EFFECTS OF THE UV-FILTER OXYBENZONE ON ESTROGEN AND TESTOSTERONE LEVELS IN ADULT ZEBRAFISH (*DANIO RERIO)*

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ABSTRACT

The usage of sunscreens has become integral in daily skincare routines due to their role in preventing sunburns and reducing the risk of skin cancer. One type of sunscreens is chemical sunscreens, encompassing compounds like Oxybenzone, which absorb and convert UV radiation. While Oxybenzone is recognized for its efficacy in UV protection, concerns regarding its safety have emerged. Furthermore, research suggests Oxybenzone may be a hormone disruptor, potentially affecting aquatic ecosystems and organisms like Zebrafish (*Danio rerio)*.

This study aimed to investigate the effects of acute exposure to Oxybenzone on hormone levels, specifically estrogen (17-beta estradiol) and testosterone, in adult Zebrafish. Significant alterations in hormone concentrations were observed through controlled exposure experiments. Testosterone levels were notably reduced in fish exposed to Oxybenzone, while 17-beta-estradiol concentrations increased, indicating potential endocrine-disrupting effects.

The findings underscore the importance of understanding the ecological implications of sunscreen ingredients beyond their intended human benefits. Further research is warranted to elucidate the broader impact of Oxybenzone on aquatic environments and wildlife, guiding regulatory measures and public awareness efforts to mitigate potential harm.

INTRODUCTION

Sunscreens became highly recommended part of everyday skincare routine following the release of studies on that show a positive correlation between sunburns and sunbaths with skin cancer rates (Berwick M, 1992). Currently there are two main types of sunscreens available in the market – physical and chemical sunscreens, which differ in terms of their active ingredients (FDA, 2021). Physical sunscreens (also called mineral sunscreen) contain Titanium Dioxide and Zinc Oxide as the active ingredient and are approved by U.S. Food and Drug Administration (FDA, 2021). They are also considered more effective in blocking UV lights (UVA and UVB) since they reflect the radiation, instead of absorbing and converting it as chemical sunscreens do (Siller, 2018). Chemical sunscreens usually contain organic filters such as Oxybenzone, Cinoxate, Dioxybenzone, Ensulizole, Homosalate, Meradimate, Octinoxate, Octisalate, Octocrylene, Padimate O, Sulisobenzone, and Avobenzone, which are also

approved by FDA, but not listed as GRASE – "Generally Recognized As Safe and Effective to use" due to lack of data on their safety (FDA, 2021).

One of the organic filters in chemical sunscreens is oxybenzone, also known as Benzophenone-3 (BP-3). Oxybenzone is a light-yellow powder that absorbs UVA and UVB lights, which makes it highly effective as a UV-protector. Absorbed UV-light is converted into energy through photochemical excitation which results in emission of longer wavelengths, decreasing the penetration of radiation into skin. (Kim, 2014). According to the Scientific Committee on Consumer Safety (SCCS), BP-3 is safe to use in the concentrations of up to 6% in face cream, hand cream and lipsticks, and in concentrations of up to 2.2% in body creams, pump sprays and propellant sprays (SCCS, 2021).

Recently, studies in Hawaii and U.S. Virgin Islands have shown that Oxybenzone results in bleaching of coral reefs *Stylophora Pistillata* (Downs, 2016). As a result, US Senate Bill 2571 was passed and enforced in 2021 to ban the sale and distribution of chemical sunscreens that contain Oxybenzone along with other ingredients in Hawaii (The Senate 29th Legislature, 2021). However, oxybenzone sunscreens are still commonly used in other states and may have potential danger to freshwater ecosystems (Zhang, 2021).

A common model organism for toxicological studies in freshwater ecosystems is Zebrafish (*Danio rerio).* Studies have shown that BP-3 can be a hormone disruptor in adult Zebrafish by altering gene expression and activating antiandrogenic (testosterone blocking or androgen antagonizing) activity (Bluthgen, 2012). Additionally, studies from 2008 suggested that BP-3 delayed hatching period and viability of Zebrafish eggs (Coronado, 2008), which can also be potentially related to endocrine-disrupting effects of Oxybenzone. Estrogen (17-beta estradiol) and testosterone are the main hormones in regulating reproductive development and behavior in zebrafish (Bluthgen, 2012). Estrogen is crucial for the maturation of reproductive organs, regulating the reproductive cycle, and maintaining bone health (Boueid, 2023). Testosterone is primarily involved in masculinization, spermatogenesis, and influencing mating behaviors (Bluthgen, 2012). Disruptions to these hormones, such as exposure to endocrine-disrupting chemicals like oxybenzone, can adversely affect reproductive success, development, and overall health in zebrafish (Bluthgen, 2012). Therefore, this study aims to investigate the change in concentrations of estrogen (17-beta estradiol) and testosterone in adult *D.rerio* under acute exposure to Oxybenzone. The results would be helpful to understand the effects of Oxybenzone that might potentially affect the hatching delay concluded in previous studies, which is environmentally important to prevent reproductive toxicity of this sunscreen ingredient.

