International Core Journal of Engineering Volume 7 Issue 7, 2021
ISSN: 2414-1895 DOI: 10.6919/ICJE.202107_7(7).0041

Investigation into the Mechanism by which 2,3,7,8-Tetrachlorodibenzo-p-dioxin Induces Dyschromatopsia by Measuring Electroretinogram Results and Cone Cell Quantification of Mice Exposed to TCDD

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Abstract

Purpose: TCDD is an environmental toxin that is exposed to humans in dangerous amounts most often through industrial accidents or occupational exposure. Studies of health effects of workers occupationally exposed to TCDD have connected the molecule to dyschromatopsia, but the mechanism of action by which dioxin induces color vision deficiency is unknown. This study sets out to experimentally examine whether TCDD is able to induce dyschromatopsia by damage to optic nerves or induce cell death in retinal cone cells directly. Methods: Damage to optic nerves will be measured by photopic electroretinogram tests on live mouse retinas and cone cell quantification will be performed by immunolabeling of mouse retina samples using goat anti-OPN1SW and rabbit anti-opsin red/green then viewing under fluorescent microscope. Possible results: Electroretinogram tests results may yield a (1) decrease in response levels indicating damage to optic nerves or (2) similar response levels indicating no damage to optic nerves. Cone cell quantification may lead to a (1) decrease in cone cell levels indicating apoptosis of retinal cells or (2) no change in cone cell levels indicating no direct effect on retinal cone cells. Conclusions: This study is able to determine if TCDD induces dyschromatopsia by one of two mechanisms (nervous system damage or cell apoptosis) but further investigations are needed to confirm the details of these mechanisms and examine mechanisms not investigated in this study.

Keywords

TCDD; Dyschromatopsia; Electroretinogram; Color Vision Deficiency.

1. Introduction

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD) is a chemical compound classified as the most toxic of the dioxin family of compounds with a World Health Organization Toxic Equivalency Factor (TEF) classification of 1. Dioxins are persistent organic pollutants with a hydrophobic and lipophilic nature that allows these compounds to resist environmental and human metabolic degradation [1], which enables the continued spread of TCDD and similar dioxins through the food chain as animals ingest toxic compounds distributed as the result of industrial activities [2]. Industrial activities which produce dioxins as a byproduct include chlorophenol and herbicide manufacturing and improper incineration of industrial waste [3]. Though many countries have strictly regulated these industries, there still persists continued exposure of humans to dioxin compounds through dietary intake [1]. In

ISSN: 2414-1895 DOI: 10.6919/ICJE.202107_7(7).0041

addition, large-scale exposure incidents of TCDD, such as the 1976 Seveso industrial disaster [4] and widespread usage of the Agent Orange herbicide during the Vietnam war [5] have added to the amount of dioxins present in the environment. More recently, the exposure of Irish farm animals to dioxin-contaminated feed led to a recalling of pork products in 2008 [6].

TCDD is able to act on human cells by binding to the Aryl Hydrocarbon Receptor (AhR). AhR is a cytosolic complex with 2 HsP90 chaperones, 1 p23 chaperone, and 1 XAP2 chaperone. Once the dioxin ligand binds to the receptor on the PAS-B domain, all but one HsP90 molecule dissociate, and AhR moves into the nucleus and binds with Aryl Hydrocarbon Receptor Nuclear Translocator (ARNT) protein at their PAS domains, releasing the last HsP90 chaperone, to create a heterodimer that then to binds to xenobiotic response elements, which are specific nucleotide sequences attached to target genes AhR can express. Through the use of various transcriptional coactivators, the AhR-ARNT compound is then able to induce the expression of target genes leading to the negative health effects caused by TCDD exposure. In addition, dioxins are able to repeatedly activate AhR to cause binding to ARNT proteins, reducing the expression of genes signaled by other receptors which also seek to bind to the ARNT protein and in turn create a lack of proteins which are necessary for cell homeostasis [7] [8]. Though TCDD may also induce activity through the AhR receptor by non-genomic pathways, the result of these activations is most commonly inflammation and other short-term health effects [6]. The most common of the long-term negative health effects brought on by TCDD include cancer, as the compound has been classified as a carcinogen by the International Agency for Research on Cancer [9], and reproductive dysfunctions, which has been experimentally demonstrated to disrupt both the male and female reproductive systems [10] [11].

