JOURNAL OF
EMERGING INVESTIGATORS

Article

Effect of increasing concentrations of cannabidiol (CBD) on hatching, survival and development of *Artemia salina*

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SUMMARY

Cannabidiol (CBD) has gained widespread popularity for treatment of a variety of medical conditions in humans. It is readily available from multiple sources without a prescription. Previous studies have documented that the endocannabinoid system, through which cannabidiol exerts its effect on the cellular level, is present in many vertebrates and invertebrates and plays an important role in neural maturation, differentiation, and survival. CBD exposure has the potential to adversely affect the development of the body and brain. We hypothesized that exposure to CBD would have a negative impact on hatching, development and survival in a model organism that develops rapidly, brine shrimp (*Artemia salina*). To investigate this hypothesis, *A. salina* eggs were incubated in five solutions with increasing concentration of CBD. The hatching rate, survival, and development were compared with a control group. Our results indicate that CBD at low concentrations (36.25 ng/ml) enhanced survival and accelerated the development of *A. salina*, while at higher concentrations it decreased survival and slowed development. These findings are relevant to human health in that the typical oral dose of CBD prescribed for anxiety and other conditions results in blood concentrations levels close to the high concentration solutions used in this experiment.

INTRODUCTION

Cannabidiol (CBD) use is widespread in our society. In one study conducted by the University of Michigan's Institute for Social Research, 38% of high school seniors had used CBD at least once in the last 12 months (2). According to Corroon et al. (3), more than 60% of the consumers of CBD reported using the drug to treat a medical condition. The most common reasons for CBD use by adults are pain, anxiety and depression. This use occurs even though there in only one FDA approved indication for CBD (rare form of epilepsy).

Cannabinoids are a group of chemically-similar compounds that can be extracted from the plant Cannabis sativa (cannabis). To date, more than 110 naturally-occurring cannabinoids have been identified (6). Due to its psychoactive properties, the most well studied of these compounds is Δ-9-tetrahdrocannabinol (THC). Cannabidiol was first identified in 1940 and accounts for 40% of the plant's extract, being the second most prevalent cannabinoid present in marijuana plants after THC (6). Unlike THC, CBD does not produce the subjective effects of marijuana and as such, initially, it was thought to be biologically inactive (6). However, there was renewed interest in studying CBD in the 1970's and further investigation indicated that CBD modulates the effects of THC in organisms and has a wide variety of biological effects of its own separate from THC (15).

On the cellular level, cannabinoids exert their effect through the endocannabinoid system (ECS) which is a composed of cannabinoid receptors (CBRs) and cannabinoid receptor proteins. The two main cannabinoid receptors, CB1 and CB2, were first characterized in the 1990's and are found in both the brain and the peripheral nervous system (6). CBD is a weak antagonist for both CB1 and CB2 and is thought to counteract the unwanted side effects of THC by partially blocking these receptors (6). The human cannabinoid receptors are present in neural progenitor cells and control their self-renewal, proliferation, and differentiation (5). Although interference with this system through exposure to exogenous CBD has the potential to adversely affect neuronal development, this question has not been thoroughly investigated.

Clinicians have long noted the biphasic (opposite response based on exposure to low or high concentrations) effect of CBD. At low doses, CBD relieves anxiety (anxiolytic), while at high doses it induces anxiety. The cellular mechanism behind this biphasic effect is believed to be the differential stimulation of CB1 receptors on different cell types. Rey et al. demonstrated that the anxiolytic properties of low dose of cannabinoids are mediated via the CB1 receptor on cortical glutamatergic terminals (4). On the other hand, at high concentrations the stimulation of the CB1 receptor on GABAergic terminals had an anxiogenic effect (4).

