Research Article

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Effect of Alcohol and Wnt Signaling Pathway Interactions in Zebrafish (Danio rerio) Tooth Development.

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ABSTRACT

Alcohol consumption during pregnancy can negatively affect fetal development. Prenatal alcohol exposure (PAE) has been associated with various birth defects and has shared numerous dental deficits with individuals with Fetal Alcohol Spectrum Disorder (FASD), such as Decayed, Missing, and Filled Teeth (DMFT), malocclusion, caries, speech impairment, and enamel defects. While distinct developmental defects have been established, less is known about the mechanisms leading to such phenotypes in tooth development. A possible explanation could be that Alcohol interacts with signaling pathways in tooth development; thus, using zebrafish (Danio rerio), we aimed to investigate the histological arrangement and cellular changes in dental defects caused by the Alcohol and Wnt signaling pathway interaction. We treated zebrafish embryos with 1% Alcohol, 2mM LiCl (Wnt activator), 10nM WNT-C59 (Wnt inhibitor), and a combination of Alcohol and Wnt modulators. Whole mount staining and histological analysis across experimental groups revealed differential tooth/tooth germ numbers, sizes, shapes, mineralization pattern, as well as Wnt10a and Wnt10b expression levels. Interestingly, combined treatments of Alcohol with LiCl showed similarities to Alcohol alone, whereas Alcohol with WNT-C59 acted synergistically to disrupt tooth development.

Keywords : Zebrafish; teeth; Fetal alcohol spectrum disorder; alcohol; Wnt signaling pathway.

INTRODUCTION

Addictive substances are known to cause developmental defects when exposed to the fetus in utero[1]. Ethanol (Alcohol) is a widely used psychoactive substance whose societal prevalence can be attributed to its ease of production, cultural and social acceptance as a beverage, and traditional use[2-4]. It is also a potent teratogen in fetal development. Alcohol consumption during pregnancy can expose the developing fetus to the teratogen and cause significant harm to the developing fetus[5]. Various fetal birth defects have been observed from prenatal alcohol exposure (PAE). The first observations were made in 1968 by Lemoine et al. who identified a set of birth defects in 127 children exposed prenatally to Alcohol[6]. In 1973, Jones and Smith coined the term Fetal Alcohol Syndrome (FAS) to describe the constellation of birth defects resulting from PAE, including growth impairments, developmental delays, craniofacial dysmorphology, central nervous system abnormalities, heart, limb, and kidney anomalies[7,8]. Since then, a broad range of morphological and organ deformities as well as cognitive deficits have been added to the list of birth defects linked to PAE. Specifically, individuals with Fetal Alcohol Spectrum Disorder (FASD) have presented with various oral abnormalities and dental deficits, with a high prevalence of Decayed, Missing, and Filled Teeth (DMFT), malocclusion, caries, speech impairment, and enamel defects[9].

Tooth development takes place via specific and complex interactions between the first branchial arch's surface ectoderm and its underlying neural crest-derived mesenchyme[10]. Ectodermal cells differentiate into ameloblasts, the enamel-secreting cells; while odontoblasts lining the tooth pulp arise from the mesenchyme and are involved in dentin formation[11,12].

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Primary regulatory mechanisms involving communication between the epithelium and the mesenchyme are key events for epithelial placode formation and budding, mesenchyme condensation, epithelium folding and growth, all of which guide tooth morphogenesis[11]. Such regulatory mechanisms involve the secretion of signaling molecules and growth factors, namely the transforming growth factor beta (TGFβ), bone morphogenetic protein (BMP), fibroblast growth factor (FGF), hedgehog, and Wnt families[13]. The Wnt gene family in particular is crucial for cell development and differentiation[14]. Secreted as ligands, the Wnt proteins activate downstream receptor-mediated pathways that play a key role in the development of ectodermal appendages, such as teeth[15,16]. The canonical Wnt signaling pathway acts at multiple stages of tooth development and morphogenesis[16]. Experiments with a Wnt antagonist and agonist have shown that inhibition of the Wnt pathway has been associated with tooth anomalies, such as oligodontia, tooth agenesis, and crown and root dysmorphologies[17-19]; whereas constitutive activation of this pathway causes large, mishappen tooth buds and ectopic teeth[16]. Many Wnt genes are widely expressed in the oral and dental epithelia, as well as in developing teeth[16]. These genes include Wnt4, Wnt5a, Wnt6, Wnt10a, Wnt10b, to name a few[20]. In particular, mutations in Wnt10a are linked to impaired tooth development, such as hypodontia, taurodontism, and ectodermal dysplasia syndromes[17,21]. Furthermore, changes in its gene expression affect other tooth development genes, suggesting that Wnt10a may have direct and indirect effects on tooth development[18].

While tooth development is under genetic control, it is also subject to environmental factors. There is evidence of Alcohol affecting various stages of tooth development by interacting with ectodermal and mesodermal cells that give rise to dentofacial structures[22]. For example, animal studies have demonstrated that the presence of Alcohol can lead to alterations in the basal layer of the tooth germ epithelium/inner enamel epithelium, decrease calcification of the dentin matrix, and slow down cell differentiation in the tooth germ as well as eruption[23-25]. Moreover, human studies on PAE have revealed dental alterations, malocclusions, agenesis, and twisted teeth[26]. More studies are required to assess the prevalence and significance of dental caries and enamel structural anomalies in this population[9,27]. In addition to its effects on odontogenic cells, Alcohol has also been shown to disrupt cell signaling, which may lead to cleft lip with or without cleft palate or cleft palate only[26,28]. One mechanism through which Alcohol disrupts signaling pathways is by suppressing the Wnt signaling pathway, and indeed Alcohol interacts with the gene expression of Wnt10a by reducing its expression in the craniofacial region and around the pharyngeal cavity[29].

