The effect of acute exposure to caffeine on locomotion, neurochemistry, and related gene expression in C.elegans

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Abstract

The current research aims to study the effect of acute exposure to different concentrations of caffeine on locomotion (Distance, Speed, Mobility rate) and neurochemistry in Caenorhabditis elegans (C.elegans) (a free-living nematode), as well as study the gene expression of the UNC-63 gene, as the UNC-63 represents the acetylcholine gene in the C.elegans. This research discusses the effects of acute exposure to caffeine according to concentration, where three concentrations 10mM,15mM, 30mM were used. The results obtained in this study using a low concentration of 10mM of caffeine. where the distance reached (1893.13± 282.38), speed (7.46 ± 4.67) and the mobility rate (36.85 ±6.45), while the level of gene expression was (3.333 ± 0.10). Also, acute exposure to caffeine when using moderate concentration of 15mM of caffeine showed a distance (4929.9 ± 1531.71), speed (9.12 ± 2.07) and the mobility rate (38.01 ±8.04), while the level of gene expression was (1.38 ± 0.10). In addition, acute exposure showed by using a high concentration of 30mM of caffeine a distance (3039.48 ±1302.30), speed (5.06 ±1.09) and the mobility rate (22.08 ± 8.38), while the level of gene expression was (3.19 ± 0.19). The study concluded that acute exposure to caffeine may leave behavioral effects dependent on concentration, in addition to that the level of the molecular effects of the drug may be contrasting with effects on the level of behavior at the same concentration, this may be due to enzymatic reasons that may increase the ability of the gene to accept larger amounts of the drug. Generally, it seems that C.elegans could be used as a pre-clinical in vivo model for detecting the effects of exposure to drugs and assessing the neuropharmacological, behavioural and molecular changes that associated with drug exposure. In addition, for future studies, it could be suggested to use C. elegans as a model to assess the effect of chronic exposure to drugs; and to develop a pharmacological protocol for controlling and treating some common diseases in society such as addiction and neurodegenerative disease.

1. Introduction

In our world, addiction represents a serious challenge as it causes severe damage to the lives of individuals annually and causes huge numbers of deaths as a result of car accidents related to alcohol abuse, drugs, cancer and other events resulting from addiction to alcohol, nicotine, and crimes caused by drugs and overdose. In addiction studies, many vertebrates such as mice and rats were used as an experimental animal model to understand the neuropharmacological mechanism of addiction and the development of addictive behavior. However, the use of invertebrates as a pre-clinical animal model instead of vertebrates has been increasing. Different invertebrates such as fruit flies (Lin et al; 2010), honeybees (Rein et al; 2013) and planaria (Nishimura et al; 2007; Mohammed Jawad, 2018).
Which have been used to clarify the neuropharmacological bases and the role of different neurotransmitters that associated with the development of variety of behavioural responses, learning mechanisms and the mechanism behind the development of addictive behavior. C.elegans was considered outstanding model and the first animal to have a sequenced genome, having approximately 19,000 genes that share many analogues in humans (Antoshechkin and Sternberg, 2007). UNC-63 gene, a gene that performs several processes to enhance the vitality and capacity of the selective acetylcholine – associated channel in C. elegans worm which includes bringing calcium ion through the plasma membrane. This gene contributes to the enhancement of locomotor behavior by coordinating the movement of worms, it is used in many ethanol disturbance studies in C. elegans (Culetto et al; 2004).
Although, vertebrates have been used over many years to study and understand addictive behaviors, the use of C. elegans in these studies is a remarkably developed (Selling et al; 2013). Moreover, C. elegans share numbers of similarities with vertebrates in terms the structure and neurochemistry of the nervous system. C.elegans has a small nervous system and an adult hermaphrodite has about 302neurons with perfect formation of all axons (White, 2013). Neurons are connected in the head, which is the most important part

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of the worms nervous system, in addition, other numbers are spread in the ventral and tail nerve cord, where neurons communicate with each other through 7000 synapses, 900 junction gaps and 1500 neuromuscular junctions, while males have a larger nervous system than hermaphrodites, as it consists of 473 cells specialized in transmitting signals between nerves and muscles and it can have a role in mating (Hobert, 2013).