CBD has been subject of multiple animal and human studies. It has been investigated as a possible therapeutic agent for sleep disorders, addiction, Parkinson's disease, and epilepsy (7). A prescription form of CBD (Epidiolex) is currently FDA-approved for the treatment of two rare and severe forms of epilepsy (Lennox-Gastaut syndrome and Dravet syndrome) (6). However, the long-term effects of CBD use on the biologic development of living organisms are unknown. The present study investigates the effect of CBD on a model organism that has a relatively short life cycle: Brine shrimp (*Artemia salina*). We hypothesized that exposure to CBD would have an adverse effect on hatching, development, and survival of *A. Salina*. *A. Salina* provides a convenient model organism in that it develops rapidly, can be observed easily under low magnification, and its normal developmental milestones have been well documented (10). Though to our knowledge, there are no studies investigating the function of CB receptors in *A. salina*, previous studies have demonstrated that the ECS is

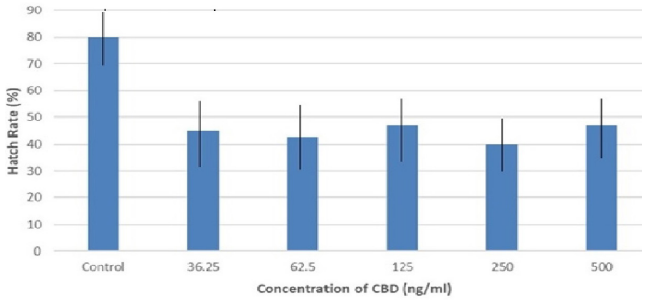

Figure 1. CBD reduces hatching of *A. salina* eggs regardless of concentration. The number of eggs hatched on day 2 was counted and represented as a percent of total number of eggs (Y-axis: hatch rate %). The presence of CBD reduced the ability of the eggs to hatch compared to controls at every CDB concentration. The hatching rate for the control group and all of the CBD groups combined were 0.80±0.07 vs, 0.43±0.03 respectively (p=0.0007). 95% confidence intervals are shown. (n=60)

present in a wide variety of invertebrates and vertebrates, and that from an evolutionary perspective it is highly conserved (15).

RESULTS

The human trials of the FDA-approved form of CBD have resulted in serum concentrations ranging from 50 ng/ml to 500 ng/ml (8). As such, *A. salina* eggs were incubated in five concentrations of CBD (36.25, 62.5, 125, 250, or 500 ng/ml) or control (salt water) and their daily development monitored. The hatching rate and percent survival were recorded, and the mean and 95% confidence intervals were calculated. The developmental phases were also documented based on a standard staging system (18).

The presence of CBD reduced the ability of the eggs to hatch as compared to controls at every CDB concentration (**Figure 1**). This effect was statistically significant and did not appear to be dose related as the reduction in the hatching rate was similar in all the solutions of CBD. On average, the hatching rate decreased by 46% in the CBD groups versus the control group. The hatching rates (**Table 1**) for the control group and all of the the CBD groups combined were 0.80±0.07 vs. 0.43±0.03 respectively (p=0.0007).

We also compared the survival of the eggs that hatched over time in the CBD solutions versus control (**Figure 2**). In the presence of CBD, a biphasic pattern of survival of *A. salina* was observed. At the lowest concentration of CBD, 36.25 ng/ml, survival of the *A. salina* was enhanced, while at higher concentrations (62 – 500 ng/ml) survival was decreased (**Table 2**). The difference in survival between the control group (0.565±0.005) and 36.25 ng/ml group (0.38±0.08) was statistically significant (p=0.008). It is also interesting to note that the rate of death of the *A. salina* in the low concentration CBD solution was lowest between days 2 and 3 and highest between days 3 and 4. For the *A. salina* in the highest concentration solution, the mortality rate was highest between days 2 to 3, after which the death rate decreased.

Furthermore, we monitored the developmental stage transitions of the embryos that hatched based on the standard staging system (18). All the embryos hatched by day 2, and no further hatching occurred after day 2. Six distinct stages of development (stages H to M) are characterized by defined morphological changes, such as size, trunk length, length of wings, and transition from one to two eyes (18). The day when each organism transitioned to the next developmental stage was monitored and compared between experimental and control groups. On day 3, the *A. salina* in the lower CBD concentration, 36.25 ng/ml solution, transitioned more rapidly from stage H to stage I (**Figure 3**). In contrast, *A. salina* incubated in higher CBD concentrations (62 – 500 ng/ml) developed more slowly compared to controls and those in 32.5 ng/ml of CBD (**Figure 3**). The fraction of *A. salina* achieving the developmental stage I on day 3 was 0.87±0.04 for the 36.25 ng/ml group and 0.52±0.08 for those in the 62.5 ng/ml (p=0.04). Higher concentrations of CBD therefore had a greater negative impact on stage transition.