MATERIALS AND METHODS:

Adult Zebrafish Assessment

A total of 98 adult zebrafish were obtained at the local pet store. 9 tanks with the volume of 10 gallons were set and filled with water, pH and ammonia levels optimized via “Ammonia Lock” and “pH up/down” solutions (API, USA). Zebrafish were put in tanks after 24 hours and observed for toxic nitrogen levels as the biproduct of their metabolism over the course of 3 days using “Freshwater Master Test Kit” (API, USA). Any abnormal changes in pH or nitrogen levels were addressed via the same kits as mentioned earlier. Fish were fed once a day with regular fish food “TetraFin Goldfish flakes plus” provided by institution (Tetra ®, USA).

Exposure to Oxybenzone

2 weeks after the tank setup, the fish were exposed to Oxybenzone. The tanks were divided into three groups, 3 tanks for control (no Oxybenzone), 3 for 500 ug/L of Oxybenzone and 3 tanks for 1000 ug/L of Oxybenzone exposure. The concentrations were chosen as Oxybenzone had chronic effects on *Danio rerio* at concentration of 191 ul/L, indicated as chronic endpoint (Kinnberg et. al. 2015) and acute effects (6 days LC50) at concentration of 2000-4000 ul (Jang et. al. 2016), thus concentrations between these two data were selected for this research. The Oxybenzone was dissolved in 0.01% DMSO solvent and introduced into the tanks. Initial reaction of the fish was recorded. The fish were observed for 6 weeks after the exposure for any abnormal behavior or increased mortality. No adjustments were made throughout the 6 weeks period. Water was added weekly accounting for evaporation of the water in tanks, but no water was removed.

Specimen collection

After 6 weeks 5 fish per tank were randomly selected for specimen collection. The fish were placed on ice for anesthesia to provide painless death. After 2-5 minutes the loss of muscle contraction was observed. The fish were dried by placing them on paper towel. Fish from each tank were pooled as 1 sample and ground via mortar and pestle for proper amount of sample for further analysis required by ELISA kit. Collected specimen samples were placed in the -80 C freezer until further evaluation.

ELISA

The ELISA plates for Testosterone and 17-beta-Estradiol were obtained from MyBioSource. The ELISA was performed following the instructions included in the manual provided by the manufacturer (MyBioSource, 2022). Testosterone standards were in concentrations of 2000-7.81 pg/ml, estrogen (E2, 17-beta estradiol) standards were in concentrations of 30000-29.3 pg/ml. Each sample was run in triplicates. The standard curve was calculated and analyzed on Excel.

Statistical Analysis

ANOVA was performed for Estrogen and Testosterone levels in each group on R Studio. Shapiro-Wilk test was performed along with plotting QQ-plot to test the normality assumption, followed by F-test for assumption of equal variance. The QQ-

plot indicated normal distribution (P>0.05, Shapiro-Wilk Test), while the F-test for variance indicated significant difference in variances. The significant results of ANOVA were further analyzed via Tukey Post hoc test on R Studio.

RESULTS

Adult Zebrafish

Over the course of the experiment, fish showed no abnormal behavior compared to normal behavior according to previous studies (Bluthgen, 2012) with only accidental mortality (nd=18) before and during exposure that was approximately evenly distributed among the treatments.

Testosterone and Estrogen analysis

The results showed significant difference in the Testosterone levels as displayed in Fig 1 (P<0.05, mean=38322.2 pg/ml, SEM=56669.65 pg/ml, n=3, ANOVA), but no significant difference in the Estrogen levels as displayed in Fig 2 (P>0.05, SEM= 203.5242 pg/ml, n=3, ANOVA). Tukey post-hoc test was performed to identify the differences in the testosterone levels of different exposure groups. The differences between each exposure group and control group were significant (P<0.05), but the difference in testosterone between the oxybenzone exposure groups was not significant (P>0.05).