Although C.elegans may lack the norepinephrine receptors (Alkema et al., 2005), it has the ability to release number of the main neurotransmitters such as acetylcholine, dopamine, serotonin and glutamate that play an active role in transmitting nerve signals between the brain and different target organs (Unwin, 2005).The presence of such neurochemical and genetic similarities in C.elegans enhances them as a valid in vivo animal model for understanding the neurochemical and molecular mechanisms that mediate drug effects and the expected targets for the development of pharmacological protocol to control and treat addiction to various addictive substances (Matsunami and Frailty, 2018).

Furthermore, C.elegans represent a valid model to study several behavioural patterns and responses, for example rapid or decreased movement, laying eggs, repeated body bends during certain periods and excretion, associative and non- associative learning, attraction to food sources; and to study the effects of some drugs on many genes (Ardiel and Rankin, 2010). Therefore, studying such behaviors, activities and understanding their regulatory pathways is a vital matter to understand the mechanisms that control different types of learning, responses and behaviour that associated with the exposure to either natural or synthetic substances. From which it can be noted that C. elegans could be a remarkable model for studying the effects of drug abuse.

Caffeine is a widespread stimulant, widely used, and many people may not dispense with it, and they enjoy consuming it. Coffee is rich in Caffeine, as well as tea and soft drinks (Uddin et al; 2017). From the above, it is clear that the roles that caffeine plays on biological activities and human health represent an urgent necessity for basic and clinical research. Specifically, a group of studies mentioned that caffeine can cause remarkable transformations in brain activities such as wakefulness, motor agitation, learning and memory (Alasmari, 2020). This effect may be a reason for treating diseases related to the brain and nervous system in general such as autism, Alzheimer's disease, Parkinson's disease (Liu et al; 2016). In order to determine the transformations, show by caffeine, it molecular mechanisms in the brain was also traced through studies conducted by several scientific laboratories. Brain cells are affected by caffeine, and indiscriminate use causes high levels of calcium (Perisse et al; 2009).

The active role of caffeine depends on communicating with adenosine receptors and suppressing these receptors that induce sleep. This effect could be a useful pathway preventing neurodegenerative diseases. However, the exact role that caffeine plays in the nervous system need more research to become clearer. In general, despite the importance of studies conducted on vertebrate animals to identify the reason behind to action of caffeine, there are many animals that can be used to understand the effect of caffeine and identify many of neurochemical effects and molecular changes it produces. Previous studies in mice indicated that acute caffeine exposure shows a dose- dependent effect and that high doses increase the level of arousal, reduce cognition and lead to impaired movement and lower doses increase the level of movement (Watson et al; 2016). In invertebrates, studies have indicated that caffeine can exhibit locomotor activity in honeybees and green insects, depending on the dose (Fernandes et al; 2012). As it has been mentioned previously, C.elegans considered valits model for conducting many studies including neurological and genetic studies, understanding the causes of diseases and exploring drugs because it has a miniature nervous system with 302 neurons and an interconnected neural network, in addition, to the complete genetic sequence that shares with human in many isotopes (Cook et al; 2019). Therefore, the aim of this study is to clarify the neurochemical and molecular effects that associated with acute exposure to different concentrations of caffeine using C. elegans as in vivo pre-clinical animal model.

2. . Materials and Methods

Strains

The strain used in the experiment is wild- type nematode (N2).

C.elegans Assays

The strain was grown on NGM medium at 21°C by using *E. coli* OP50 as a food source.

Caffeine effect

Caffeine (1.3,7 trymethylxanthine) was prepared in three concentration 10mM, 15mM, 30mM where 50 microliters of each concentration were added to the previously prepared NGM plates to which OP50 was added.