DISCUSSION

We hypothesized that CBD would have deleterious effect on the development and survival of *A. salina*, but our results did not confirm our hypothesis. Though, at higher concentrations, CBD did negatively affect the survival and achievement of developmental milestones, at low concentrations it had the opposite effect.

The presence of CBD reduced the ability of the eggs to hatch compared to controls at every CBD concentration (**Figure 1**). The hatching rate for the control group and the CBD groups were 0.80±0.07 vs. 0.43±0.03 respectively (p=0.0007). This effect was not dose related as the relative decrease in the hatch rates was similar in all CBD concentrations tested vs. the control.

In addition, our data indicates that at low concentrations

Groups	Mean	Std. Error
Control (salt water)	0.80	0.07
36.25 ng/ml	0.38	0.08
62.5 ng/ml	0.43	0.10
125 ng/ml	0.46	0.03
250 ng/ml	0.40	0.06
500 ng/ml	0.46	0.06

Table 1. The number of *A. salina* eggs hatched on day 2 was counted and represented as a percent of total eggs (hatch rate %). The presence of CBD reduced the ability of the eggs to hatch compared to controls at every CDB concentration.

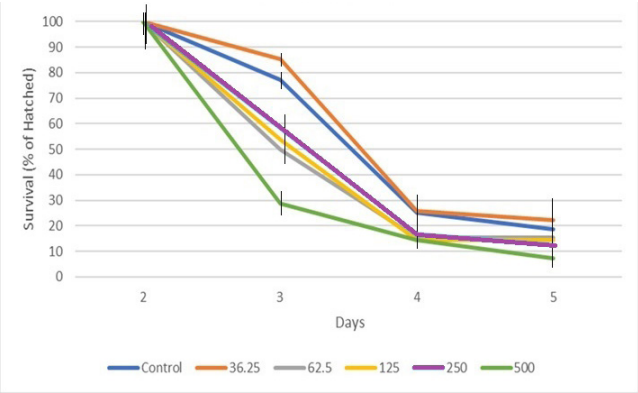

Figure 2. CBD significantly enhances survival at low concentration but decreases survival at higher doses. The number of living *A. salina* were recorded over each successive day and represented as a percent of total number of eggs hatched (Y-axis: Survival rate %). The 36.25 ng/ml solution of CBD significantly enhanced the survival of the *A. salina* (p=0.008) on day 3 compared to the control, while higher concentrations led to a decrease in survival. 95% confidence intervals are shown. (n=60)

(36.25 ng/ml), CBD enhanced the survival of the *A. salina* compared to the control group. The difference in survival between the control group (0.565±0.005) and 36.25 ng/ml group (0.38±0.08) was statistically significant (p=0.008). However, with increasing CBD concentration there was a trend toward decreased survival (**Figure 2**). This deleterious effect on survival was dose-related with the highest concentration solution (500 ng/ml) causing the greatest decrease in the relative survival (0.135±0.035).

We also examined the effect of CBD on development of *A. salina* (**Figure 3**). The *A. salina* in the 36.25 ng/ml solution developed more rapidly compared to the organisms in the control solution, while the development of those in the higher concentration lagged. The fraction of *A. salina* achieving the developmental stage I on day 3 was 0.87±0.04 for the 36.25 ng/ml group and 0.52±.08 for those in the 62.5 ng/ml (p=0.04). The increase in the rate of reaching developmental milestones when exposed to the low concentration of CBD oil was most pronounced during the early days of development (Day 3) but persisted into full maturation. Furthermore, for all concentrations above 36.25 ng/ml, there was a trend for the exposure to CBD to reduce the rate of development.

Our data raises some questions that could be subject of further investigation. This experiment could be repeated to examine the effects concentrations of CBD below 36.25 ng/ml to better determine the optimal concentration to enhance *A. salina* survival and development. Furthermore, based on our findings, one could hypothesize that low dose CBD decreased hatching, but enhanced maturation and survival due to the differential effects of CBD on different cell types (possibly due to stimulation of different receptor types). However, this hypothesis would have to be the subject of further detailed studies.