While various craniofacial defects have been associated with PAE, and Alcohol can negatively affect tooth development as seen in FASD, the mechanism through which these processes occur is still largely unknown[7-9,22]. Due to the complexities and limitations of PAE in human research, we have turned to the use of zebrafish as animal models[30-32]. Zebrafish (*Danio rerio*) has emerged as a popular and viable model organism in biological research, offering reproducible experiments with genetic and environmental control[33,34]. Its advantages include rapid development, external fertilization, easy maintenance, and evolutionarily conserved Alcohol metabolizing genes[35-37]. Zebrafish share genetic and physiological similarities with humans, with 70% of functional genes related to human diseases[38,39]. Despite differences in morphology and histology from mammals, the transparency and rapid development of zebrafish embryos facilitate observation and experimentation[40]. Thus, zebrafish can model human development and allow researchers to study the teratogenic effects of Alcohol[37]. Our lab has previously published the effect of Alcohol on zebrafish tooth development[41]. Key findings demonstrated that embryonic Alcohol exposure caused abnormal tooth formation, defects in palatal cartilage development and differentiation, and disruptions in the patterning of melanocytes, suggesting the possible teratogenic effects of Alcohol on neural crest cell-derived structures[41]. However, the mechanisms leading to such phenotypic changes require further investigation as they may result from Alcohol-induced changes, Alcohol and gene interactions, or activation or inhibition of complex metabolic mechanisms in cells[41]. In this study, we aim to investigate the Alcohol and Wnt signaling pathway interaction in zebrafish tooth development at the histological level.

MATERIALS/METHODS

Zebrafish Rearing and Breeding

Wild-type zebrafish (WT-AB) were maintained in the water parameter regulated by the Tecniplast rack system at the Bannatyne campus, University of Manitoba, and the zebrafish breeding colony was originally purchased from The Hospital for Sick Children (SickKids), University of Toronto. The fish colonies were maintained according to the established institutional guidelines. Adult zebrafish were fed a diet of Gemma 300–supplemented live shrimp and maintained on a 14/10 day/night cycle. Embryos were obtained from natural spawning. The eggs were transferred into a clean Petri dish containing embryo media and raised in an incubator at 28.5˚C for five days. At 5 days post fertilization (dpf), they were transferred into the larval-rearing nursery tanks of the rack system.

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Alcohol and Wnt pathway modulators Treatment and Embryo Fixation

For this study, samples were treated with five different chemicals, including 1% Alcohol (Cat. No. HC13001GL, Fisher Scientific, USA), 2 mM Lithium chloride (LiCl, Cat. No. 866405-64-3, TCI, USA), 10 nM WNT-C59 (Cat. No. 500496, Sigma Aldrich, USA), and a combined treatment of 1% Alcohol with 2 mM LiCl and 1% Alcohol with 10 nM WNT-C59 at the age of 10 hours post fertilization (hpf). The concentrations used were previously determined to be effective in fish research[42,43]. After 12 hr., the treatments were terminated, and the embryos were washed several times in fish raring water and raised according to the standard operating procedures. At different time points, embryos were euthanised by 1% tricaine methanesulphonate (MS222) (Cat. No. 118000500; Acros Organics, USA) and fixed overnight in 4% paraformaldehyde (PFA), then stored in phosphate-buffered saline (PBS) for analysis.

Whole-Mount Double Staining

Acid-free double cartilage and bone staining was performed for tooth analysis. Standard protocol for Alcian blue and Alizarin red staining was used for the experiment[46]. All specimens were processed through an ascending series of glycerol in 1% KOH and then transferred to the storage solution (100% glycerol)[47].

Electron Microscopy (EM)

6 dpf zebrafish were treated with 1% Alcohol, 2mM LiCl, 10nM WNT-C59, and 1% Alcohol combined with 2mM LiCl or 10nM WNT-C59. Fixed fishes were placed in the fixative of 3% Glutaralde in 0.1M Sorensen's buffer. The samples were prepared and sectioned, for toluidine blue and TEM sections. Images of the morphology, degree of enameloid and dentin mineralization, and pulp cells of the functional tooth and tooth germs were assessed.

Wnt10a Immunofluorescence Protocol for Whole-Mount Staining of 10dpf Zebrafish

The above treated 10 dpf zebrafish were processed for whole mount immunofluorescence staining with Wnt10a antibodies according to standard protocol with the following modifications. The PBS was replaced with PBS-Tween (0.1 Tween 20) and stored in 4°C overnight. The samples were rinsed with distilled water for less than 1min. and stored in acetone in -20°C for 20mins to be fixed. They were rinsed again with distilled water for 1min. Samples were transferred into 1% DMSO/1% TritonX in PBS (PBSDT) in room temperature (RT) overnight to be perforated, allowing the infiltration of antibodies later on. PBSDT was replaced with 5% Normal Goat Serum in PBSDT blocking solution for 1h. Tubes with positive control, Alcohol, LiCl, WNT-C59, Alcohol + LiCl, and Alcohol + WNT-C59 were incubated with primary Wnt10a antibodies (1:1000 dilution in PBSDT), and negative control was kept in PBSDT. All samples were stored in 4°C for 5 days. They were then washed 8 times (approximately 15mins) with PBSDT and incubated with secondary Alexa488 antibodies (1:800 dilution in PBSDT) for 3 days in 4°C on a shaker. Over labelled samples were washed with PBS-Tween for an additional 15mins. Samples were stored in 35% glycerol in PBS-Tween in 4°C.

All immunostained 10 dpf zebrafish samples were dissected with fine glass/tungsten needles to expose the pharyngeal tooth bearing regions. They were then observed in PBS-Tween on a fluorescence microscope with the ZEN Microscopy Software.

Whole-Mount In Situ Hybridisation (WMISH)

Probes were prepared according to the manufacturer's instructions (DIG RNA Labeling kit, SP6/T7, Cat. No. 11175025910; Roche). The probe strength was detected using the dot blot techniques according to the standard procedure [44]. WMISH was performed on 48 hpf wild-type zebrafish embryos[45]. After incubating in Hyb (-) solution, samples were exposed overnight to *Wnt10a* and *Wnt10b* probes in Hyb (+) solution at 70 °C. Colourimetric detection of RNA was achieved using NBT/BCIP staining, and images were captured using a stereomicroscope in a 50% Alcohol and 50% glycerol solution (Zeiss Discovery V8).