In a study tracking the neurological and neurophysiological effects of TCDD exposure on a small cohort of workers exposed to TCDD from 1965-1967, dyschromatopsia, specifically blue-yellow color vision loss, was detected in 6 of 15 exposed individuals 35 years after the exposure [12] and was detected in all 7 remaining individuals 50 years after exposure [13]. This link is supported by current literature which correlates occupational exposure to other toxins with the development of dyschromatopsia among affected populations [14][15][16]. However, it has not been experimentally studied by what mechanism TCDD specifically is able to induce dyschromatopsia. In similar toxins, direct optic nerve damage is most often attributed to be the direct cause of dyschromatopsia and subsequent vision acuity decline in what is considered toxic optic neuropathy [17].

The current study presented in this paper hypothesizes that mice exposed to TCDD will develop color vision deficiency by mechanism of damaging the related retinal nerves as can be examined through electroretinography and will also lead to a decline in the overall cone cell count, implying that TCDD does affect visual perception through causing retinal cell death directly.

2. Materials and Methods

2.1 Materials

32 C57BL/6 mice will be used in this experiment, with 16 male and 16 female animals at an age range of 12-15 weeks to insure maturity. Due to the well-established electroretinography [18] and cell quantification [19] methods for the C57BL/6 mice strain, it was selected for use in this experiment. This experiment will strictly abide to AAALAC guidelines in its treatment of lab animals. Animals will be euthanized immediately if excessive discomfort is detected.

Purchased TCDD will be titrated to determine its original concentration, then diluted with corn oil into 3 different concentrations (50 μg/kg, 100 μg/kg, and 500 μg/kg) to suit the three different dosage groups in this experiment.

Chemicals necessary for performing electroretinography include an solution of 100mg/mL ketamine hydrochloride and 100mg/mL xylazine hydrochloride, 1% atropine sulfate, 2.5% phenylephrine hydrochloride, 0.5% proparacaine hydrochloride, and 2.5% hypromellose.

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Materials necessary for cell quantification include sodium pentobarbital, 4% paraformaldehyde, 0.5% Triton X-100, goat anti-OPN1SW antibody, rabbit anti-opsin red/green antibody, donkey anti-goat Alexa Fluor 488 antibody, and donkey anti-rabbit Alexa Fluor 488.

2.2 TCDD Exposure

The mice are split into 4 groups of eight, each with an equal number of males and females and similar weight range. Each group will either be delivered 50 μg/kg, 100 μg/kg, or 500 μg/kg of TCDD solution with the remaining negative control being delivered corn oil only. A positive control was not established due to the lack of experimental evidence in current literature that a solution is able to certainly cause dyschromatopsia when introduced to mice. Solutions will be delivered through oral gavage at a ratio of 10 mL per 1 kg of mouse weight. 96 hours will be observed between the time of solution delivery and the next step of experimentation on each animal.

