Investigating the Apoptotic Induction Potential of Various Chemical Food Preservatives on *Caenorhabditis elegans*

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Abstract

Despite the ubiquity of food preservatives in modern-day cuisine, the holistic range of their effects has yet to be adequately explored. The purpose of this experiment is to examine whether these different food preservatives can induce caspase-based apoptosis in animal tissue specifically due to Caspase-3-related protein expression in *Caenorhabditis elegans* (chosen because of the homologous conserved pathways that it shares with humans). The methods involved a unique protocol: *C. elegans* were incubated with preservatives of concentrations from 6.25-50 mM (similar to physiological concentrations found in regulated packaged foods) and a negative control of distilled water for 6 hours, and subsequently fixed with ethanol, permeabilized using antibody wash with detergent, and imaged with anti-tubulin and Caspase-3 antibodies applied. As a result, this experiment uniquely tests preservatives *in vivo* utilizing three different preservatives with four concentrations each, resulting in a holistic evaluation of apoptotic induction with a sufficient amount of treatment groups. Another crucial part of the protocol was the antibody staining, which involved quantifying the Caspase-3 expression through primary and secondary antibodies binding to specific sites on the protein. However, a potential limitation of this step is that non-specific binding of the antibodies, even with the blocking serum mitigating its effects, would mean that background fluorescence may potentially impact the results. Protein expression was then measured using confocal microscopy and the ImageJ imaging program to calculate the fluorescence of the imaging assay, representing the level of caspase expression. Citric Acid had significantly more Caspase-3-related protein expression than Sodium Benzoate and Sodium Nitrate. Additionally, most of the preservatives induced more expression than the control, except for some Sodium Nitrate treatment groups. Statistical analysis was done using an Unbalanced Two-way ANOVA and Tukey Multiple Comparison tests. The results insinuate that the common food preservatives Citric Acid, Sodium Benzoate, and (to a lesser extent) Sodium Nitrate induce Caspase-3-related protein expression leading to apoptosis. This has implications for not only the safety and regulation of overconsumption of food preservatives, but also the field of oncology, as inducing apoptosis in cancerous cells is a novel potential treatment for fighting cancer. For example, Citric Acid in particular may play a critical role in preventing cancer cell growth. As a result, preservatives have a clear impact on human health, with certain preservatives and preservative concentrations having a stronger effect than others.

Introduction

Preservatives are becoming increasingly more common in U.S. foods, with almost 60% of foods purchased including some type of additive (Dunford et al., p. 889-901), with overall negative effects on health including obesity and hypertension (Nardocci et al., 20). However, little is known about their specific effects on animal cells and tissue. Previous studies in this field include experiments of individual preservatives on different cell lines, including the HaCaT cell line with Citric Acid and HCT116 cells with Sodium Benzoate. Increased FAS and caspase protein expression indicate that they have the potential to cause apoptosis, pointing to apoptosis as a potential effect of chemical food preservatives.

Apoptosis (programmed cell death) is triggered by the expression of caspases (proteins involved in carrying out apoptosis) culminating in the expression of Caspase-3 in humans (Elmore, 07). Apoptosis additionally results in the breakdown and restructuring of tubulin (a protein involved in the structure of microtubules) in the cytoskeleton (Twomey et al., 2018). Apoptosis can occur through either an extrinsic pathway (formation of the DISC complex) or an intrinsic pathway (formation of the apoptosome) which both eventually lead to the activation of Caspase-3

Caenorhabditis elegans (*C. elegans*) is a nematode up to 1.5 mm in length. The apoptotic pathway in *C. elegans* is similar to that of humans, and the proteins involved are homologous to human proteins as well (Caspase-3 has a 50% positive match with CED-3, an executioner caspase in *C. elegans*, in BLAST sequencing), making it ideal for study (Candé et al., 2004).

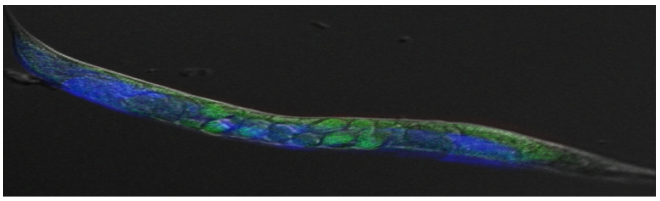
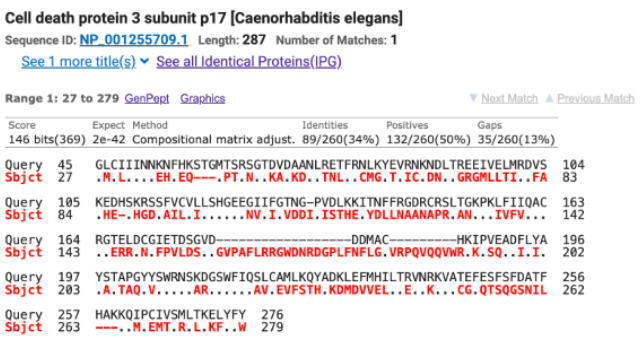


