Perspective
Non-essential dietary factors: from test tube to lifespan

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Abstract: The agriculture and food industries have an opportunity to change the future of food production by providing foods containing non-essential dietary factors (NEDFs) that improve health beyond a freedom from disease. In order to accomplish this in a safe and time-effective manner, however, each NEDF needs to be tested on a number of levels before it is incorporated into foods. *In vitro* assays are useful for discovering potential beneficial NEDFS, but *in vivo* studies are needed to both confirm positive effects and to detect negative effects. The genetic and metabolic similarities between humans and *Caenorhabditis elegans*, combined with the ease with which this nematode can be manipulated in the laboratory make it an attractive candidate to fulfill the role of an early stage *in vivo* model. Measuring the lifespan of *C. elegans* cultured in the presence of a range of concentrations of a NEDF would be of both mechanistic and strategic value in identifying those NEDFs that warrant further testing.
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INTRODUCTION

Traditionally, the role of agriculture and the food industry has been to supply the population with the essential nutrients needed to sustain life. This has been done very successfully in the past, and the efficiency of agriculture and the variety of foods available are increasing on a continuing basis. Recently, however, with all of the essential nutrients discovered and their Dietary Reference Intakes established (Institute of Medicine, http://www.iom.edu), more and more nutrition-related research is moving towards investigating the potential benefits of non-essential dietary factors (NEDFs) – compounds, such as red wine polyphenolics, that are beneficial to health but that are not necessary to sustain life. Following this research trend, agriculture is seeking to improve the value of commodities and products by adding or increasing the levels of many of the NEDFs reported in the literature to affect health beneficially.

Although the addition of beneficial NEDFs to foods may achieve health benefits, most of the components that are considered to be NEDFs emerge from focused bioactivity screens for activities against a specific disease target. When a molecule is identified in a focused bioassay as having an apparently beneficial effect on a particular metabolic pathway, the process of establishing whether it is likely to be truly beneficial to human health has just begun. Agriculture is only just developing strategies for discovering health benefits that will need to be analogous to, but distinct from those well established for the pharmaceuticals. There is a fundamental difference between the food and pharmaceutical industries, specifically the time scale on which their products operate and the end goal of the products they deliver to the public. Pharmaceutics focuses on curing diseases in as short a time span as possible; thus, they can tolerate a certain level of chronic toxicity in their compounds. Food and agricultural enterprises, on the other hand, are developing diets to improve overall health over the entire lifespan of a human,[1] and as a result can tolerate neither acute nor chronic toxicity in their products. Assays for toxicity would best be performed in a whole organism with easily measured, reliable endpoints. Mammalian models, although similar to humans in terms of both metabolism and genetics, would be prohibitively expensive and time consuming to use in testing every NEDF that appeared to be beneficial. Use of the nematode *Caenorhabditis elegans* as a model, and its lifespan as an easily measured endpoint, is an attractive approach for studying this problem. This model is already being used in the field of toxicology for similar purposes.[2] Although this article uses polyphenolics as examples of NEDFs, they are not the only class of molecules that fall under this category. Other potentially beneficial NEDFs include

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conjugated linoleic acid, oligosaccharides, and other phytochemicals that do not fall into the classification of polyphenolics.

IN VIVO COMPARED WITH *IN VITRO* ASSAYS

A likely route for a research program to identify beneficial NEDFs is through a systematic approach using specific selection criteria followed by testing using targeted bioassays.[3] An identification scheme such as this would be useful, but the results obtained from *in vitro* assays may not necessarily be applicable *in vivo*, and results need to be confirmed using *in vivo* experiments. A good example of this is the research surrounding the polyphenolics found in wine. Known as the 'French Paradox,' daily consumption of red wine in conjunction with a high-fat diet, specifically saturated fats, is associated with a lower than expected incidence of atherosclerosis. This effect is commonly attributed to the antioxidant properties of the polyphenolics found in red wine.[4] The first paper to put forth this hypothesis reported on an *in vitro* study of human LDL oxidation in the presence and absence of red wine polyphenolics.[5] Although the interpretation of this study is perfectly valid when applied to *in vitro* studies, there is a lack of *in vivo* evidence in the literature to support any health claims associated with the antioxidant properties of dietary polyphenolics. However, in 2003, a small-scale screening of various polyphenolics showed that resveratrol, a polyphenolic found in red wine, extended the lifespan of the yeast *Saccharomyces cerevisiae* by up to 70%.[6] Resveratrol was subsequently reported to extend the lifespan of both *C. elegans* and *Drosophila melanogaster*, and the mechanism of action of resveratrol and other similar compounds was not antioxidant activity, but genetic regulation.[7] Similar research has shown that resveratrol can improve the health and survival of mice fed high-calorie diets.[8] Although this work does not disprove the theory that polyphenolics improve health through *in vivo* antioxidant activity, it does provide strong evidence that a mechanism other than antioxidant activity is responsible for the health benefits associated with this class of molecules. If the mechanism of action in humans is shown to be gene regulation and not antioxidant activity, then over a decade's worth of scientific research will have been falsely based on the assumption that *in vitro* mechanisms of actions are necessarily applicable to *in vivo* actions. This is far from the death knell of *in vitro* studies, however. They are an indispensable part of target identification and hypothesis formulation. Such a finding would simply demonstrate the need for *in vivo* studies to back the claims formulated from previous *in vitro* studies.