The biphasic effect of CBD on living organisms has been previously documented (4,16). It is not possible to come to a definitive explanation for this finding based on our study; however, other studies have shown that CBD does exhibit a biphasic effect at a molecular level (4). CB1 receptors on various types of cells are known to react differently to CBD concentrations. The anxiolytic properties of low dose of cannabinoids are mediated via the CB1 receptor on cortical glutamatergic terminals. On the other hand, at high concentrations the stimulation of the CB1 receptor on the GABAergic terminals has an anxiogenic effect (4). Clinical studies have documented this biphasic effect of CBD on feeding behavior, motor activity, motivational processes and anxiety (16). It would be tempting to speculate that the biphasic response to CBD on the molecular level could lead to the biphasic clinical response, but this theory would have to be tested by future studies.

Also, it would be of interest to evaluate the effect of low dose CBD on survival and maturation of cells in higher organisms. For example, in mammals, the onset of certain innate behaviors (those behaviors that are instinctive and not learned, such as certain reflexes) during development are linked to maturation of a minimum number of neuronal cells in particular areas of the brain (13). It would be useful to see if exposure to CBD during critical periods of early development in mammalian brain would hasten or delay the onset of such behavior. Furthermore, CBD is known to have cytotoxic effects at higher doses and has been investigated as a potential chemotherapeutic agent for treatment of cancer (14). Studies looking at the cytotoxic effects of CBD on neuronal cells would further our knowledge in this field.

Previous studies have demonstrated that cannabinoid receptors, CB1 and CB2, are present in the tissues of a variety of vertebrates including mammals, birds, reptiles, and fish (9). More recent studies have documented the existence of these same receptors in nematodes, onychophorans,

Groups	Mean	Std. Error
Control (salt water)	0.565	0.005
36.25 ng/ml	0.385	0.015
62.5 ng/ml	0.215	0.015
125 ng/ml	0.25	0.02
250 ng/ml	0.23	0.02
500 ng/ml	0.135	0.035

Table 2. The number of living *A. Salina* were recorded over each successive day and represented as a percent of total hatched eggs (Survival rate %). The 36.25 ng/ml solution of CBD enhanced the survival of the *A. salina*, while higher concentrations led to a decrease in survival.