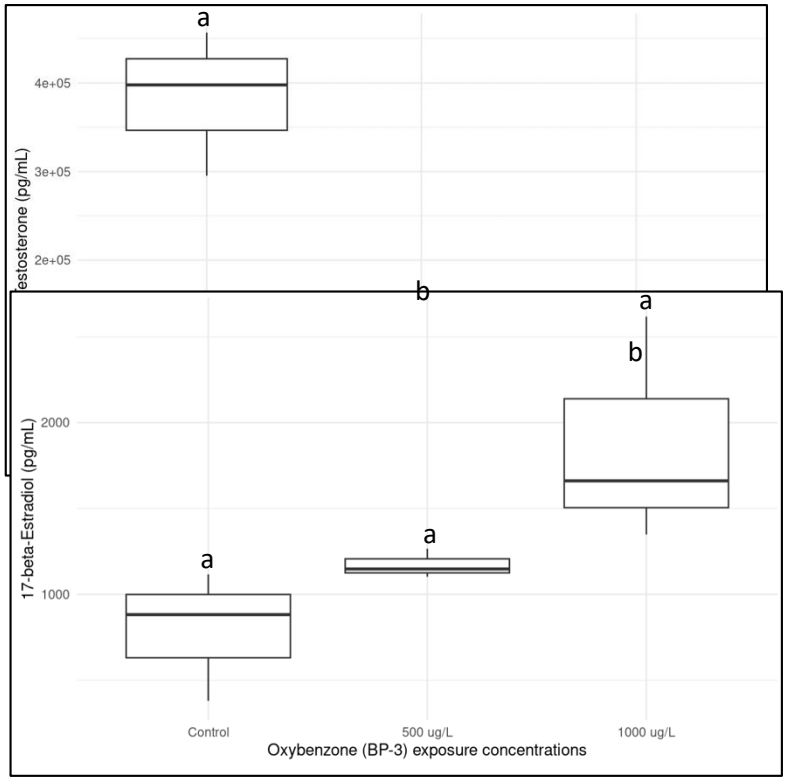

Figure 1 The boxplot shows the concentrations of Testosterone based on different Oxybenzone exposure groups. The control group has the highest concentration of Testosterone (mean=383322.2 pg/ml), the high exposure group has the lowest (mean=35305.5 pg/ml).

Figure 2 The Boxplot shows the concentrations of Estradiol over several Oxybenzone concentration exposure groups, with highest levels of 17-beta-estradiol (mean=1875.56 pg/ml) and lowest in control group (mean=792.67 pg/ml)

DISCUSSION

The purpose of this experiment was to investigate the effects of acute exposure to Oxybenzone on hormone levels, specifically estrogen (17-beta estradiol) and testosterone, in adult Zebrafish..

The observed effects of high concentrations of Oxybenzone in this study showed a significant decrease in Testosterone levels (fig 1). This aligns with previous research indicating Oxybenzone's estrogen-mimicking property and its ability to suppress Testosterone in Zebrafish by interfering with the expression of transcripts involved in hormonal and steroidogenic pathways (Bluthgen, 2012). These findings raise concerns about the potential demasculinization of testes and reproductive disturbances in the environment (Caspillo, 2014), emphasizing the importance of understanding the broader ecological implications of Oxybenzone exposure.

This study showed no significant difference in the estrogen levels between the control group and oxybenzone exposure groups (fig 2). This result is consistent with the previous study conducted on fathead minnows (Kinz et al., 2006) and male zebrafish (Bluthgen, 2012), but opposite to the results of the studies on Japanese medaka and rainbow trout (Coronado et al., 2008). This may be due to the differences in fish species, as well as the studies based on genders of the fish, which suggests the future studies to be designed with separating the fish by genders for accurate analysis.

Another crucial consideration was the potential for hormonal disruption from the solvent used (0.01% DMSO). While DMSO has been reported to have endocrine-disrupting effects on Zebrafish (Mortensen et al., 2006), previous studies incorporating solvent controls in Oxybenzone research demonstrated insignificant changes in hormonal levels (Bluthgen, 2012), (Downs, 2016). Nevertheless, it is noteworthy that Oxybenzone dissolved in any solvent, including ethanol or acetone, has been shown to exhibit hormone-disrupting effects (Coronado 2008), suggesting the need for further exploration of solvent controls in future research studies.

In conclusion, our study demonstrates that acute exposure to high concentrations of Oxybenzone decreases testosterone in adult Zebrafish, indicative of potential reproductive disturbances. The study did not find any significant changes in the estrogen levels, yet further studies with different experimental designs are necessary to understand this correlation better. These findings underscore the importance of comprehending the broader ecological implications of Oxybenzone exposure, particularly its role as a hormone disruptor in aquatic environments. Further research is needed to explore alternative solvents and refine experimental methodologies. By enhancing our understanding of Oxybenzone's ecological footprint, we can inform regulatory measures to minimize its environmental impact and safeguard aquatic ecosystems.

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