CAENORHABDITIS ELEGANS: A GENETIC AND METABOLIC MODEL FOR HUMAN HEALTH

As mentioned above, it is necessary to test every potentially beneficial NEDF *in vivo*, and such testing requires a model organism. Obviously, performing the testing in humans would be ideal, but would be neither feasible nor ethical. A lower mammal would be the next best option after humans, but for reasons of cost, time, and other complications, even the most simplistic mammal model isn't practical. Bacteria and yeast would be suitable models for assays of certain pathways, but they are not similar enough to humans to be an effective model for testing all NEDFs. An attractive model is the highly studied nematode, *C. elegans*. Although *C. elegans* as a model has drawbacks, it is a good metabolic and genetic model for humans, and is a common model for studying many different aspects of genetics, biology, and metabolism. It shares many common biochemical pathways with humans, such as those of lipid metabolism, for example.[9,10] Its small size (*ca* 1 mm in length) and ease of culture means that large numbers of nematodes can be grown fairly inexpensively in a relatively small amount of space. Its short lifespan (<1 month) enables researchers to investigate how an added compound affects the total lifespan on a relatively rapid basis. Longevity screens of large numbers of nematodes are already common among research groups investigating aging and longevity to identify genetic mutants with altered lifespan,[11–13] and these analyses can be automated to further increase analytical throughput.[14]

Identification of mechanisms of action is simplified in this nematode as compared with higher organisms. The numbers of genes, pathways, physical structures, and cells are considerably fewer in a nematode as compared with those in a mammal, thus the number of possible targets of a specific molecule is smaller. Genetic manipulation using either RNA interference (RNAi) or mutational knockouts is common among investigators already studying *C. elegans*; such manipulations can further aid the elucidation of the mechanism of action of a NEDF. The linking of diet and genetics is currently of great interest for the health and nutrition community. Nutrigenomics is a rapidly expanding field in nutrition, and *C. elegans* could prove a useful tool to investigate the interaction of diet and genetics. Clearly, certain conditions that are specifically associated with higher mammals, such as cardiovascular disease, may be impossible to model in either a nematode or any other non-mammalian model, but it may be possible to investigate the beginning stages of disease progression, e.g., altered lipid metabolism, in *C. elegans*.

Some concerns related to cell culture assays – such as the lack of phase I and phase II metabolic enzymes, and non-biologically-relevant concentrations of molecules present in the culture media[15] – can be overcome using *C. elegans* as a model organism. *Caenorhabditis elegans* has been shown to possess genes homologous to human phase I and phase II metabolism genes.[16] Although it has yet to be shown whether these genes produce enzymes with similar metabolic activity, the presence of homologous genes

suggests that phase I and phase II metabolism is indeed similar to that in higher organisms. Unpublished results from this laboratory, as well as data from the literature,[7] show that *C. elegans* takes up compounds added to the medium in a concentration-dependent manner, and a saturation effect is observed at higher concentrations, beyond which there is no additional effect. Thus, by varying concentrations in the medium, it is possible to determine a range of concentrations over which an added compound elicits an effect, as well as the concentration beyond which toxic effects occur. Unfortunately, because *C. elegans* is presumably able to absorb nutrients not only through its simple digestive tract, but also through its outer cuticle, how NEDFs distribute throughout its body is not known, and warrants further investigation.

LIFESPAN AS A MEASURABLE ENDPOINT

As stated above, *in vitro* assays are useful, but limited for studies related to human health, and results from such studies should be backed up with *in vivo* investigations; however, it is important that *in vivo* studies measure the proper endpoint to assess overall health, regardless of whether or not a specific mechanism of action is under investigation. For example, Schramm *et al*. used *D. melanogaster* to investigate meiotic chromosome segregation in flies raised on a high quercetin diet.[17] An earlier report stated that quercetin could affect meiotic non-disjunction *in vitro*, so Schramm *et al*. focused their study on this aspect. From their observations, they concluded that a high quercetin diet had no negative consequences for meiosis. Although the researchers were able to conclude that quercetin is indeed non-toxic, the procedures used were complicated, specific to one class of molecules, and are not conducive to large-scale or high-throughput screening. Longevity analysis of *C. elegans*, however, is at the same time general to any class of molecules, uncomplicated, and conducive to large-scale screening.

