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Research Article

Zebrafish (Danio rerio)

Dose-dependent Effects of Ethanol on Caudal Fin Regeneration in Zebrafish (Danio rerio)

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Ethanol is a well-established teratogen known to disrupt developmental signalling pathways; however, its effects on adult tissue regeneration remain insufficiently understood. This study investigates the dose-dependent impact of ethanol on the regenerative capacity of the caudal fin in adult zebrafish (Danio rerio). Adult zebrafish (mean body length ~40 mm) underwent caudal fin amputation and were subsequently exposed to sub-lethal ethanol concentrations of 0.01%, 0.02%, 0.04%, and 0.08% (v/v), alongside a control group. The fish were maintained under these conditions and monitored daily until complete regeneration was observed in the control group.

The results demonstrate a clear concentration-dependent effect of ethanol on regeneration. Lower ethanol concentrations exhibited regeneration patterns comparable to the control, whereas higher concentrations resulted in delayed and markedly reduced fin regrowth. These findings suggest that elevated ethanol exposure may interfere with oxidative balance and key morphogenetic signalling pathways essential for wound healing and tissue regeneration.

This study provides foundational insights into the influence of environmental toxicants on regenerative processes and highlights the need for further mechanistic investigations.

Keywords: zebrafish (Danio rerio), caudal fin regeneration, ethanol toxicity, dose-dependency, regenerative biology, teratogen

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1. Introduction

The ability to regenerate lost or damaged tissues is a fundamental biological phenomenon that varies significantly across the animal kingdom. In mammals, regenerative capacity is generally limited, often resulting in fibrotic scar formation and incomplete functional recovery (Alibardi, 2022; Akimenko et al., 2003). In contrast, certain vertebrates, including teleost fish and urodele amphibians, exhibit remarkable regenerative capabilities, enabling the complete restoration of complex tissues and structures (Liu et al., 2021; Marques et al., 2019). Understanding the mechanisms underlying such regenerative potential holds significant promise for advancing regenerative medicine (Beffagna, 2019; Gamba et al., 2014).

The zebrafish (*Danio rerio*) has emerged as a powerful model organism for studying regeneration due to its exceptional ability to restore multiple tissues and organs, including the heart, brain, spinal cord, retina, and caudal fin (Gemberling et al., 2013; Kizil et al., 2012; Marques et al., 2019; Sehring & Weidinger, 2022; Lenkowski et al., 2013). Its rapid development, small size, optical transparency during early life stages, and amenability to genetic manipulation make it an invaluable system for investigating complex biological processes (Gemberling et al., 2013; Marques et al., 2019). Notably, the regenerative capacity of the zebrafish caudal fin is robust and remains largely unaffected even after repeated amputations (Azevedo et al., 2011).

Caudal fin regeneration in zebrafish is a classic example of epimorphic regeneration, characterized by the formation of a proliferative structure known as the blastema (Cao et al., 2022; Mateus et al., 2012; Poss et al., 2000; Sehring & Weidinger, 2022). Following amputation, rapid wound closure occurs within hours through epidermal coverage, followed by blastema formation beneath the wound epidermis within 1–3 days under normal conditions (Mateus et al., 2012; Petrie et al., 2014). The blastema consists of proliferative, lineage-restricted progenitor cells, including dedifferentiated osteoblasts, which contribute to the regeneration of bone, vasculature, nerves, and connective tissues (Knopf et al., 2011; Lee et al., 2020; Sousa et al., 2011).

Key signalling pathways, such as Fibroblast Growth Factor (FGF) and Wnt signalling, play critical roles in regulating blastema formation and regenerative outgrowth (Lee et al., 2020; Poss et al., 2000; Kudoh et al., 2002). Complete restoration of fin size and pattern is typically achieved within approximately two weeks (Marques et al., 2019; Pfefferli et al., 2014; Sehring & Weidinger, 2022; Shao et al., 2011).

Despite the well-characterized intrinsic regenerative capacity of zebrafish, external environmental factors can significantly influence regenerative outcomes. Ethanol, a widely prevalent environmental and biological compound, is known to induce oxidative stress and disrupt developmental processes, particularly in zebrafish embryos (Alsakran & Kudoh, 2021; Soares et al., 2014; Tsedensodnom et al., 2013). Ethanol metabolism has been associated with cellular dysfunction and tissue damage, especially in metabolically active organs such as the liver and muscle (Coffey et al., 2018; Voordeckers et al., 2020). Given that regeneration requires precise coordination of cellular proliferation, differentiation, and signalling pathways, it is likely to be sensitive to such metabolic and oxidative perturbations.