Tooth measurements

The above Alcohol and Wnt modulator treated 15, 20, 25, and 30 dpf zebrafish were dissected using a Nikon-SMZ 10A dissecting microscope to expose the lower pharyngeal jaw. The tooth number counts and measurements were carried out according to the established protocols.

Statistical Analysis

The data were subjected to the independent-samples t-test, one-way analysis of variance (ANOVA), Tukey's pair-wise comparison, and chi-square analysis using SPSS Statistics version 21 (IBM Corp., USA). A P-value < 0.05 was considered statistically significant.

RESULTS

Differences of Tooth Mineralization between Control and Chemical Treatment Samples in 15, 20, 25 and 30 dpf

The acid-free double-stained tooth-bearing pharyngeal jaws of zebrafish and stereomicroscope observation of color intensity was performed to study the effect of different chemicals on tooth mineralization (Fig. 1). The Alcohol-treated samples showed a reduction in the intensity of alizarin red staining at 15 and 20 dpf compared to control (Fig. 1 E, F), while it was the same at 25 and 30 dpf (Fig. 1 G, H). The

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reduction in color intensity indicates teeth were not fully mineralized until 25 dpf. More intensity reduction was observed in LiCl-treated (Fig. 1 I, J) and Alcohol + LiCl-treated samples (Fig. 1 M, N) compared to control at 15 and 20 dpf. These results showed that tooth mineralization was not complete until 25 dpf in these two treatment groups. In WNT-C59-treated samples, the color intensity reduction was observed at 15, 20, and 25 dpf (Fig. 1 Q, R, S), and tooth mineralization started to complete at 30 dpf (Fig. 1 T) compared to control. In the Alcohol + WNT-C59 treatment, alizarin red was scarce and limited to the tip of the teeth at 15 and 20 dpf (Fig. 1 U, V). At 25 dpf, tooth mineralization was observed in most of the teeth but was not completed at 30 dpf (Fig. 1 W, X).

Figure 1

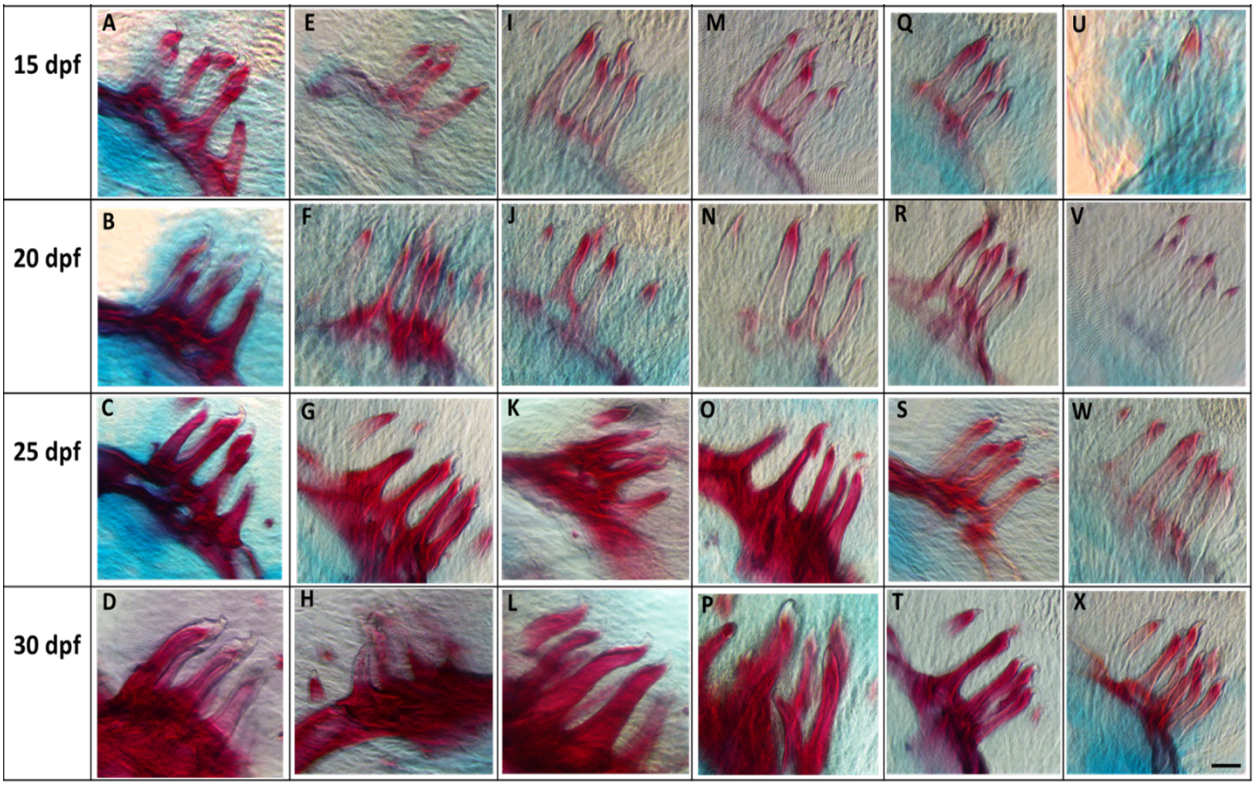

Figure 1: Acid-free double-stained tooth-bearing pharyngeal bones of zebrafish at 15, 20, 25, and 30 dpf. (A-D) Control samples that show tooth-bearing lower pharyngeal bones with six teeth in each bone. These teeth are unicuspid and directly attached to the underlying bone. The teeth are fully mineralized at this stage. (E–H) Samples exposed to 1% Alcohol, (I-L) 2mM LiCl, (M-P) 1% Alcohol + 2mM LiCl, (Q-T) 10nM WNT-C59, and (U-X) 1% Alcohol + 10nM WNT-C59 at 10 hpf show malformed and hypo-mineralized teeth. Scale bar: 20 µm.

