Assessment of the effects of cannabidiol (CBD) in the alternative toxicity model *Caenorhabditis elegans*.

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Abstract

Many pathways and signaling cascades involved in organismal development and neuronal function, including endocannabinoid synthesis and signaling systems, are well conserved from worms to people. This study in progress is being conducted using *C. elegans* to help fill data gaps on the effects of cannabinoid compounds and a hemp extract. Effects in *C. elegans* on oxidative stress response (OxStrR), developmental timing, juvenile spontaneous locomotor activity, and reproductive output have so far been assessed for CBD at exposures of up to 500µg/mL. In *C. elegans* adults, CBD at 25-500µg/mL (0.08-1.59mM) reduced OxStrR in a dose-response manner. No effects on exposed adult gross morphology or progeny-to-adult ratios were identified at exposures up to 500µg/mL. In juvenile *C. elegans*, there was mild hypoactivity at 200-500µg/mL (0.64-1.59mM) CBD exposures, and a non-significant trend to developmental delay only at the highest tested exposure of 500µg/mL.

Introduction

The development and evaluation of faster, cheaper, and more reliable toxicity testing strategies using alternative models and new approach methodologies is mandated by Congress and outlined in the FDA's Predictive Toxicology Roadmap. Recently, great progress has been made in developing human cell based *in vitro* and microphysiological systems, but they cannot yet fully model complex biological processes such as organismal development and chemically induced neurobehavioral effects. *C. elegans* are microscopic nematodes with a 3-day lifecycle. Numerous genetic pathways involved in organismal development, neuronal function, and responses to toxic chemicals are conserved across phyla, suggesting that toxicity data from small model organisms such as *C. elegans* may contribute to weight of evidence assessments for safety evaluation. Though a simple organism, this model can provide more rapid and less costly toxicity and mode of action information relative to testing with laboratory mammals, but more testing is required before data from small alternative animal models can be used for regulatory purposes.

Endocannabinoid signaling systems are conserved from worms to humans. Endocannabinoid transmitters 2-arachidonyl glycerol (2-AG) and anandamide (AEA) have been found throughout the animal kingdom, and *C. elegans* encode members of each of the seven TRP ionotropic cannabinoid receptor subfamilies. The human cannabinoid receptor CB1 gene can substitute for its functional ortholog *npr-19* in *C. elegans* behavior assays. 2-AG and AEA have indirect effects on monoaminergic (serotonin, dopamine, and GABA) neurotransmitter signaling in rodents and *C. elegans*. Taken together, these findings suggest that *C. elegans* may be a good model for evaluation of the effects of hemp derived cannabinoids.

Materials and Methods

Exposure Method: In this study, *C. elegans* were maintained and exposed in *C. elegans* Habitation Medium, a mixture of 80% chemically defined nutrient medium and 20% non-fat cows' milk. Early chemical evaluations found that CBD was not stable in this medium. 10x nanoemulsions, produced with a high-pressure microfluidizer and composed of 5% sesame oil and 1% polysorbate 80 (Poly-80, a food-grade surfactant) in non-fat cows' milk (contains emulsifying proteins), were found to be an effective exposure method, based on conserved stress response pathway effects. Evaluations of organismal uptake and emulsified cannabinoid adsorption to plastic are currently underway. Experiments used two negative controls, a. vehicle emulsion (0.5% sesame oil exposure concentration), and b. Poly-80 in milk (0.1%Poly 80 exposure concentration as a negative control for sesame oil exposure). Cannabinoid chemicals purchased from Cayman Chemical (Ann Arbor, Michigan), hemp extract was provided by the University of Mississippi.

Evaluation of Conserved Genetic Pathways of Effect: Transgenes in *C. elegans* strains CL2166 (*gst-4*p::GFP, Figure 1) and CL2070 (*hsp-16.2*p::GFP) were used as biomarkers for conserved Nrf2-mediated oxidative stress response and unfolded protein response, respectively. Synchronized cohorts were exposed for 24h, from the fourth (last) larval stage to adulthood. Effects were evaluated using a microfluidic/laser instrument like a flow cytometer but designed for *C. elegans*.