Fig. 1. *C. elegans* stained with DAPI staining and anti-tubulin antibodies



Cell death protein 3 subunit p17 [Caenorhabditis elegans]

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See 1 more title(s) See all Identical Proteins(IPG)

Range 1: 27 to 279 GenPept Graphics Next Match Previous Match

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Fig. 2. Figure demonstrates BLAST Sequencing, comparing Caspase 3 with CED-3 (executioner caspase in *C. elegans*), highlighting homology between human and *C. elegans* apoptotic pathway proteins.

Because of the lack of research regarding various chemical preservatives on specifically animal tissue through *in vivo* models, it is crucial to study their effects on inducing apoptosis or programmed cell death caused by Caspase-3. By utilizing the model organism *C. elegans*, we ensure that the findings of this study are applicable to animal tissue as a whole and that the amount of apoptosis induced is reflective of the effect that chemical preservatives have. *C. elegans* offer numerous advantages as opposed to other model organisms, making them the ideal model organism for studying the cellular process of apoptosis under confocal microscopy. Specifically, the ability of *C. elegans* to self-fertilize and cross-fertilize means that their rate of reproduction resulted in a substantial amount of worms for the study. Additionally, the transparency of *C. elegans* and constant cell number mean that it is the ideal organism to observe under a microscope (Riddle et al. 97). In our experiment, *C. elegans* were incubated in the chemical food preservatives Citric Acid, Sodium Benzoate, and Sodium Nitrate in order to determine potential apoptosis.

The purpose of the experimentation is to determine whether preservatives induce caspase-induced apoptosis in animal tissue and if so, whether the type of preservative influences the magnitude of caspase expression in order to understand their apoptotic effects on human cells and tissues.

Hypothesis

If *C. elegans* are exposed to common food preservatives, then there will be an increased expression of Caspase-3-related proteins. Moreover, suppose the type of food preservative is related to caspase-induced cell death in the nematode *C. elegans*. In that case, the citric acid will achieve the highest magnitude of apoptosis due to its ability to cause cellular stress, followed by sodium benzoate and sodium nitrate.

Since CED-3, the main executioner of apoptosis in *C. elegans*, is directly related to Caspase-3 and 9 in humans, a Caspase-3 assay would detect the most protein production in *C. elegans* exposed to citric acid since it increases Caspase-3 expression the most, sextupling the protein's quantity after 24 hours at 12.5 mM compared to a control (Ying et al., 2013). Very similar in magnitude, yet below that of citric acid would be sodium nitrate and sodium benzoate. Sodium nitrate was shown to induce quantities of Caspase-3 1.2 times that of the control after a 6-hour exposure at 12.5 mM. Since at a 3-hour exposure, it induced caspase production 1.1 times that of the control, it is reasonable to assume that it would induce Caspase-3 production 1.8 times that of the control over 24 hours (Liu et al., 2015). Sodium Benzoate was also shown through fold changes to produce Caspase-3 around 1.8 times that of the control at 12.5 mM over 24 hours (Yilmaz & Karabay, 2018). Thus, they would be equal in magnitude of Caspase-3-related protein expression.

Literature Review

There has been a sharper focus in recent years on studying the effects of different synthetic chemicals on apoptosis in common laboratory-standard cell lines. For instance, Liu et al.'s study on the impact of sodium nitrate on Human Gastric Adenocarcinoma Epithalia (AGS) cells revealed that the chemical can raise caspase levels to a significant degree and potentially pose a cytotoxicity threat to such cells. Moreover, an investigation conducted by Yilmaz and Karabay uncovered the effects of Sodium Benzoate on HCT116 cells, including caspase activation and Bim protein activation. The papers highlight support for the hypothesis that preservatives can cause apoptosis and regulatory protein expression in cell lines, and as humans consume many preservative-laden foods daily, these effects should be given more attention. However, the studies also demonstrate a lack of research into apoptosis at the broader tissue level, arguably more applicable to humans, as all experiments related to apoptosis and preservatives till now have both been only associated with cell lines and have only investigated apoptotic effects through one preservative. This investigation seeks not only to uncover the apoptotic effects of preservatives at the specificity of tissues but also to compare cytotoxicities of different common food preservatives concerning the same type of tissue and cells.