Analysis of Tooth Number between Control and Chemical Treatment Samples in 15, 20, 25, and 30 dpf Zebrafish

By counting the attached teeth in the acid-free double-stained sections of the pharyngeal jaw at each time points, we found that Alcohol-treated samples had significantly less teeth than the control at 15 dpf (Fig. 2). Interestingly, while hypodontia was observed at 15 dpf in Alcohol-treated samples, tooth numbers at 20, 25, and 30 dpf (Fig. 2) were similar to the control. An increase in tooth number was observed in the LiCl treatment group at 25 dpf, while this parameter was significantly less than the control at 15 and 20 dpf (Fig. 2). This finding revealed that LiCl may regulate the tooth number. The Alcohol + LiCl treatment group showed a significant decrease in tooth number compared to the control at 30 dpf but not at 15, 20 and 25 dpf (Fig. 2). Notably, the WNT-C59 treatment decreased the tooth number significantly at 25 and 30 dpf (Fig. 2), while the Alcohol + WNT-C59 samples had decreased tooth numbers at all time points compared to the control, making it the most severely affected (Fig. 2).

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Figure 2

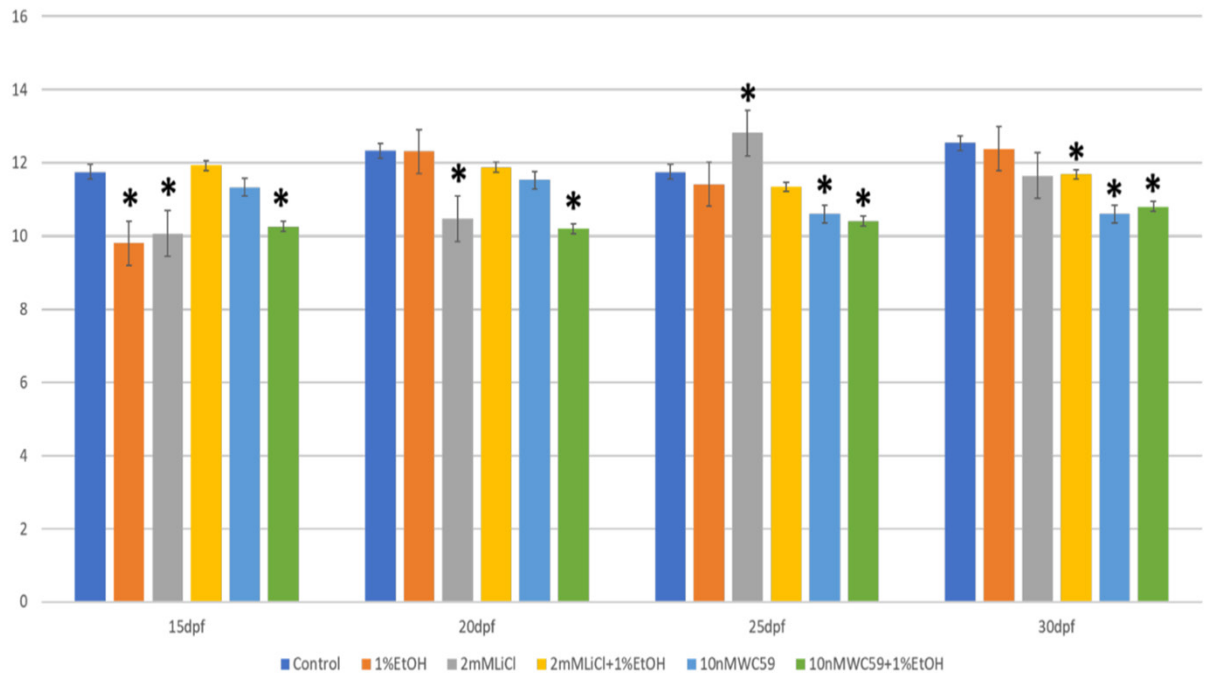

Figure 2 : Comparing tooth number between the control and different chemically treated samples at 15, 20, 25, and 30 dpf. Asterisks show $P<0.05$.

Analysis of Toluidine Blue Staining-Functional Teeth

Erupted functional teeth are directly attached to 5th ceratobranchial arch in zebrafish. The hard tissues and pulp of the different experimental groups can be visualized by toluidine blue stain, which has a high affinity for acidic tissue components as well as glycosaminoglycans in mineralized tissues[48]. Compared to the control (Fig. 3 A), all treatment groups presented a larger pulp with increased vacuoles. Significant changes in the tooth size, shape, and stain intensity of hard tissues can be seen across each group. Alcohol-treated samples showed lighter staining of cusp tips (Fig. 3 B). In the LiCl group (Fig. 3 C), the stain intensity in the functional tooth was comparable to the control, though a wider and longer pulp with increased vacuoles as well as a change in tooth shape can be appreciated. When LiCl was combined with Alcohol (Fig. 3 D), however, the functional tooth became larger while maintaining a similar shape as the control. The staining in WNT-C59 samples (Fig. 3 E) was less intense at the cusp tip. When WNT-C59 was combined with Alcohol (Fig. 3 F), the tooth shape was significantly changed and blunted. Furthermore, the stain intensity in hard tissues was decreased. In all the chemically treated groups, mineralization was disrupted, resulting in either a thin collar enameloid or an overall less intense staining in hard tissues. More notably, the attachment to bone was interrupted in some cases (Fig. 3 B, C, D, F).