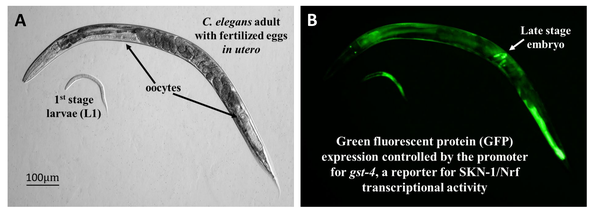

Figure 1. *C. elegans* strain CL2166 expresses the transgene *gst-4*p::GFP. It is a bioreporter for SKN-1 transcription factor activity. SKN-1 is the *C. elegans* ortholog of Nrf regulators of oxidative stress and inflammatory response. A. Brightfield image. B. Fluorescence image.

The worm Development and Activity Test (wDAT): This assay uses wild type N2 *C. elegans* to measure developmental milestone acquisition and developmental stage specific spontaneous locomotor activity. Age-synchronized cohorts are exposed from hatching to adulthood (3 days) in 12-well plates and assessed with an activity tracking instrument placed inside a hot/cold incubator.

Progeny Ratio Assay: The *C. elegans* progeny-to-adult ratio assay measures the number of progeny relative to the number of adults in control and exposed populations, and also provides information on adult microfluidic/laser technology morphometry indices of Time-of-Flight (TOF, a measure of size), and Extinction (EXT, a measure of optical density). Age-synchronized cohorts were exposed via their nutrient media from the onset of reproductive maturity and evaluated two days later when the oldest progeny were approximately halfway through juvenile development.

Results and Discussion

C. elegans transgenics can provide rapid bioassays for evaluation of changes in gene expression in conserved pathways of chemical response. The *C. elegans glutathione S-transferase 4* gene (*gst-4*) is used as a bioreporter for activation of SKN-1, the *C. elegans* ortholog of Nrf transcription factors that regulate oxidative stress response (OxStrR) and inflammatory pathways (Figure 1). A high fat diet is associated with oxidative and inflammatory stress in humans and laboratory mammals. The vehicle nanoemulsion exposed the *C. elegans* to 0.5% sesame oil, a high fat diet for this model. With exposure to the control emulsion, the bioreporter for SKN-1/Nrf regulated *gst-4* gene expression was increased about three-fold over the matched control of 0.1% polysorbate 80 (Poly-80) in milk without emulsified sesame oil (Figure 2A). CBD has been shown to decrease markers of inflammatory and oxidative processes in human cell cultures and laboratory mammals. In *C. elegans* exposed to CBD or a hemp extract (exposure concentrations normalized to the CBD content), the bioreporter for SKN-1/Nrf transcriptional activity was reduced in a dose response manner (Figure 2A).

High fat diets increase the expression of heat shock and unfolded protein response (UPR) genes in rodents. In a single experiment each so far, cytoplasmic chaperone UPR bioreporter for *heat shock protein 16.2* (*hsp-16.2*) gene expression was increased about two-fold with exposure to the vehicle control emulsion (Figure 2B). Human cell cultures exposed to CBD show decreases in UPR, and preliminary results indicate that this may also be the case in *C. elegans* (Figure 2B).