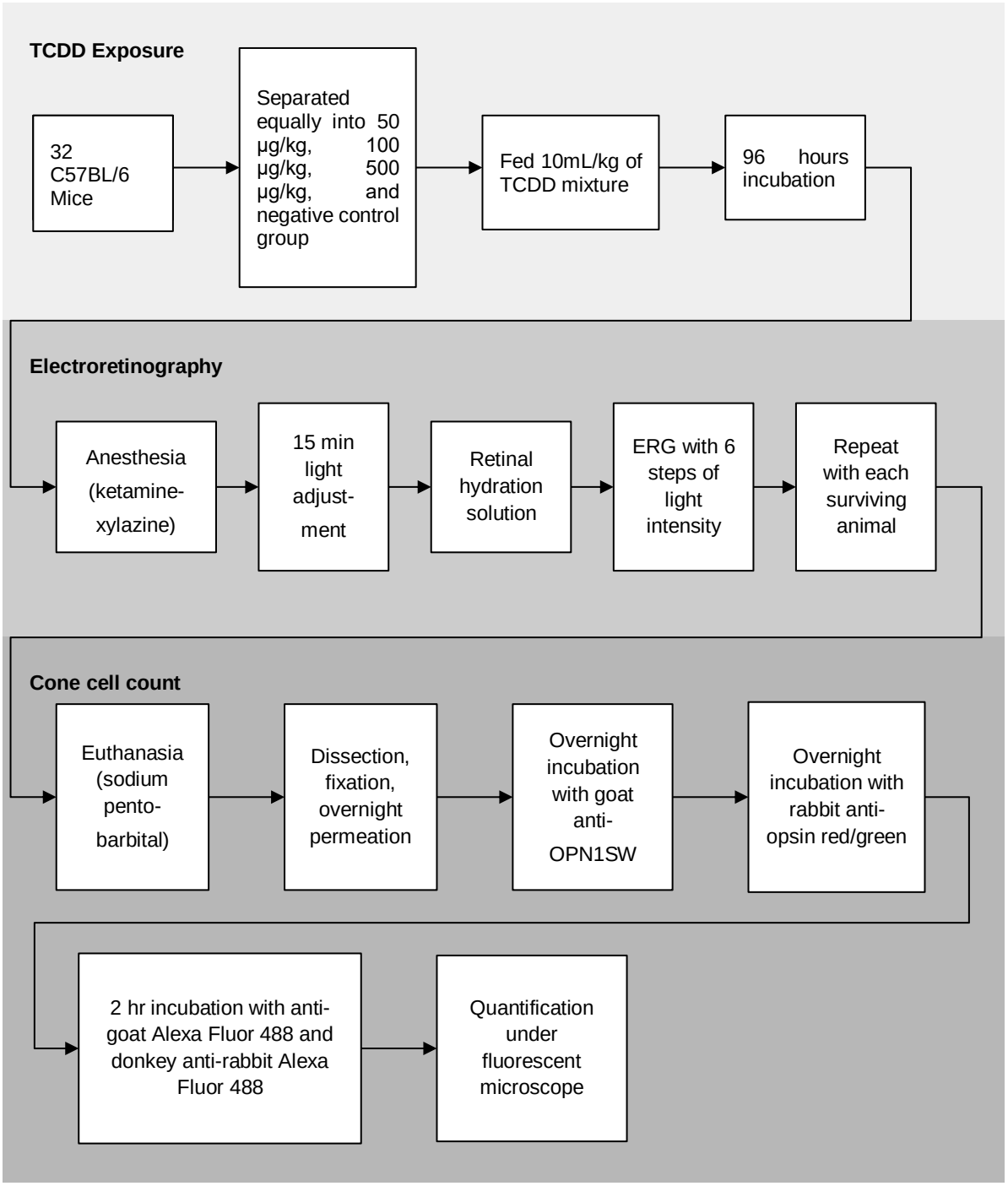

Figure 1. Flowchart of experiment method

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2.3 Electroretinography

After TCDD exposure, the mice retinas will be scanned using electroretinography (ERG). Photopic ERG will be performed on all 32 animals according to procedures established by Benchorin et al. 2017 [20].

Anesthesia should be prepared by a mixture of 80 mg/kg ketamine and 13 mg/kg xylazine. Mice will first be light-adapted for 15 minutes at a light intensity of 20 cd sec/m^2 then moved away from other animals onto a clean and dry heating pad for administration of the ketamine-xylazine solution. 1% atropine sulfate, 2.5% phenylephrine hydrochloride, 0.5% proparacaine hydrochloride, and 2.5% hypromellose ophthalmic solutions will be dripped onto the exposed mice eye for hydration and assisting with fixing electrodes in place. 29 gauge, 12mm needle electrodes will be placed with the ground electrode at the base of the tail and the reference electrode below the skin in between the animal's eyes followed by contact lens electrodes placed directly on the animal's eyes. A Ganzfeld stimulator and a visual electrophysiology system will then be used to run 6 steps of light intensity (ranging from 0.78 cd sec/m^2 to 20 cd sec/m^2) and photopic ERG responses will be recorded. Electrodes will be removed and cleaned with a cotton swab and water.

2.4 Cone cell count

Mice retinal cone cells will be quantified as according to procedures established by Ortín-Martínez et al. 2014 [19].