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Figure 3

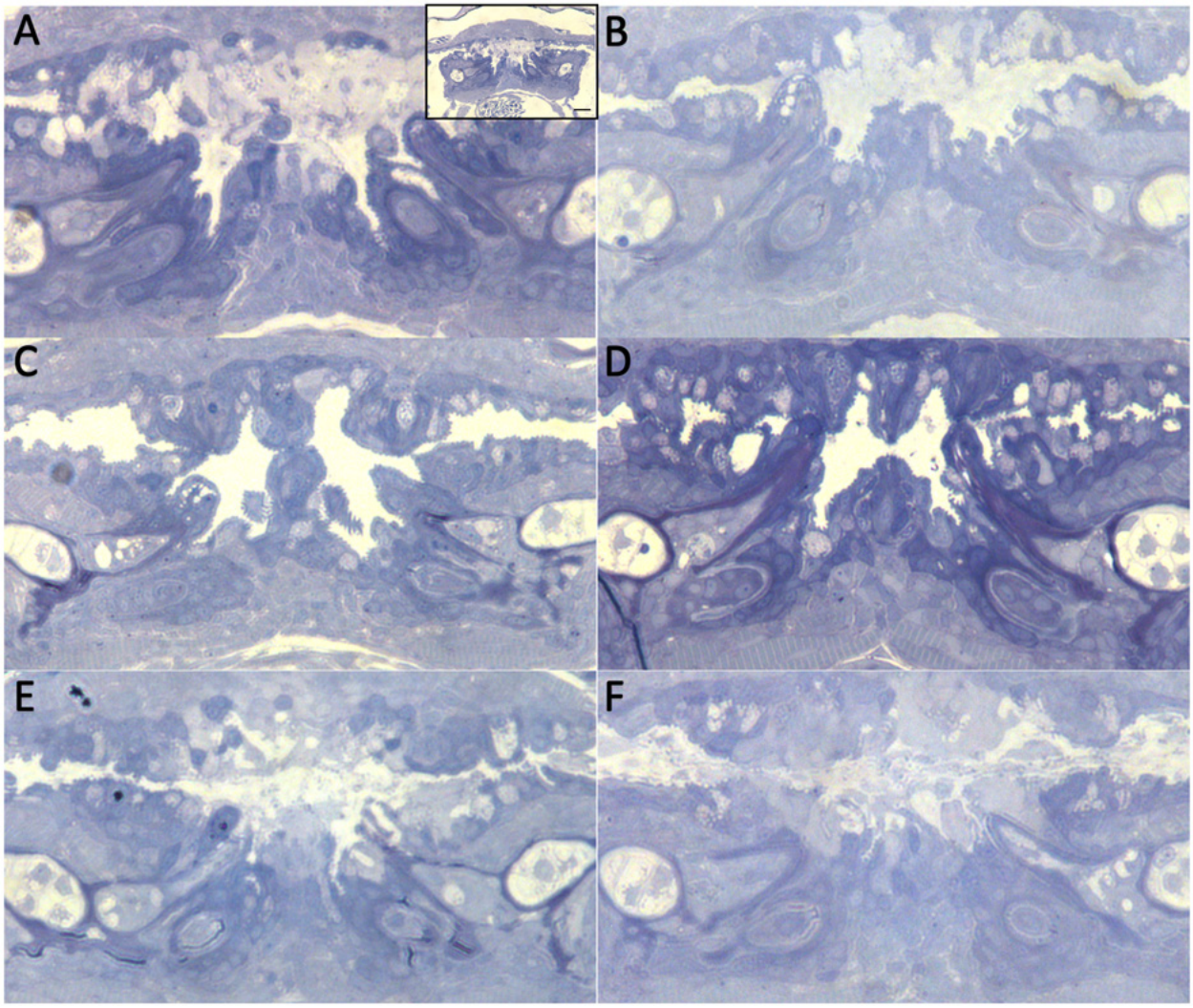

Figure 3: Toluidine blue cross sections of functional tooth (bracketed) and tooth germs (circled) of zebrafish at 6 dpf. (A) Control sample with minimal spaces in the pulp and high intensity staining in hard tissues. Samples exposed to (B) 1% Alcohol, (C) 2mM LiCl, (D) 1% Alcohol + 2mM LiCl (E) 10nM WNT-C59, (F) 1% Alcohol + 10nM WNT-C59 show altered pulp density, tooth size and shape, and stain intensity in hard tissues. pb: pharyngeal bone, p: pulp, d: dentin, e: enameloid, asterisk marks interrupted tooth attachment to bone, arrowhead marks vacuoles/matrix vesicles in pulp. Scale bar: 1000nm. A-F images were enlarged for clarity.

Analysis of Toluidine Blue Staining and EM Images-Developing Tooth Germs

Cross sections of the pharyngeal region in 6 dpf zebrafish further allowed us to visualize the developing tooth germs. Additional analysis of tooth germs under EM permitted us to observe the hard tissue mineralization pattern and layers. The tooth germs in untreated fish samples showed a double layer epithelial cells surrounding the dental papilla (Fig. 4 A). At higher magnification revealed a high-density mineralization front with an intense mineralized outer enameloid (Fig. 4 C). The Alcohol-treated samples had a similar likeness under the light microscope, though the tooth germ sizes were decreased while the dental papilla sizes were increased. Furthermore, the tooth germs were more numbered compared to the control (Fig. 4 B). EM images showed differences including a thicker predentin and a thicker mineralization front of moderate density (Fig. 4 D). A notable feature of LiCl-treated samples was an increased proliferation and disorganization of epithelial cells along with increased number of tooth germs of comparable size to the control (Fig. 4 E). This disorganized pattern was also seen in the thicker mineralization front (Fig. 4 G). The mineralization in the periphery was significantly denser, while the predentin in the innermost layer was relatively thinner. Similarly, Alcohol + LiCl samples showed increased epithelial proliferation and tooth germ numbers but of smaller size compared to control (Fig. 4 F), having a similar likeness to Alcohol-treated samples. This attribute was consistent in the EM sections with a slightly lower density mineralization front (Fig. 4 H). The tooth germs in WNT-C59-treated samples were scattered, smaller, and more numerous (Fig. 4 I). A closer look revealed a dense enameloid

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periphery and a large mineralization front of very low density (Fig. 4 K). Consistently showing the most significant changes, the combined Alcohol and WNT-C59 treatment led to considerably smaller tooth germs with a single layer epithelial cells surrounding the dental papilla. Epithelial proliferation was also disrupted, leading to more scattered cells, which may appear to increase tooth germ numbers (Fig. 4 J). Furthermore, its innermost layer of predentin around the pulp was the thinnest among all groups. Within the low-density mineralization front, an irregular mineralization pattern with few protein particles and a lower density enameloid periphery can be observed (Fig. 4 L).