Although the developmental toxicity of ethanol has been extensively studied, its effects on adult regenerative processes remain less well understood. In particular, the dose-dependent impact of ethanol on the rate, extent, and underlying cellular mechanisms of caudal fin regeneration in adult zebrafish has not been comprehensively investigated.

Therefore, the present study aims to evaluate the effects of varying ethanol concentrations on caudal fin regeneration in adult *Danio rerio*. We hypothesize that increasing ethanol concentrations will progressively impair regenerative efficiency by delaying regenerative onset and reducing overall tissue outgrowth. By elucidating how ethanol influences regenerative processes, this study contributes to a broader understanding of environmental modulation of regeneration. Such insights are relevant to environmental toxicology, the health of aquatic ecosystems, and the identification of pathways that may be targeted in regenerative medicine.

2. Materials and Methods

1. Experimental Design and Animal Maintenance:

This study was designed to evaluate the effects of varying ethanol concentrations on caudal fin regeneration in zebrafish (*Danio rerio*). A total of 120 adult zebrafish were procured from a local aquarium supplier and acclimatized under laboratory conditions prior to experimentation.

To ensure experimental reliability, key environmental variables—including water temperature and pH were carefully controlled and maintained at constant levels throughout the study. These parameters are known to influence metabolic activity and physiological processes such as growth and regeneration; thus, maintaining stable conditions minimized external variability that could confound the observed effects of ethanol exposure.

2. Selection and Grouping of Experimental Animals:

To account for the potential influence of body size on regenerative capacity, zebrafish were pre-screened and grouped based on size. Previous studies have indicated that smaller individuals may exhibit faster regenerative responses compared to larger ones (Uemoto et al., 2020). Accordingly, fish of comparable body size were selected to minimize size-related variability in regeneration rates.

The experimental population included both sexes. Fish were randomly assigned to five groups (n = 24 per group): one control group and four treatment groups exposed to ethanol concentrations of 0.01%, 0.02%, 0.04%, and 0.08% (v/v), respectively.

3. Acclimatization and Ethanol Exposure:

Prior to fin amputation, zebrafish underwent a gradual acclimatization process to ethanol. Individuals in the treatment groups were exposed to incrementally increasing ethanol concentrations until the target concentrations (0.01–0.08% v/v) were reached. Absolute ethanol was used for solution preparation.

This stepwise acclimatization was implemented to reduce acute stress responses that could otherwise interfere with physiological processes, including regeneration. Gradual exposure allowed the fish to adapt to experimental conditions, thereby improving the reliability of subsequent observations.

4. Caudal Fin Amputation Procedure:

Caudal fin amputation was performed under sterile conditions using sharp surgical scissors. Fish were handled with care, and gloves were worn throughout the procedure to maintain aseptic conditions.

Amputations were standardized by making transverse cuts parallel to the dorso-ventral axis of the fin, ensuring consistency in the level of tissue removal while avoiding damage to underlying musculature. This standardization was essential to ensure comparability of regenerative responses across all experimental groups.

5. Measurement of Fin Regeneration:

Fin regeneration was assessed using a standardized measurement protocol. Initial fin area was recorded prior to amputation by tracing the caudal fin onto graph paper. Subsequent measurements were taken daily by tracing the regenerating fin area, allowing for quantitative assessment of regrowth over time.

This approach provided a consistent and reproducible method for monitoring changes in fin size. Standardization of measurement techniques minimized observational error and enhanced the accuracy and comparability of the collected data.

6. Maintenance and Exposure Conditions:

Fish were maintained in individual holding containers under controlled laboratory conditions. Ethanol solutions were replaced every 24 hours to maintain consistent exposure concentrations and prevent the accumulation of metabolic waste products.

Containers were covered with muslin cloth to minimize contamination while allowing adequate aeration. Small perforations ensured sufficient oxygen exchange. Regular renewal of solutions also contributed to maintaining optimal water quality, thereby reducing potential confounding effects.