Behavioral Test

The developing worms were transferred at 21°C to a plate containing 50 µL of caffeine and these plates were distributed according to the three concentrations used in the study, which are 10mM,15mM,30mM, then the locomotor activity (distance, speed and mobility rate) of the worms was tracked and recorded for a 20 minutes session by a camera connected to a microscope equipped with the Omax Toup View program. The video results were analyzed using Tox Trac software (Sweden).

Quantitative RT- PCR Analysis

The worms are picked up from an agar plate, and 20 µL of water is added to it wash them from bacterial residues, then collected in the 15ml test tubes and frozen in liquid nitrogen -196°C, then they are ground using a mortar and pestle, where the RNA is extracted using Trizol reagent and according to the instructions the manufacturer, and determination of the yield of RNA by

Nano – drop spectrophotometer, where the total RNA extracted from the worms was processed using (iNtRON biotechnology), then 2mg of treated RNA is used as a template for the synthesis of (cDNA) in a reaction system, 20 µL using script (cDNA) supermix, according to the manufacturer protocol, then PCR tests using RT-PCR system with RealMOD™ Green SF 2X QPCR mix (iNtRON Biotechnology) under the conditions follows: 40 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 20- 60 s, extension at 72 °C for 30 s. The DNA sequences of PCR primers are listed in Table 1. The levels of each mRNA were measured using the Ct method and were normalized to the *ama*-1gene to select for the effect of caffeine on the *UNC-63* gene.

Source or Reference	Sequence (5'–3)'	Amplicon (bp)	Primer
(Mello et al; 1991)	TTTTGGCCCGGGATGTGTTGTTGGGGATCG TATTTGGCATGCTCTGTGACTGCCTATGG	502	UNC-63 F UNC-63 R

Procedure

During the acute exposure to caffeine, 40 worms were used in this experiment. The worms were divided into four groups (each group contains 10 worms): control, 10mM caffeine, 15mM caffeine and 30mM caffeine. In addition, the worms were equally divided and tested into two different contexts: agar only (20 worms, 5 worms from each group); and agar with sand (20 worms, 5 worms from each group). The worms in different experimental groups were had only a one single exposure to the drug to assess the effect of acute exposure to different concentration of caffeine. Moreover, animals in the experimental groups were exposed individually to the drug and tested in a 35mm petri dish; the petri dish could be contained agar only or agar with sand to offer two contextual cues during the experiment. One drop of the drug (50 microliter) is placed and spread on the plate and left for one to two hours until the homogeneity between the drug and the surface of the dish and then dry to allow the worms to move freely. When the worms habituated to the new environmental cues, it begins moving around and covered different parts of the petri dish. the movement of the worm is tracked and recorded for a 20 minutes session using a camera connected to a microscope. After that, the recorded video is analyzed using Tox Trac software and the data for the locomotor activity (distance, speed and mobility rate) were obtained and analyzed., After completing the experimental session, every individual worm in each group was washed with distilled water two to three times in order to remove any bacteria residues or any particles from the agar or the drug; then, the worms are placed in special test tubes and rotated by the centrifuge device for one minute, where the worms gather at the bottom . The worms is withdrawn from the bottom of the tube and crushed using a mortar and pestle, then the RNA is extracted and of stages of gene expression are completed (Albeg et al., 2011). The animals in the control group were exposed and examined in the same experimental cues that used with other experimental groups, except that they were not exposed to the drug.