Materials and Methods:

Materials

C. elegans ordered from Carolina Labs, NGM Growth Media plates, OP50 strain of *E. coli*, 50 mM stock solutions of Citric Acid, Sodium Nitrate, and Sodium Benzoate, 24-well plate, Leica SP8 WLL Confocal Microscope, alpha Tubulin Antibody (B-5-1-2) sc-23948 mouse monoclonal, Anti-Caspase 3 Antibody active (cleaved) form AB3623 Rabit Polyclonal, 20% Tween 20 detergent, Bovine Serum Albumin blocking serum, VECTASHIELD Antifade Mounting Medium

Procedures

1. *C. elegans* were transferred onto over 20 NGM Growth Media plates and 300-500 uL of the OP50 strain of *E. coli* was added onto each petri dish, before being incubated for 3 days.
2. Roughly 200 individual *C. elegans* per treatment (to ensure a sufficiently large sample size after harsh fixing and staining procedures) were incubated in 800 uL of concentrations from 6.25-50 mM (6.25 mM, 12.5 mM, 25 mM, and 50 mM) of three different common food preservatives: citric acid, sodium nitrate, and sodium benzoate (12 treatment groups in total), along with a negative control group of distilled water. These concentrations are similar to physiological concentrations found in regulated packaged foods, systematically increased to elicit a possible effect of a hypothetical overdose of preservatives. This was done in a 24-well plate.
3. After 6 hours of incubation, *C. elegans* were fixed with ethanol in the same well plate overnight at 4 degrees C. In the experiment's original procedure, the freeze-cracking method was utilized, which involved deep freezing *C. elegans* between two slides, separating them, and fixing with formaldehyde instead of ethanol. However, due to the difficulty of accurately performing the procedure, a revised procedure that involved directly permeabilizing the cuticles was employed instead. A future implication could potentially be to find a more stable method to crack the protocols.
4. The worms were then treated with antibody wash with 20% Tween-20 detergent to permeabilize the cuticles for antibodies to be added, and blocking serum was added to

prevent non-specific binding and reduce background noise. The primary antibody added was Anti-Caspase 3 Rabbit Polyclonal, and the Mounting Medium used to wash the excess secondary antibody was Antifade Vectashield.

5. The worms on the slides were imaged for Caspase-3 quantity and anti-tubulin (using Leica SP8 WLL Confocal Microscope with Objective 20x and Magnification 1x-2x), two trials per condition, and expression for Caspase-3 like proteins were measured through fluorescence (range 0-255) of the assays using the ImageJ program on 3 ROIs per trial to obtain 6 samples per condition, ensuring that there were sufficient samples which would increase precision. The higher the fluorescence, the greater the signaling of Caspase-3-like proteins. Background fluorescence was managed through a combination of standardization of non-worm areas in the image to a value of 0 in the fluorescence scale and separation of ROIs such that visible background discrepancies would not be included in data collection.

Results

In Figure 4, the *C. elegans* were imaged for Caspase-3 associated proteins, with increased fluorescence corresponding to increased production, where the number 1 represents 6.25 mM (millimolar) of the preservative, 2 is 12.5 mM, 3 is 25 mM, and 4 is 50 mM (and A is the control, B is Sodium Nitrate, C is Sodium Benzoate, and D is Citric Acid). The *C. elegans* incubated with preservatives showed significantly more protein production than the control, with Citric Acid showing the most production followed by Sodium Benzoate and Sodium Nitrate. The red circles are examples that indicate areas of high caspase protein concentration. Holistically, protein production was directly proportional to the concentration of preservatives in each treatment group, and the control demonstrated little to no caspase activity.

The numbers refer to concentrations, where 1 is 6.25 mM, 2 is 12.5 mM, 3 is 25 mM, and 4 is 50 mM. The letters refer to preservative type, where A is the control, B is Sodium Nitrate, C is Sodium Benzoate, and D is Citric Acid. The tubulin antibody imaging in Figure 5 indicates the degradation of the microtubule structure through apoptosis as shown by limited tubulin expression in the preservative treatment groups.

Figure 6 visually portrays the differences in apoptotic induction potential between the preservatives and the control. *C.elegans* were introduced to an environment with preservatives in differing concentrations for 6 hours and imaged using confocal microscopy and Caspase-3 assays. Citric Acid was shown to affect the caspase activity the most, while all of the preservatives seem to be statistically significantly greater ($p<0.05$) in inducing caspase expression than the control (using ±2 SEM error bars).