7. Ethical Considerations:

All experimental procedures were conducted following approval from the relevant institutional ethics committee. The study adhered to established guidelines for the care and use of laboratory animals. Strict compliance with ethical standards ensured the humane treatment of zebrafish throughout the experiment and enhanced the scientific credibility and reproducibility of the study.

8. Statistical Analysis:

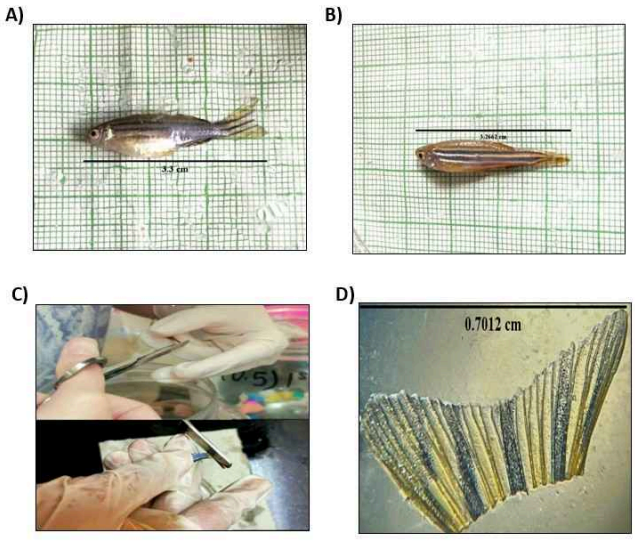
Data obtained from the experiment were analyzed using one-way analysis of variance (ANOVA) to assess differences in regeneration rates among the control and ethanol-treated groups. ANOVA was selected as an appropriate statistical method for comparing means across multiple groups and determining the significance of ethanol concentration on regenerative outcomes. Statistical significance was evaluated at a confidence level ($p < 0.05$).

3. Results

The results demonstrate a clear dose-dependent effect of ethanol on caudal fin regeneration in zebrafish (*Danio rerio*). Regeneration time varied significantly across treatment groups depending on ethanol concentration.

Fish in the control group, as well as those exposed to low ethanol concentrations (0.01% and 0.02% v/v), exhibited substantial and complete fin regeneration within 13 days post- amputation. Regenerated fins in these groups showed restoration of both length and structural integrity comparable to the control.

In contrast, higher ethanol concentrations resulted in impaired regenerative responses. The 0.04% group exhibited delayed regeneration, with measurable regrowth observed only during the later stages of the observation period. The 0.08% group showed minimal to negligible regeneration, with little evidence of fin outgrowth even after 13 days.



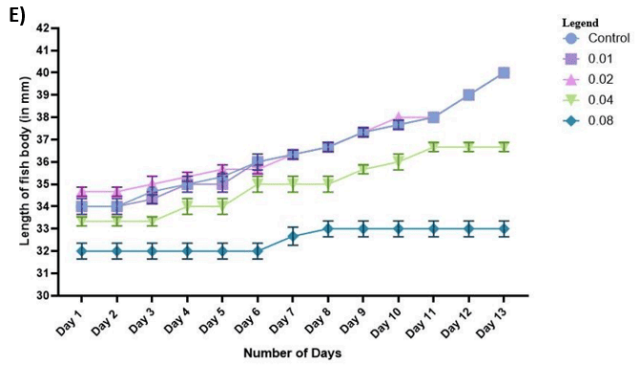


Figure: Graphical representation of caudal fin regeneration following amputation in zebrafish (*Danio rerio*).

A-D) Zebrafish were handled with care during tail amputation under strictly maintained sterile conditions. Sterile gloves and fine, sharp surgical scissors were used to ensure precision and minimize tissue damage. An equal length of the tail was excised from each specimen to allow consistent assessment of regeneration. Following the procedure, the fish were immediately transferred to their respective globes for further observation.

E) The x-axis indicates time post-amputation (days), while the y-axis represents total fin length (mm). Distinct colour codes correspond to the control group and each ethanol concentration, illustrating comparative regenerative trends across treatments. Graphical analysis (mean ± SD; n = 8 per group, averaged from triplicates) revealed a progressive decline in regenerative capacity with increasing ethanol Statistical analysis using one-way ANOVA followed by Dunnett's multiple comparisons test ($\alpha = 0.05$) indicated that the 0.08% ethanol group differed highly significantly from the control (****P< 0.0001), confirming a strong inhibitory effect of ethanol at higher concentrations. These findings support the hypothesis that increasing ethanol concentrations adversely affect both the rate and extent of caudal fin regeneration.