3. Results and Discussion

Locomotor activity assay

In the current study, the effects of acute exposure to caffeine was examined and the changes in locomotor activity (distance, speed and mobility rate) were assessed during a 20 minutes session using C. elegans. In general, the data are presented in figure 3.1 showed there is no significant increase in distance covered by the animals in all experimental groups (group10mM caffeine, group15mM caffeine, group 30mM caffeine) compared to the distance covered by the animals in the control group (6786.16 ±1281.16). However, the distance covered by the animals during acute exposure to a low concentration of caffeine (10mM) and high concentration of caffeine 30mM was significantly decreased compared to the distance covered by the animals in both group 15mM caffeine and the control group (P=0.034). This could indicate that there is a weak response to the drug at a low and high concentrations and that the dopaminergic receptors shown incompatibility with these concentrations, which could affect the production of dopamine quantities that mediate motor activity and thus we noted that the distance through which the worms move has decreased in comparison with the data of the control group (Ettarh et al; 2000). Moreover, the distance covered by the animals in the group 15mM caffeine and animals in the control group was not significantly differed; and the animals in both groups was showed a similar level of distance during the acute exposure session. Thus, this result may suggest there is an increase in the ability of the worms to accept additional amounts of drug that can stimulate excitement and locomotor activity (Machado et al, 2019). However, it was direct target of caffeine is acetylcholine receptors (nAChRs), so the interaction of caffeine with neuronal receptors in high concentrations may inhibit the flow of acetylcholine at the neuromuscular synapse region, which stimulating the secretion of adenosine A1; and this could promote an inhibition of acetylcholine release. Thus, reducing motor activity and increasing the level of curvatures with appearance of muscle spasms, which can be interpreted as neurotoxicity, which may reduce alertness, attention and impair the level of cognition (Min et al., 2017). This result is consistent with previous studies indicating acute exposure to high concentrations of caffeine can reduce dopamine production at the neuromuscular synapse, thereby inhibiting motor activity (McIntire et al, 1993).

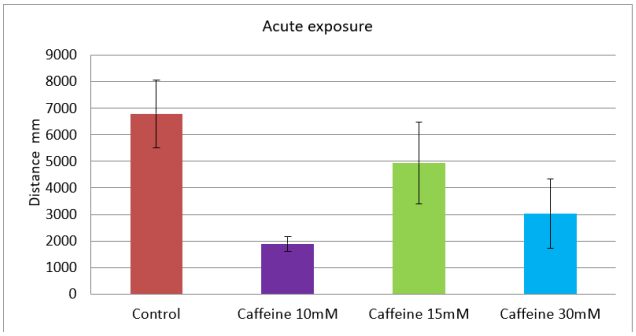

Figure (3.1): Shows the mean (± SE) of distance covered by the animals in different groups during acute exposure session.

The data for the speed of worms in different groups during acute exposure session is presented in Figure 3.2. The data showed there is a slight increase in speed of animals in group 15mM caffeine compared with speed of the animals in control group and other two experimental groups (10mM caffeine and 30mM caffeine), however, this increase was not significant. Also, there is no significant difference in speed covered by the animals among all groups. These, data could indicate that there is a low response to the drug, and that caffeine did not stimulate the activity of dopaminergic neurotransmitters, this may be due to the rapid metabolism to the drug or its weak link with its receptors and thus its effects decreased (Basset *et al*; 2014). In addition, the slight increase in speed that was shown by the animals exposed to 15mM caffeine could suggest that there is an improvement in locomotor activity when exposed to a moderate concentration of caffeine and that the nerve receptors showed a response to the drug, which reduces the active of adenosine A1 and increase the activity of dopamine and glutamate transporters and thus may be enhances memory and learning (Flint *et al*; 2006). Furthermore, the reduction in speed of animals in group 30mM caffeine is consistent with previous studies indicating that high concentrations of caffeine can reduce the locomotor activity (Fisone *et al.*, 2004).

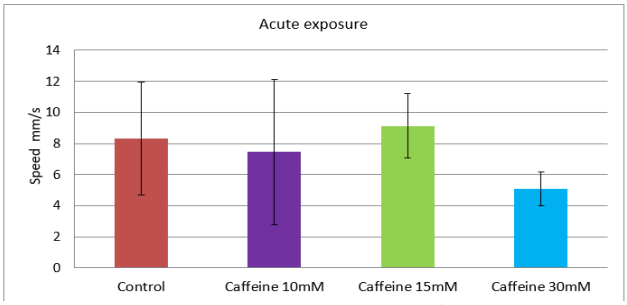

Figure (3.2): Shows the mean (±SE) of speed by the animals in different groups during acute exposure session.

