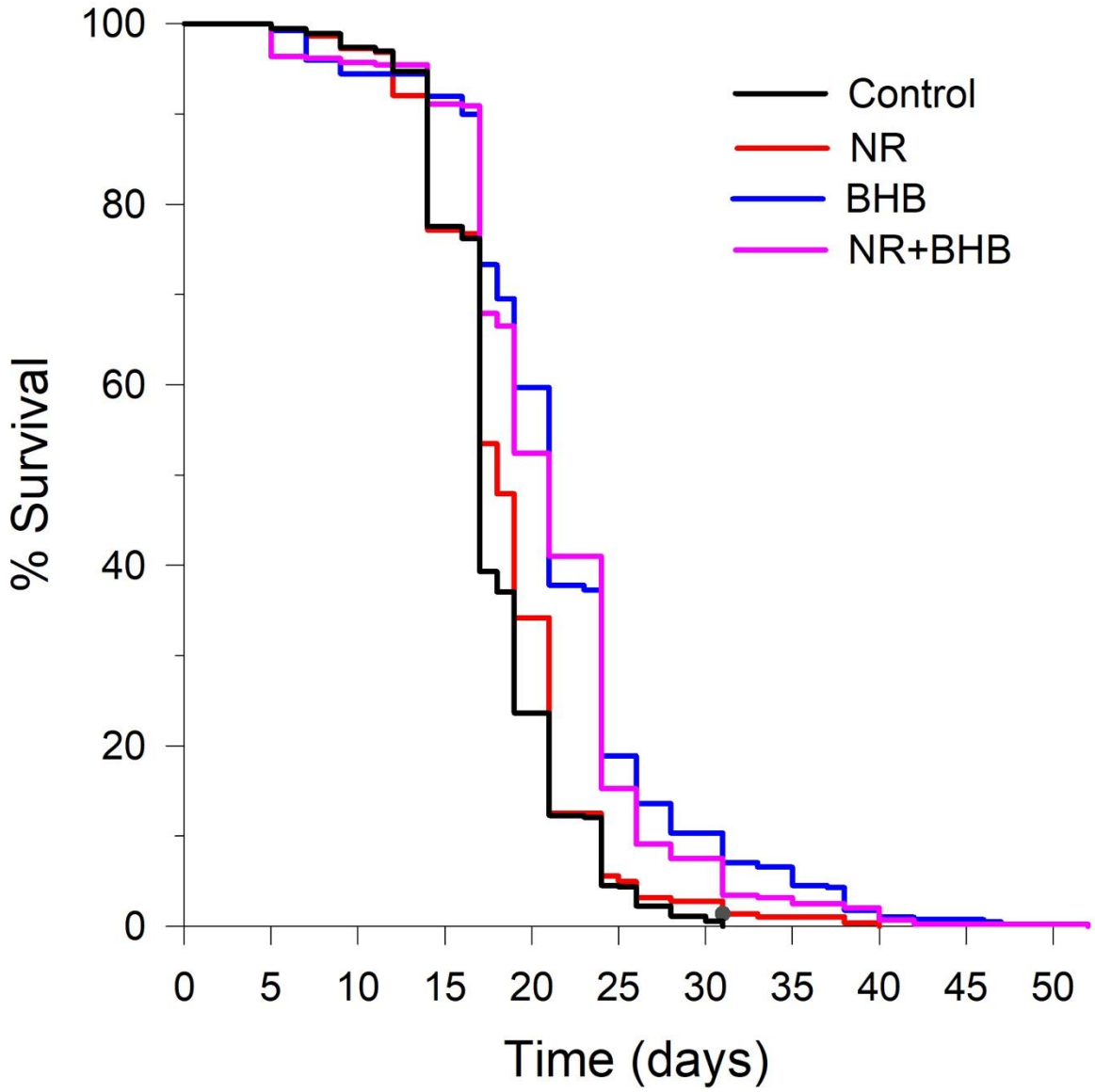

Figure 3: Survival analysis when NR, BHB, or both were first administered at L4

Table 1: Mean lifespan when treatment was initiated at L1

Treatment	Mean Survival Time
Control	18.384
NR	21.992
BHB	10.962
NR+BHB	14.381

Table 2: Mean lifespan when treatment was initiated at L4

Treatment	Mean Survival Time
Control	17.854
NR	18.433
BHB	21.584
NR+BHB	20.977

Table 3. Percent change in mean lifespan when NR, BHB, or both were administered

Treatment	L1 First Treatment	L4 First Treatment
NR	+20%	+3%
BHB	-40%	+21%
NR+BHB	-22%	+18%

Our data indicate that NR is almost exclusively extending lifespan by acting during the larval stages and BHB is exclusively shortening lifespan during this time as well. BHB appears to be extending lifespan exclusively during adulthood. Therefore, it also appears that NR and BHB are functioning through independent pathways. The ability of NR to greatly extend (by 31%) the BHB-shortened lifespan when both compounds were first administered at L1 further supports this conclusion. A further lifespan experiment showing additive effects where NR is administered starting at L1 and BHB is administered starting at L4 would strengthen this hypothesis.