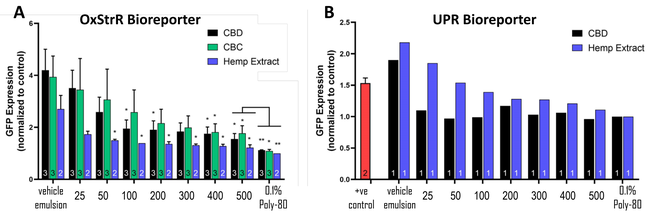

Figure 2. Transgenic *C. elegans* for higher throughput conserved mode of action detection. A. Bioreporter for oxidative stress response (OxStrR). B. Cytoplasmic unfolded protein response (UPR) bioreporter. The positive (+ve) control for UPR was mild heat shock (37°C). Green fluorescent protein (GFP) relative to the 0.1% polysorbate 80 (Poly-80) negative control on the y-axes. Concentrations in µg/mL on the x-axes.

A study from the National Toxicology Program of over 200 chemicals found that *C. elegans* larval growth can predict chemically induced developmental effects in rats and rabbits nearly as well as those two species predict developmental effects in each other [1]. That, and the conservation of endocannabinoid signaling, suggests that *C. elegans* may be useful for modeling the developmental effects of cannabinoids. With continuous exposure from the first larval stage, CBD showed a non-statistically-significant trend towards developmental delay only at the highest concentration tested, 500µg/mL (1.59mM, Figure 3A). Spontaneous locomotor activity was slightly decreased at all concentrations tested, though not in a dose-response manner, and only at the fourth and last larval stage (L4, Figure 3B).

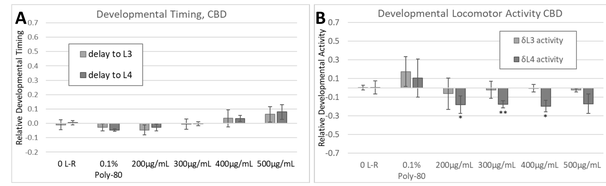

Figure 3. The worm Development and Activity Test (wDAT) for assessment of *C. elegans* developmental timing and locomotor activity. A. The relative timing for exposed *C. elegans* to reach the third (L3, light gray) and fourth (L4, dark gray) larval stages. B. Changes in population spontaneous locomotor activity during development relative to the vehicle emulsion control. '0 L-R' indicates the difference between the vehicle emulsion controls in two side-by-side plates, giving an indication of experimental variability.

In *C. elegans*, CBD at concentrations of up to 500µg/mL did not alter the number of progeny per adult in exposed populations (Figure 4). Adult morphological parameters of time of flight (TOF, a measure of size) and extinction (EXT, a measure of optical density) were also not affected.

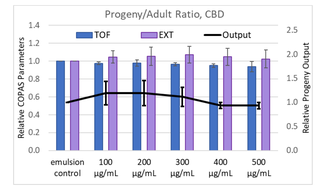

Figure 4. The Progeny Ratio Assay. Relative to the vehicle emulsion control, no significant effects on reproductive output (Output = progeny 'n' ÷ adult 'n') or adult morphometry parameters were found at concentrations of up to 500µg/mL CBD.

Conclusion

This study is still in progress. So far, we have established that, as in mammals exposed to a high fat diet, oxidative stress is upregulated in *C. elegans* exposed to nanoemulsions composed of sesame oil, polysorbate 80, and milk. CBD has been shown to reduce markers of oxidative stress and inflammation in humans and laboratory rodents. Nrf transcription factors regulate oxidative stress and inflammation, and a biomarker for transcriptional activity of SKN-1, the *C. elegans* Nrf ortholog, was reduced in a dose dependent manner by CBD. In developing *C. elegans*, CBD induced a non-statistically-significant trend towards developmental delay, but only at 500µg/mL. CBD exposure was associated with mild developmental hypoactivity at all concentrations tested, but only during L4, the last juvenile stage prior to adulthood. Further experiments will assess these endpoints with cannabichromene and a hemp extract, as well as organismal uptake of cannabinoids.

1. Boyd, W.A., et al., *Developmental Effects of the ToxCast Phase I and Phase II Chemicals in* Caenorhabditis elegans *and Corresponding Responses in Zebrafish, Rats, and Rabbits.* Environ Health Perspect, 2016. **124**(5): p. 586-93.