Figure 4

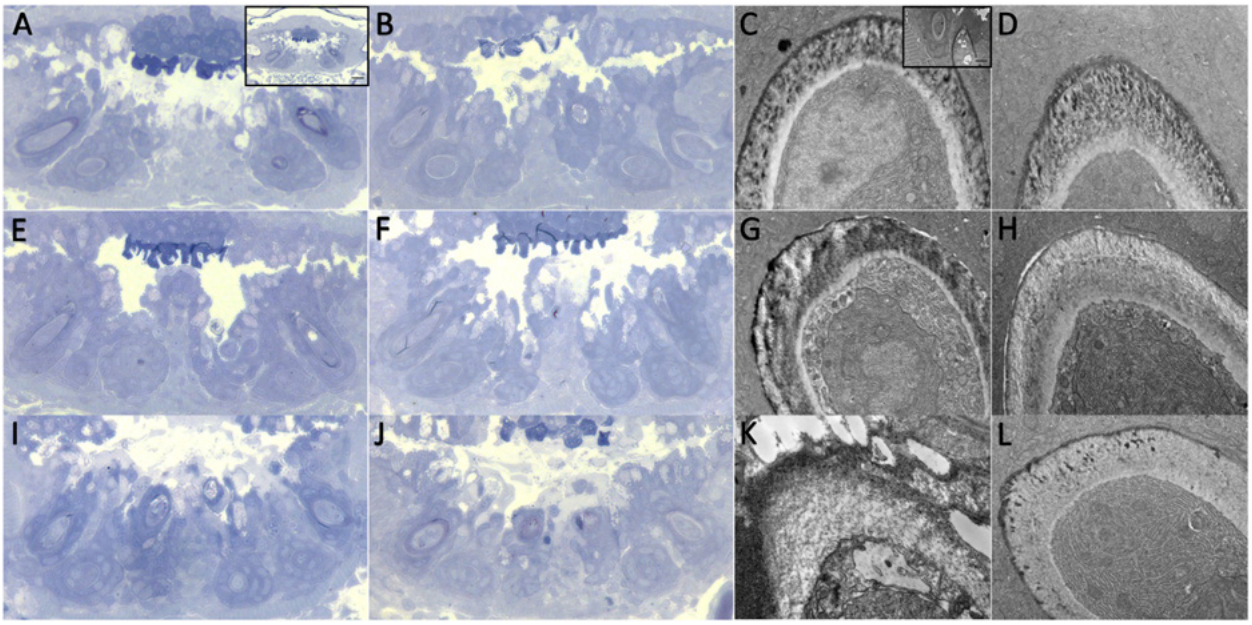

Figure 4: Toluidine blue and EM cross sections of tooth germs (circled) of zebrafish at 6 dpf. (A, C) Control sample with double layer epithelial cells surrounding dental papilla and a high-density mineralization front with a highly mineralized outer enameloid. Samples exposed to (B, D) 1% Alcohol, (E, G) 2mM LiCl, (F, H) 1% Alcohol + 2mM LiCl, (I, K) 10nM WNT-C59, (J, L) 1% Alcohol + 10nM WNT-C59 show altered epithelial cell organization, tooth germ size and numbers, as well as mineralization pattern and density in hard tissues. dp: dental papilla, e: enameloid, p: pulp, pd: predentin, arrow marks hard tissues, bracket marks mineralization front. Scale bar: 1000nm (toluidine blue), 6 □m (EM). A-L images were enlarged for clarity.

WMISH

We utilised WMISH to evaluate the impact of different treatments on the expression of the *Wnt10a* and *Wnt10b* genes in zebrafish larvae. We observed *Wnt10a* and *Wnt10b* expression, denoted by purple colouration, in the developing craniofacial region and pharyngeal cavity of 48 hpf larvae (Fig. 5). The negative control sample, without the *Wnt10a* and *Wnt10b* probes, showed no such colouration (Fig. 5 A). Specifically, the LiCl-treated sample demonstrated increased expression (Fig. 5 F, G), while the other treatments led to a reduction, underlining the influence of the treatments on Wnt signaling genes in the context of tooth development (Fig. 5 B, C, D, E, H, I, J, K, L, M).