The data represented in Figure 3.3 also showed the mobility rate of nematodes in different groups during acute exposure to caffeine, and the results showed that there were no significant differences between all groups. Despite that, there was a clear decrease in the mobility rate during exposure to caffeine in the 30mM group compared to the control group and the other experimental groups 10mM,15mM. Also the motor activity of the worms was slightly decreased when exposed to a low concentration of caffeine 10mM, this decrease although not significant, can give a slight effect of the drug on the movement of the worms due to the rapid metabolism, which reduces the effect of the drug and thus dopaminergic neurotransmitters associated with movement can show a slight response to the drug, which may give biphasic effects between being affected by the drug and creating a response and between being unaffected by the drug (Fisone et al., 2004). Furthermore, worms exposed to 15mM caffeine showed a slight decrease in worms mobility rate may be very close to that of the control group, which may enhance the activity of dopaminergic receptors in sending nerve signals that may stimulate muscle activation the body and therefore motor activity may increase, which is a key role that caffeine can play at moderate concentration, this result is consistent with previous studies in rats which indicated that moderate concentrations of caffeine can stimulate locomotor activity (Machado et al, 2019). In addition, exposure to a high concentration of 30mM caffeine reduced the mobility rate of worms, which may inhibits the activity of acetylcholine in transmitting signals at the neuromuscular contact region, thus enhances motor rest and increasing the activity of the adenosine A1 receptor. (Halldner et al., 2004).

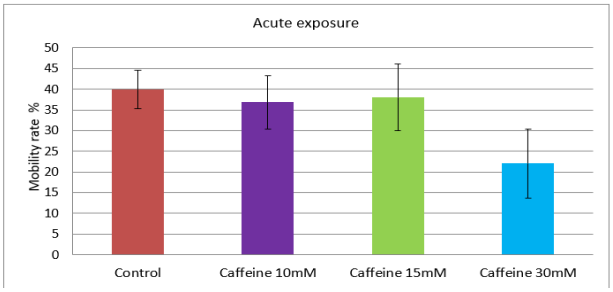

Figure (3.3): Shows the mean (±SE) of mobility rate by the animals in different groups during acute exposure session.

3.2 Gene expression assay

Gene expression assay was conducted to identify the responses shown by the UNC-63 gene to caffeine at different concentrations 10Mm,15Mm,30mM, as well as to compare changes at the genetic level with the neurological changes, when using a low concentration of caffeine showed a significant increase in the level of gene expression compared to with the high concentration, moderate concentration and control sample, where the expression rate was (3.333 ± 0.10),(Figure, 3.4),(P< 0.001) which explains that there is an excessive response to the drug and a significant increase in the secretion of mRNA amounts that may enhance the caffeine rush in the UNC-63gene. Thus , caffeine may accumulate at the UNC-63 and since the UNC-63gene represents the neurotransmitter acetylcholine gene in nematodes, this may lead to the blockade calcium channel that stimulates the release of acetylcholine at the neuromuscular junction, which may affect motor activity and suppress feelings of excitement and stimulation. Furthermore, acute caffeine exposure