4. Discussion

The present study provides strong evidence for a dose-dependent suppression of caudal fin regeneration in adult zebrafish (*Danio rerio*). While low ethanol concentrations (0.01% and 0.02%) did not significantly alter regenerative outcomes, higher concentrations (≥0.04%) resulted in delayed or severely impaired regeneration, with near-complete inhibition observed at 0.08%.

This gradient of response is consistent with previous studies demonstrating that low levels of ethanol are relatively well tolerated, whereas higher concentrations disrupt key developmental and cellular processes, including morphogen signalling pathways such as Sonic hedgehog (Shh) and fibroblast growth factor (Fgf) signalling (Alsakran & Kudoh, 2021; Bilotta et al., 2004; Sidik et al., 2021).

A key mechanism underlying these effects is likely ethanol-induced oxidative stress. Reactive oxygen species (ROS), particularly hydrogen peroxide (H_2O_2), play an essential role in early regenerative events such as wound epidermis formation and blastema initiation (Ray et al., 2012). However, excessive ROS production—commonly associated with ethanol metabolism—can overwhelm antioxidant defenses, leading to lipid peroxidation, protein oxidation, and DNA damage (Heaton et al., 2002; Smith et al., 2005).

In the highest exposure group (0.08%), elevated ROS levels likely disrupted wound healing and inhibited blastema formation, thereby preventing the transition to the proliferative phase of regeneration. The delayed regeneration observed in the 0.04% group may reflect partial physiological adaptation to ethanol exposure, allowing limited recovery of regenerative processes over time.

In addition to oxidative stress, ethanol may impair regeneration by disrupting key signalling pathways. Fgf signalling is critical for blastema formation, epithelial–mesenchymal transition, and regenerative outgrowth (Poss et al., 2000), while Shh signalling regulates patterning of the fin rays (Romero et al., 2018). Ethanol-mediated downregulation of these pathways could reduce mitogenic signalling and impair tissue organization.

Furthermore, Wnt/β-catenin signalling—an essential regulator of blastemal cell proliferation and osteoblast differentiation—is also sensitive to oxidative stress and upstream pathway disruptions (Wehner & Weidinger, 2015; Tang et al., 2019). Suppression of Wnt signalling at higher ethanol concentrations would further limit cell proliferation and regenerative capacity.

The absence of significant differences between the control, 0.01%, and 0.02% groups suggests the presence of a threshold effect.

Ethanol concentrations at or below 0.02% appear insufficient to induce oxidative or signalling disruptions that impair regeneration, whereas concentrations of 0.04% and above exceed this threshold, resulting in measurable biological effects.

Overall, these findings extend the known teratogenic effects of ethanol to adult regenerative biology and highlight the sensitivity of regenerative processes to environmental stressors.

5. Conclusion

This study demonstrates that ethanol exerts a dose-dependent inhibitory effect on caudal fin regeneration in adult zebrafish (*Danio rerio*). Low ethanol concentrations (0.01% and 0.02%) did not significantly affect regenerative outcomes, with complete fin restoration observed within 13 days, comparable to the control group. In contrast, higher concentrations (0.04% and 0.08%) resulted in delayed and severely impaired regeneration, with near-complete inhibition observed at 0.08%.

These results indicate the presence of a critical threshold between 0.02% and 0.04%, beyond which ethanol significantly disrupts regenerative processes. The observed effects are likely mediated through mechanisms involving oxidative stress and interference with key signalling pathways such as Fgf, Shh, and Wnt/β-catenin.

The findings underscore the broader toxicological impact of ethanol on tissue regeneration and highlight the importance of minimizing environmental exposure to preserve regenerative capacity. Future studies should focus on elucidating the underlying molecular mechanisms and exploring potential mitigation strategies, such as antioxidant supplementation, to restore regenerative function under ethanol-induced stress conditions.

Conflict of Interests

The authors declare that they have no conflict of interest.

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