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Figure 5

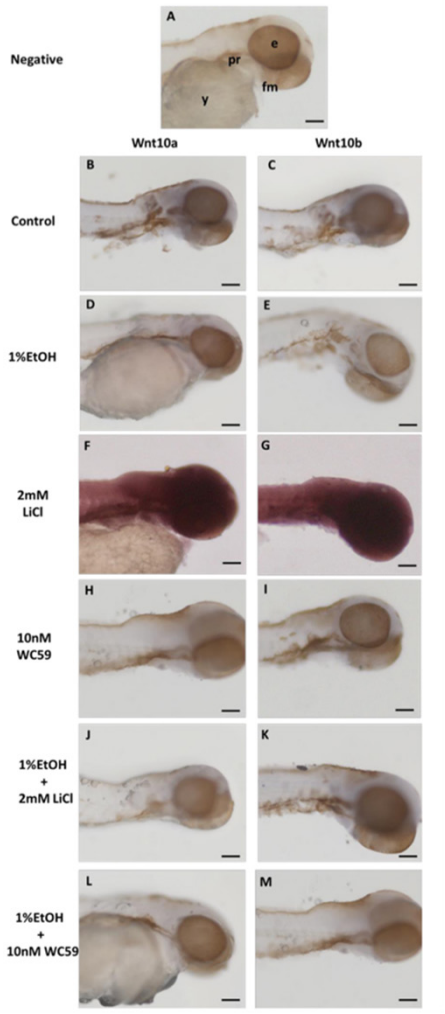

Figure 5: Whole-Mount in situ Hybridization of 48 hpf zebrafish for Wnt10a and Wnt10b probe detection around the pharyngeal cavity and developing craniofacial regions. (B, D, F, H, J, L) Samples of Wnt10a probe. (C, E, G, I, K, M) Samples of Wnt10b probe. (A) Negative control, (B, C) positive control, (D, E) 1% Alcohol, (F, G) 2mM LiCl, (H, I) 10nM WNT-C59, (J, K) 1% Alcohol + 2mM LiCl, (L, M) 1% Alcohol + 10nM WNT-C59. e: eye, pr: pharyngeal region, fm: future mouth, y: yolk sac. Scale bars: 50 µm.

Analysis of Wnt10a Immunofluorescence in Dissected 10 dpf Zebrafish

Using Alexa488 antibodies against Wnt10a and exposing the zebrafish pharyngeal region, we could visualize the expression of this gene via immunofluorescence in the tooth-bearing areas. All treatment groups excluding the negative control, which provided baseline fluorescence (Fig. 6 A), were incubated with Wnt10a antibodies and showed varying degrees of fluorescence intensity. The positive control showed fluorescent tooth-bearing regions and pharyngeal bones (Fig. 6 B), in which other Wnt genes have been reported to be expressed (https://www.sciencedirect.com/science/article/pii/S0925477310000833). This fluorescence was strongly increased in the LiCl-treated sample, revealing the most intense fluorescence level out of all the treatment groups (Fig. 6 D). Contrarily, the remaining treatment groups demonstrated either a reduced or comparable fluorescence intensity as the negative control (Fig. 6 C, E, F, G).

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Figure 6

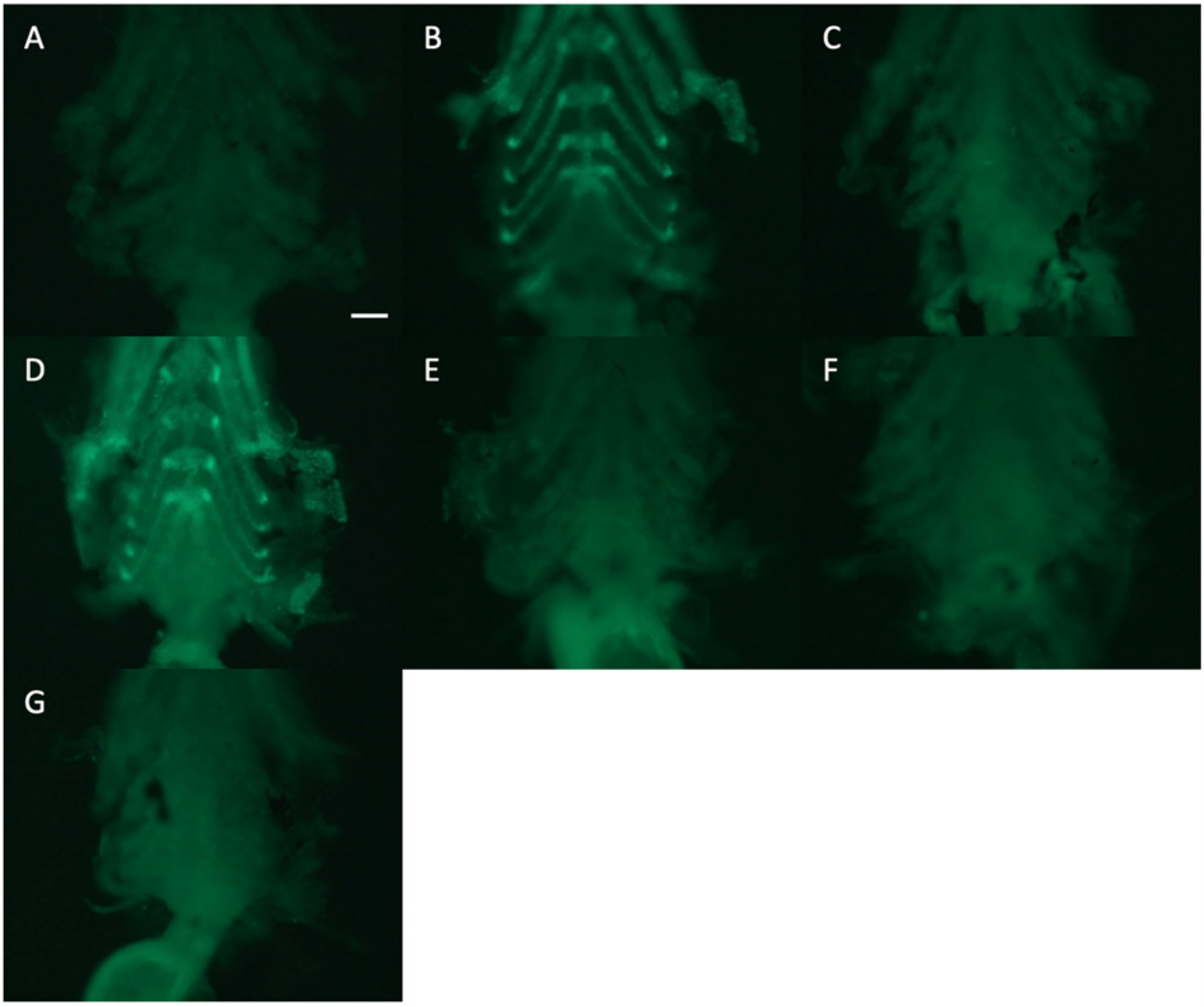

Figure 6: Whole-Mount Immunohistochemistry of 10 dpf zebrafish in the pharyngeal tooth-bearing regions. (A) Negative control, (B) positive control, (C) 1% Alcohol, (D) 2mM LiCl, (E) 1% Alcohol + 2mM LiCl, (F) 10nM WNT-C59, (G) 1% Alcohol + 10nM WNT-C59. Arrowheads indicate pharyngeal bone with teeth. Scale bar: 50 μm.