Measuring lifespan as an endpoint in an assay has advantages and disadvantages. One major drawback of measuring lifespan as the only endpoint in a screen of NEDFs is that certain undesirable side effects that do not result in altered lifespan would not be detected. However, such effects that may be missed when using *C. elegans* as a model could be identified in later mammalian screens, as discussed below. Longevity studies also would not reveal information about the mechanisms that would cause a change in lifespan with NEDF supplementation, but they would be very useful in identifying which compounds or mixtures of compounds should be investigated further. From that point, only those NEDFs found to have no negative effects or to increase the longevity of *C. elegans* would be moved to the next phase of testing using a mammalian model so that the same effect could be observed in a higher organism. In all cases, a final human trial should be conducted to show that the molecules proven beneficial in earlier screens are also beneficial to humans.[18] This is not to say that *C. elegans* itself would not be useful for investigating side effects and mechanisms, just that they would not be apparent during the course of a longevity screen, and could be investigated using a different assay.

Lifespan is a holistic measurement of metabolic health integrated over all stages of growth and development, meaning that either a positive or negative effect in any metabolic pathway or at any stage in development may result in either a lengthening or a shortening of lifespan. A more traditional assay relying on a biomarker as an endpoint may identify a molecule as being beneficial for one pathway, but a negative effect on another pathway that is not being monitored may be overlooked. If the overall effect of such a molecule is positive, then it should bear out as either an lengthening or no alteration of lifespan. Conversely, if the overall effect is negative, then it should result in a shortened lifespan. Regardless of the molecule of interest and the pathway, or pathways, affected, lifespan analysis will, at the very least, identify those NEDFs that are safe and warrant further investigation. The focus of this paper is on NEDFs that can improve health, but certainly compounds found to shorten the lifespan of *C. elegans* would be of great interest. Such compounds could be further investigated in terms of what not to eat or as possible neutraceuticals.

Another advantage that lifespan has as a measurable endpoint over measurement of a biomarker is that it is easily determined, requiring no complicated, fallible biochemical or analytical techniques or assays, thereby reducing costs for screens and increasing efficiency in terms of both money and time. Lifespan as an endpoint can also be used regardless of the molecule under study. With a biomarker approach, each molecule or class of molecules would need its own biomarker and set of assays. Moreover, health is difficult to define in quantifiable terms that everyone or even a majority of those in health-related fields can agree to. Therefore, determining levels of a biomarker that are 'healthy' or 'unhealthy' is nearly impossible, with the exception of the more studied markers such as blood lipids or glucose. Most researchers would agree that a measurable, statistically significant increase in lifespan of an organism as a result of treatment with an NEDF is an indication that it has a positive effect on health.

Care must be exercised when conducting lifespan analysis using *C. elegans* due the formation of a hypobiotic state known as a dauer under conditions of stress.[19,20] When the conditions that cause dauer formation are reversed the nematodes continue a normal life cycle. Dauers can exist for months with feeding, and as such a nematode that enters the dauer state will appear to have a much longer lifespan than one that does not; however, this is not a true extension of lifespan, merely a suspension of metabolic activity followed by a resumption of a normal life cycle. As *C. elegans* can only enter the dauer state during the second of the four larval stages, this complication can

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be avoided by conducting lifespan analysis after the second larval stage.

CONCLUSIONS

Caenorhabditis elegans is well-suited as a model organism to use in the process of determining those NEDFs that exhibit beneficial health effects and those that do not. This can be accomplished in much the same way as toxicologists have proposed using *C. elegans* to study toxicants.[2] Although this nematode model alone is not sufficient for discovering and validating beneficial NEDFs, it could be indispensable as part of a larger identification and testing scheme. Lifespan analysis of *C. elegans* cultured with potentially beneficial NEDFs that have been identified from previous *in vitro* assays can confirm beneficial effects and reveal unanticipated negative effects. In this way, time and money can be spared by preventing lengthy mammalian trials of harmful compounds.

In light of the growing awareness of the public that diet affects health, the food and agricultural enterprise has an unprecedented opportunity to change the face of agriculture. By delivering foods containing NEDFs that have been proven to provide a benefit to human health, they can improve the health of the population beyond a mere freedom from disease. However, there needs to be many levels of testing between identification of potentially beneficial NEDFs and human trials. The use of *C. elegans* to address this need as described in this article can be integrated into any existing research and development program, whether in academia or the private sector. Thus, the process of identifying, testing the efficacy and safety of NEDFs, and then incorporating only those compounds that were found to be beneficial into the diet would be relatively simple and cost effective.

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