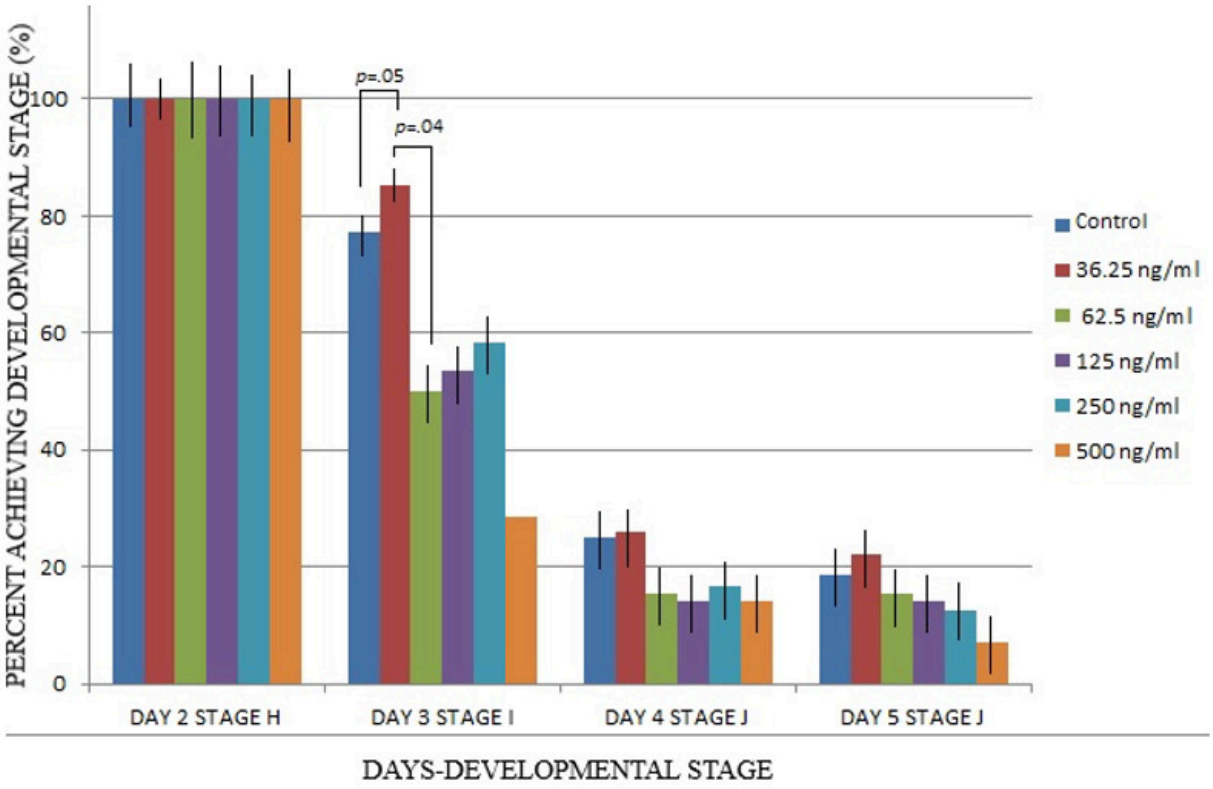

Figure 3. CBD accelerates development of *A. salina* at low concentrations but retards development at higher doses. The number of *A. salina* reaching a specific developmental stage on each day was recorded and represented as a percentage of all hatched eggs for each solution (Y-axis: % achieving developmental stage). On day 3, higher percentage of the *A. salina* in the 36.25 ng/ml solution achieved stage I (0.86±.02 compared to those in the control solution (0.77±.02, p=0.05), while the development of the organisms in the higher concentration lagged. The fraction of *A. salina* achieving the developmental stage I on day 3 was 0.86±0.02 for the 36.25 ng/ml group and 0.52±0.08 for those in the 62.5 ng/ml (p=0.04). 95% confidence intervals are shown. (n=60)

and crustaceans (15). To our knowledge, no studies have investigated the presence of these receptors in *A. salina*, but our data provides strong indirect evidence that the endocannabinoid system is present in *A. salina* and plays a role in hatching and development.

METHODS

Five solutions of increasing concentrations (32.25 ng/ml, 62.5 ng/ml, 125 ng/ml, 250 ng/ml and 500 ng/ml) of CBD (10 mg/ml stock solution, American Shaman Co., batch number 09122020) were prepared using serial dilution in a salt solution. The salinity was kept constant across the control and all experimental groups. The CBD in the stock solution was water soluble, so the CBD was dissolved in water and not oil, and as such, salt water was used as the control solution. Thirty *A. salina* eggs (NOASTORE brand) were added to each concentration of CBD and control. A second independent experiment was run simultaneously for a total of 60 eggs per concentration and control solution. The eggs in the solutions were housed in a 36-compartment clear plastic box with a lid. *A. salina* were monitored daily (at approximately 5:00 PM) under a dissecting microscope and observations were recorded for the number of eggs hatched, their developmental stage over time, and their survival. All the eggs that eventually hatched, had done so by day 2. The organisms were considered dead if they were immobile and floating in the solution. The developmental stages were recorded using a standard staging system (18) based on observed gross morphology. All solutions were kept at room temperature and exposed to regular day and night cycles. All statistical analysis was performed using Graphpad Prism 8 software (Graphpad.com). Standard errors and 95% confidence intervals were calculated for all figures. The hatching data (**Figure 1)** was analyzed using one-way ANOVA to look for a statistically significant difference. Post hoc testing was performed using t-test to determine whether a statistically significant difference existed between the individual experimental groups. The survival (**Figure 2**) and development data (**Figure 3)** for the *A. Salina* were normalized to day 2. Two-way NOVA was performed and the difference in the individual experimental groups were assessed using repeated t-tests.

ACKNOWLEDGEMENTS

This research was performed under the supervision of Dr. Paula Monaghan Nichols, Associate Dean for Research, University of Missouri Kansas City School of Medicine.

Received: April 10, 2020
Accepted: June 22, 2020
Published: July 27, 2020

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