The animals will be euthanized using an IP injection of 200mg/kg sodium pentobarbital then perfused transcardially using 4% paraformaldehyde (PFA) followed by retina dissection, mounting, and fixation in PFA. Immunolabeling of the mounted species will then be conducted at room temperature by permeation using 0.5% Triton X-100 for 15 minutes, overnight incubation at 4°C with goat anti-OPN1SW and rabbit anti-opsin red/green, 3 washes of PBS, 2 hour room temperature incubation with donkey anti-goat Alexa Fluor 488 and donkey anti-rabbit Alexa Fluor 488, followed by mounting using mowiol mounting medium on a viewing slide for fluorescent microscope use. Scanned images will be automatically quantified using open-source software.

3. Possible Results

3.1 ERG result comparisons of mice exposed to TCDD and negative control

3.1.1 Reduced photopic ERG responses are measured in the majority of mice in one or more dosage groups

The photopic responses of mice exposed to TCDD during the electroretinography experiment are more than 25% lower in response peak and latency compared to the negative control of mice not exposed to TCDD or completely undetectable. This pattern occurs in the majority of mice in at least one of the three different dosage-separated groups.

3.1.2 Reduced photopic ERG responses are not measured in the majority of mice in one or more dosage groups

The detected photopic responses of TCDD-exposed mice using electroretinography are detectable and less than 25% different from the latency and peak heights of the control group.

3.2 Cone cell count comparisons of mice exposed to TCDD and negative control

3.2.1 Cone cell counts of mice exposed to TCDD stay constant

Less than a 10% average difference is detected between the cone cell counts of mice exposed to TCDD and mice in the negative control.

3.2.2 Cone cell counts of mice exposed to TCDD decline

More than a 10% average decline is detected between the cone cell counts of mice exposed to TCDD and mice in the negative control.

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3.2.3 Cone cell counts of mice exposed to TCDD increase

More than a 10% average increase is detected between the cone cell counts of mice exposed to TCDD and mice in the negative control.

3.3 Majority of mice die from TCDD poisoning

The majority of mice used for this experiment die. As a result, the sample size becomes too small and the trends concluded by the surviving animals are less credible.

4. Discussion

4.1 ERG result comparisons of mice exposed to TCDD and negative control

4.1.1 Reduced photopic ERG responses are measured in the majority of mice in one or more dosage groups

This result would confirm the hypothesis that dyschromatopsia is induced by damage to the nervous system that severs the electrical responses measured by ERG, agreeing with current literature indicating dyschromatopsia is one of the symptoms of toxic optic neuropathy caused by exposure to toxic substances [17]. This result only indicates that harming retinal nerves is a definite mechanism by which TCDD affects vision, however does not exclude the possibilities that other mechanisms, such as damage or death to the retinal cells directly, are occurring concurrently. However, both the retinal and neural physiology of mice is significantly different from that of humans, so further investigations must be conducted to further verify whether neural pathways are a definite mechanism of action for TCDD inducing color vision deficiency.

4.1.2 Reduced photopic ERG responses are not measured in the majority of mice in one or more dosage groups

This result would suggest that TCDD does not induce dyschromatopsia through affecting pathways in the nervous system. This result does not indicate that dyschromatopsia can not be induced by TCDD and this result does not exclude the possibility that TCDD may have caused non-functional defects to occur in retinal cone cells, resulting in communication to the nervous system being normal but cell function being abnormal, thus this effect can not be detected using electroretinography. This effect in which dyschromatopsia is induced but damage to the nervous system can not be detected would be similar to results shown by congenital color vision deficiency, in which electroretinogram responses would still display normally [21]. This result may have also been reached by experimental design or execution mistakes, including not enough time between TCDD administration and the measuring of results. In addition, even if the correlation between TCDD and dyschromatopsia through the nervous system can not be proven in mice, it may still be present in humans because of the physiological differences between the two species. Further investigation and repetition of the experiment would need to be conducted to verify that there is likely no connection between TCDD and color vision deficiency by damage to the nervous system.