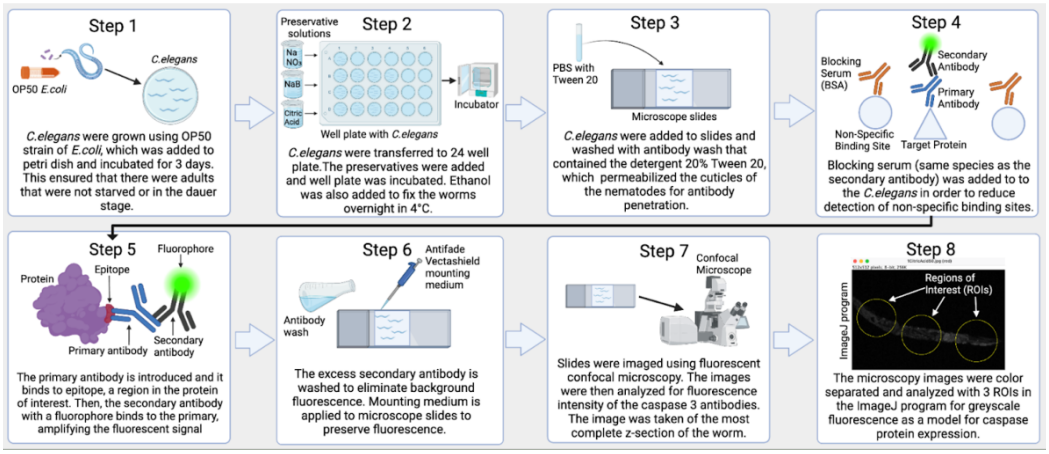


Fig. 3. Figure visually exemplifies the procedures used in the experimentation, beginning with growing the *C. elegans* and incubating them with preservatives, to antibody staining and imaging with confocal microscopy and gathering data using ImageJ.

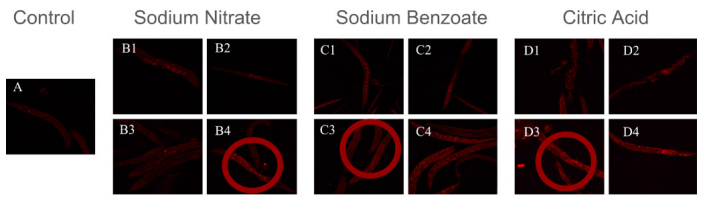


Fig. 4.

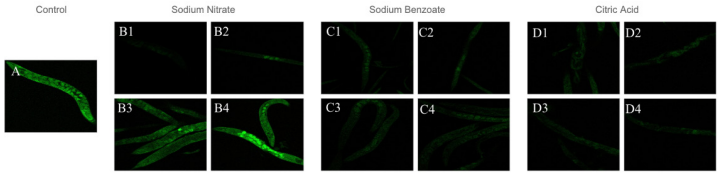


Fig. 5.

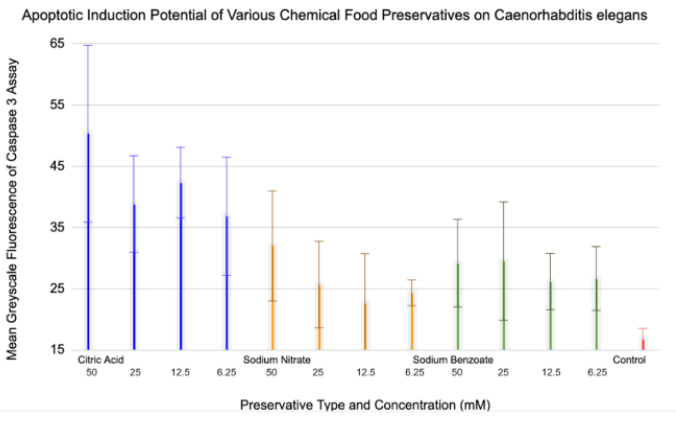
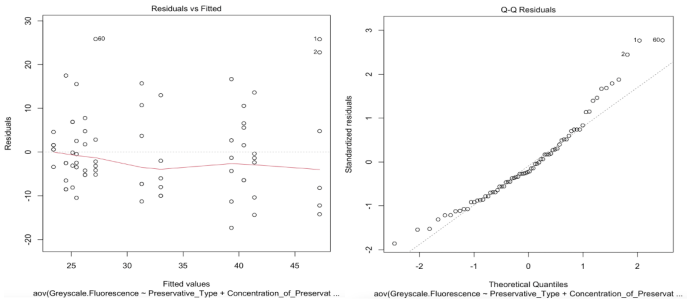


Fig. 6.