may give mismatched genetic and behavioral effects depending on the ability of the gene to absorb additional amounts of the drug and thus may give genetic defects that can make the muscles hypersensitive to the drug, which may cause muscle stiffness (Dolezelova et al; 2007). While, when using a moderate concentration of caffeine 15mM, the level of gene expression decreased compared to low concentration and high concentration and increased compared to the control sample amounted to (1.38 ± 0.10),(Figure 3.4), which indicates that there is a response to the drug at the UNC-63gene and that the amount of mRNA have increased significantly and therefore this response may enhance the activity of acetylcholine receptors that regulate motor activity, which indicates that intake large amounts of extra caffeine at moderate concentrations did not show an accumulation of the drug at the UNC-63 gene and thus may enhance locomotor activity (Everitt and Robbins; 1997). when a high concentration of caffeine was used, the level of gene expression increased significantly, as shown in,(Figure, 3.4), and reached to (3.19 ± 0.19), which indicates sensitivity to the drug, and this sensitivity may provoke excessive responses at the UNC-63gene may lead to accumulation of caffeine in the UNC-63 gene, and since the UNC-63gene represents the motor excitation gene in nematodes, this may cause an imbalance in the transmission of dopamine signals in the neuromuscular synapse region, which reduces motor activity, this results is consistent with the result obtained during the locomotor activity assay, which indicates that the use of a high concentration of caffeine may reduce locomotor activity and gene expression in nematodes (Kaas et al, 2010).

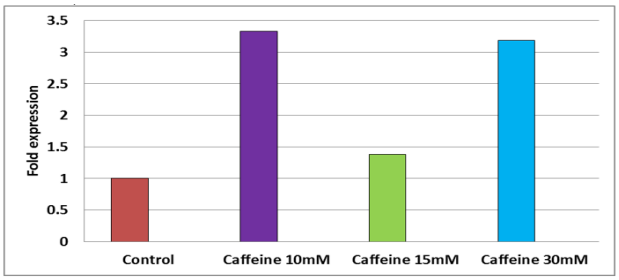

Figure (3.4): Shows the gene expression level of UNC-63 gene during acute exposure to caffeine at different concentrations, the gene expression folds were calculated using the ct value method.

4. Conclusion

The study concluded that caffeine in moderate concentration enhances the locomotor behavior of the worms by modulating the neurotransmitter systems dopamine, acetylcholine and serotonin and accelerating the firing of neuronal signals that activate the movement. We suggest that this acceleration of neurotransmitter release may be due to the activation of *UNC-2* dependent calcium influx, which modulates the postsynaptic response. In addition, assay the gene expression of *UNC-63* gene showed a response to the drug and the mRNA levels does not accumulate, indicating an increase in the activity of acetylcholine after acute caffeine exposure, and this is in agreement with previous studies that indicated the moderate concentration shows traits that enhance memory, learning and motor activity. On the contrary, the high concentration of caffeine did not show motor activity and the worms speed decreased significantly with the level of distance and the mobility rate and therefore the high concentration of caffeine may reduce the movement of the worms in general as a result of the decrease in dopamine signals at the neuromuscular junction region due to exposure to the drug. Therefore, the decrease in dopamine may enhance the activity of adenosine A1 that inhibits the release of neurotransmitters, which may generate muscles convulsions or neurotoxicity. Furthermore, the *UNC-63* gene expression assay showed an increase in the level of gene expression that may explain caffeine accumulation and thus drug sensitivity. The use of a low concentration of caffeine showed little response to the drug by the worms and this may not be consistent with previous studies as current study showed that caffeine at a low concentration can show a biphasic effect where it may show a slight response to caffeine or may not show any response. Interestingly, the gene expression assay of the *UNC-63*gene showed an excessive response to the drug and a large accumulation of caffeine at the *UNC-63*gene. This leads to inhibition of calcium influx released by the *UNC-2*gene and thus calcium channel blockade may lead to a defect in the release of acetylcholine and dopamine signals, which may cause inhibition of motor activity. Furthermore, it was observed that the worms motor activity was highly dependent on concentration, while gene expression showed activity that may not depend on concentration directly, but rather on the ability of the gene to receive drug quantities, and this may be due to changes in transcription or modification in histone activity. Therefore, it could be recommended to exposed the animals to the drug for several days (chronic exposure) instead of one single exposure (acute exposure); that could be important to assess the effect of chronic exposure to the caffeine on the development of addictive behavior and studying the effect of drugs on addiction- related genes. In addition, these results could have future applications in terms of developing pharmacological approach for the treatment of addiction and other diseases.

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