4.2 Cone cell count comparisons of mice exposed to TCDD and negative control

4.2.1 Cone cell counts of mice exposed to TCDD stay constant

This result would suggest that TCDD affects color vision through the nervous system or causes defects in cone cells which cause them to lose their function instead of directly inducing cell death in retinal cells, disagreeing with the hypothesis of this report, and disagrees with current literature that indicates TCDD causes cell death in areas it reaches [23]. This result may also have been reached by experimental design or execution mistakes, including not enough time between TCDD administration and the measuring of results, the use of decomposed TCDD in the experiment, or the failure of TCDD to properly transport to the nervous system and cone cells. Whether or not this result was caused by the use of decomposed TCDD can be tested by examining the sample through mass spectrometry and comparing the sample's peaks to what is standard for the TCDD molecule.

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4.2.2 Cone cell counts of mice exposed to TCDD decrease

This result suggests that toxins may directly affect the eye and induces apoptosis in cone cells, thus reducing color detection and eventually overall visual ability. However, this result does not exclude the possibility that defect damage to cone cells or nervous system damage is occurring concurrently. This conclusion agrees with the hypothesis of this study and is supported by literature stating TCDD shows high rates of cell apoptosis, especially in the eye region [22]. The experiment should be repeated with a different euthanasia method in case improper IP injection led to accidental death of cone cells.

4.2.3 Cone cell counts of mice exposed to TCDD increase

This result is unlikely as TCDD has been shown to cause cell aging and death instead of causing cell growth in the eye and brain regions [22][23]. This result may be caused by experimental design or execution mistakes, including mistakes by automatic quantification software, mistakes in the immunodetection procedure of one or more samples (e.g. the accidental disposal of a portion of a sample during washes, insufficient incubation times), or contamination of the control that caused cell death in the cone cells of the control mice and resulting in an inaccurate reference for control.

4.3 Majority of mice die from TCDD poisoning

This result would invalidate any results obtained from the experiment because of the resulting small sample size and is likely because the dosage of TCDD was too high and became quickly lethal for many animals. The experiment should then be repeated with lower concentrations of TCDD in the administered solution. However, this result is highly unlikely as previous experiments [24] have administered higher or similar doses without the mice dying in less than 4 days.

Since each possible result includes and/or excludes certain mechanisms of action TCDD could take to impact vision, a summary of which mechanisms of action could be possible is listed in Table 1 based on what combination of results from category 1 (electroretinogram results) and category 2 (cell count results) are achieved. Likely mechanisms are mechanisms suggested to be the cause of achromatopsia by the experiment results, possible mechanisms are mechanisms that are not investigated through the experiment and have a unknown likelihood of contributing to TCDD inducing achromatopsia, and unlikely mechanisms are mechanisms suggested to not contribute to TCDD inducing achromatopsia by the experiment results. The third result in category 2 was excluded from the table because it does not give clear implications of the mechanism of action by which TCDD induces dyschromatopsia.

Table 1. Possible results of experiment and conclusions that can be made from results

	Electroretinogram results show reduced cone cell response	Electroretinogram results show normal cone cell response
Cone cell count declines after exposure	Likely mechanisms: Damage to nervous system Induces apoptosis in cone cells Possible mechanisms: Induces defects which stop cone cell function but does not kill cone cells	Likely mechanisms: Induces apoptosis in cone cells Possible mechanisms: Induces defects which stop cone cell function but does not kill cone cells Unlikely mechanisms: Damage to nervous system
Cone cell count remains the same after exposure	Likely mechanisms: Damage to nervous system Possible mechanisms: Induces defects which stop cone cell function but does not kill cone cells Unlikely mechanisms: Induces apoptosis in cone cells	Possible mechanisms: Induces defects which stop cone cell function but does not kill cone cells Unlikely mechanisms: Damage to nervous system Induces apoptosis in cone cells

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5. Conclusion

In conclusion, this study would experimentally investigate the mechanism by which the environmental toxin TCDD induces dyschromatopsia, or color vision deficiency, in species exposed to through the electroretinography measurement and cone cell quantification of mice exposed to varying doses of TCDD and the subsequent quantification of cone cells. The results of this experiment would be able to narrow down which mechanism TCDD damages visual ability through, whether it is damage to the nervous system, limited damage to cone cells, inducing apoptosis in cone cells, or a combination of the aforementioned causes. Investigation into the relationship between TCDD and dyschromatopsia in animals with greater physiological similarity to humans in the nervous and visual systems would increase the applicability of this study's results to humans and thus may shed light on potential ways to negate or recover from the visual effects of TCDD and similar toxins.

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