Statistical Analysis



There is a significant difference in the amount of Caspase-3 associated protein production between the control and the treatment groups through the Unbalanced Two-Way ANOVA test ($p=9.322e-08 < 0.05$). The data was backed by Levene's test for Homogeneity of Variance ($p=6.636e-1 > 0.05$) and proven to have normal distribution by the Q-Q Residuals (Figure 8) and Residuals vs Fitted Graphs (Figure 7). Using the Tukey multiple comparisons of means (without outliers) test, it was shown that both Citric Acid and Sodium Benzoate were significantly greater than the control ($p=6e-7 < 0.05$ and $p=2.43073e-2 < 0.05$ respectively). Sodium Nitrate was not significantly different from the control ($p=6.87399e-2 > 0.05$). Tukey multiple comparisons of means test comparing Citric Acid to Sodium Benzoate and Sodium Nitrate also portrayed that Citric Acid was significantly greater than both of them ($p=7.86e-5 < 0.05$ and $p=5.4e-6 < 0.05$ respectively).

Discussion

Qualitative analysis of anti-tubulin imaging reveals that in general, structural tubulin degradation occurs in a dose-dependent manner, supporting the idea that the preservatives used induced apoptosis since the cell death breaks down the microtubule structures that the tubulin is an integral part of.

In support of this, the control expressed clear and ordered microtubule tubulin structure, or the normal microtubule structure found in most animal cells, while most of the treatment groups demonstrated scattered and limited tubulin structure, indicating the occurrence of apoptosis. However, Sodium Nitrate 25 and 50 mM were outliers in the data in that they portrayed microtubule structures similar to the control.

The results corroborate published studies such as those done by Ying et al. and Yilmaz and Karabay about the effect of preservatives on inducing apoptosis or caspase protein production by supporting the theory that they decrease cell viability and increase caspase expression.

The data supported the hypothesis, with the first part being fully proven through analysis of the data. Most of the treatment groups elicited increased Caspase-3 homologous protein production compared to the control (with the exception of certain concentrations of Sodium Nitrate). The second part was also validated by the data as the Citric Acid treatment induced the highest quantity of protein expression (as demonstrated by Figure 1), and the difference of caspase protein expression between Sodium Benzoate and Sodium Nitrate was not significantly different as shown by the multiple comparison of means test (yet lower than Citric Acid)

The research question was answered as most of the preservatives in the treatment groups tested do induce apoptosis to a significant degree, and the type of preservative does influence the magnitude of protein expression. The results are significant in the research areas of cytology and animal histology because they prove that preservatives, most notably Citric Acid, induce Caspase-3-related protein expression which leads to apoptosis and tissue degradation, and degrades tubulin structures (which are found in all animals).

Conclusion

Overall, preservatives do appear to elicit a significant increase in the amount of Caspase-3-related protein expression and thus apoptosis, with Citric Acid inducing the most apoptosis followed by Sodium Benzoate and Sodium Nitrate, which overall serves to elucidate the apoptotic effect that chemical preservatives have on animal tissue.

This work can be applied to explain the effects of preservative overdosing due to overconsumption of certain food items, including canned fruits and processed meat. The results lend themselves to a plausible interpretation that long-term and high-concentration exposure to preservatives can lead to tissue degradation and the involuntary activation of the intrinsic apoptotic pathway. This is supported by previous research done on Citric Acid's effects on the HaCaT cell line through flow cytometry of morphological and cell cycle changes, which concluded similar implications. Additionally, preservatives with greater apoptotic induction potential such as citric acid could be utilized in cancer therapy drugs to kill off cancer cells, as targeting the apoptotic pathway of cancerous cells is becoming an increasingly popular therapy method according to research done by Fulda et al. Further research can include identifying methods of triggering non-caspase-induced apoptosis with preservatives, in order to better target cells with knocked-down genes for caspase production. Additional studies can be focused on: innovating a novel procedure for permeabilizing the cuticle of the *C. elegans* in order to facilitate ease of access of antibodies for fluorescent confocal microscopy; inhibiting apoptosis, specifically through the anti-apoptotic members of the Bcl-2 protein family such as Bcl-XL that inhibit through sequestering caspases or preventing release of factors such as cytochrome c; and identifying how to increase protein expression in *C. elegans* to reduce apoptosis and tissue degradation

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