The ability of BHB to exclusively extend lifespan during adulthood is consistent with a mechanism of lifespan extension through the activation of the DAF-16/FOXO and SKN-1/Nrf2 transcriptional regulators that function during adulthood to extend lifespan [21-22]. The ability of NR to extend lifespan almost exclusively during the larval stages is consistent with its activation of the mitochondrial unfolded protein response (UPR^{mt}) [7], which functions exclusively during the larval stages to extend lifespan [23]. The ability of 0.5 mM NR to extend lifespan was blocked by knockdown of *ubl-5*, a mediator of UPR^{mt} [7]. However, 0.5 mM NR-

mediated lifespan extension was also blocked in *daf-16* mutant worms. This suggests that activation of DAF-16/FOXO is shared by both BHB and NR treatments.

How does BHB decrease lifespan when administered during the larval developmental stages? It is likely that the ability of BHB to function as an HDAC inhibitor mediates this function. BHB functions as a class I HDAC inhibitor [24]. *C. elegans* has three class I HDAC genes: *hda-1*, *hda-2*, and *hda-3*. Partial knockdown of *hda-2* or *hda-3* increased lifespan and prevented BHB from further extending lifespan, while full knockout of *hda-2* or *hda-3* decreased lifespan [16]. This data, together with the ability of a lower 10 mM dose of BHB than the 20 mM dose used here, to extend *C. elegans* lifespan when administered starting at L1, suggests that when BHB only partially inhibits HDAC function during development, lifespan can be increased by HDAC inhibition during adulthood. But when BHB completely inhibits HDAC function during development, lifespan is decreased and cannot be increased by HDAC inhibition during adulthood.

To support the conclusions drawn from our data, further experiments could be performed where NR, BHB, or NR+BHB are added only from the L1 through L4 larval stages and not during adulthood. It is hypothesized that nearly the same results on lifespan would occur as those found here when adding the compounds from L1 until the end of the lifespan. Experiments could also be performed where these compounds are only administered from mid-adulthood onward. The results of such experiments would better model the effects of these compounds when taken by middle-aged humans. NR and BHB have different temporal requirements for lifespan extension in *C. elegans*, with NR functioning almost exclusively during development and BHB functioning almost exclusively during adulthood. BHB, at the high concentration administered here, was toxic to larval *C. elegans*. The concentration of BHB administered here (20 mM) is

roughly equal to the levels in human patients with very severe ketoacidosis, but the level inside the worms is likely 10-fold lower, roughly the level occurring in humans after a 24-hour fast [24].

Further NR supplementation experiments could be performed to determine the optimal time frame for NR treatment to increase the lifespan, while preventing wasteful treatment during times it does not affect lifespan. These experiments could start by treatment of NR only during the developmental stages and then transition into further treatment during early adulthood.

To better understand the best time to initiate BHB supplementation in order to optimize lifespan extension, further experiments could be performed initiating BHB administration at the L2 or L3 larval stages. Lifespan experiments could also be performed using an RNAi-hypersensitive worm strain to determine if a more complete knockdown of the *hda-2* or hda-3 HDACs from the L1 larval stage decreases lifespan, as this treatment increased lifespan in the normal N2 worm strain. If lifespan is decreased by knockdown in the RNAi-hypersensitive strain, RNAi treatment could be initiated at the L2, L3, or L4 larval stages using this strain to determine the larval stage in which HDAC inhibition is toxic. This would allow a temporal understanding of the effects of HDAC inhibition during the larval stages on lifespan, which would likely suggest the optimal time to initiate BHB supplementation in order to maximize lifespan.

Due to the toxic effects of 20 mM BHB administration during larval development and the apparent requirement of NR administration during larval development for lifespan extension, the experiments performed here were not able to directly address our major research hypothesis that BHB and NR have additive effects on lifespan extension. To directly address this hypothesis our data suggest that NR treatment should be initiated at L1 and continue until at least L4, after

which NR treatment will likely have minimal effect on lifespan. Our data also suggest that L4 is the developmental stage where 20 mM DL-BHB administration should be initiated and treatment should be continued throughout the rest of the lifespan for maximal extension of lifespan.

The future experiments described above were designed to find the temporal "sweet spot" where combined NR and BHB addition maximize longevity. Positive results in these experiments would support the hypothesis that these compounds have additive effects when added at the optimal times, while also identifying the maximal lifespan extension provided by these two compounds. Optimization of the concentrations and temporal requirements of these compounds will allow for the future testing of multi-compound cocktails, which function by multiple mechanisms to augment lifespan in *C. elegans*, and eventually be tested in mammals, including human clinical trials.

Conclusions

Overall, the data suggests the ability of NR and BHB addition during different stages of life to enhance lifespan, which could also likely lead to the delay of age-related diseases. The research also demonstrates how a compound can have drastically different effects on lifespan depending upon the developmental stage at which it is first applied. As such, published results should always explicitly indicate the life stage that treatments are first applied. Now that the temporal requirements of these compounds for maximizing longevity have been identified, these compounds can be added together with other novel longevity interventions and administered to *C. elegans* to identify potent anti-aging cocktails. This can be accomplished with minimal cost and time. These experiments could yield profound results that can be scaled up for the treatment of many human aging-associated diseases.

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