DISCUSSION

PAE has detrimental effects on various organs and body systems, including teeth[15,41]. While mechanisms leading to presentations of various tooth anomalies are not known, it has been hypothesized that Alcohol may act directly or indirectly on cells by interacting with cell signaling pathways important in tooth development[41]. In this study, we utilized zebrafish treated with Alcohol, LiCl (Wnt activator), and WNT-C59 (Wnt inhibitor), as well as combinations of Alcohol with the Wnt modulators to investigate the mechanisms by which Alcohol interacts with the Wnt signaling pathway to impart the tooth defects seen in individuals with PAE.

Whole mount staining of Alcohol groups revealed a reduced stain intensity of alizarin red (Fig. 1 E-H) and toluidine blue (Fig. 3 B). This indicates a reduced production of calcium and glycosaminoglycans, to which alizarin red and toluidine blue bind to, respectively[46,48]. Furthermore, our results showed a lower density mineralization front in developing tooth germs (Fig. 4 D) and a delayed point of complete mineralization at 25 dpf (Fig. 1 E-H). Contrarily, untreated zebrafish samples begin mineralization in their first pair of teeth at 82 hpf and attain full mineralization at 4-6 dpf[49-51]. Notably, these observations are consistent with the existing literature documenting the negative impact of *in vivo* PAE on the secretory function of ameloblasts and dentin matrix formation[9,22]. Therefore, we can infer that Alcohol has an inhibitory effect on tooth mineralization, thereby delaying and decreasing the overall degree of mineralization in functional teeth. Consequently, this may disrupt their attachment to the pharyngeal bone (Fig. 3 B, C, D, F), which correlates with early tooth exfoliation[41]. This inhibitory effect further extends to the

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sizes of tooth germs and functional teeth. Our previous study reported Alcohol-treated samples showing straight tooth cusps with a decreased tooth height and width[41]. Similarly, the tooth numbers at 15 dpf were also reduced (Fig. 2). A return of tooth numbers to normal levels at subsequent time points (Fig. 2) supports the hypothesis that adverse effects of Alcohol are especially significant in first-generation teeth and gradually decrease through successive tooth replacement cycles[44,52].

The Wnt signaling pathway is of particular importance in the early tooth development process, and mutations of this pathway result in various tooth anomalies[17-19]. Indeed, experimentation with WNT-C59, showed a greater inhibitory effect than Alcohol alone. We report an increased reduction (Fig. 3 E) and delay in functional tooth mineralization, only beginning to attain complete mineralization at 30 dpf (Fig. 1 Q-T). Further supporting this finding, the developing tooth germ displayed a thick mineralization front of very low density (Fig. 4 K). Therefore, WNT-C59 may suppress ameloblast secretion as well. Interestingly, the histological results of all chemically treated groups revealing enlarged pulps (Fig. 3) may provide insight into our previous finding of reduced cusp length to tooth length ratio in WNT-C59-treated samples. Finally, the noticeable hypodontia at 25 dpf and 30 dpf (Fig. 2) as well as a study on the chronological difference in tooth length and width indicate that the inhibitory role of WNT-C59 becomes more pronounced with successive tooth replacement cycles. Our experimentation with LiCl revealed an overall increase in cellular activity and Wnt gene expression (Fig. 5 F, G, Fig. 6 D). Continuous activation of the Wnt signaling pathway led to a disordered proliferation of epithelial cells (Fig. 4 E). This correlates with an observation by Handrigan et al. who stated that the dental epithelial cell proliferation may be promoted by canonical Wnt signaling, thus enabling tooth replacement in snakes[53]. Furthermore, a gradual increase in tooth number to hyperdontia at 25dpf in LiCl-treated samples (Fig. 2) is consistent with a study that observed an eruption delay in multiple teeth after treating cichlid embryos with LiCl[54]. Interestingly, while LiCl functional teeth also showed enlarged pulps (Fig. 3 C), the cusp length to tooth length ratio was increased instead. This indicates that the increase in tooth length was in fact the result of increase in cusp length. Indeed, EM analysis of LiCl-treated tooth germs revealed a haphazard high-density mineralization front and a significantly denser enameloid periphery (Fig. 4 G). Thus, we can infer that LiCl acts as an activator by stimulating the epithelial and odontoblast-like dental hard tissue secreting cells and augmenting enameloid secretion. Previous studies found the role of Wnt signaling in controlling dentin thickness, and Wnt10b specifically regulating odontoblast differentiation and expression of noncollagenous dentin proteins[55-57].

Our results from the combined treatments of Alcohol and Wnt modulators conclude that there is an interaction between Alcohol and the Wnt signaling pathway in PAE tooth development. With the inhibitory effect of Alcohol and the activating effect of LiCl, one would expect the combined treatment to present an average of the two. However, these samples displayed a resemblance to the Alcohol-treated zebrafish. An explanation for this manifestation could be that Alcohol increases Tyr phosphorylation of GSK3b, which promotes the degradation of β-catenin, a key downstream Wnt signaling pathway protein[58]. Another study showed that chronic high-dose Alcohol exposure inhibits Wnt signaling[59]. In fact, the Wnt10a and Wnt10b expression levels were significantly reduced (Fig. 5 J, K, Fig. 6 E). Similarly, Alcohol + LiCl tooth germs revealed a shared likeness with the Alcohol group (Fig. 4 B, F) but with a lower density mineralization front (Fig. 4 H), leading to an increased disruption of tooth mineralization (Fig. 1 M-P). The functional tooth number and cusp shape were found to be affected in later tooth replacement cycles in Alcohol + LiCl samples compared to the early presentation in Alcohol-treated samples (Fig. 2), which explains the relatively larger, yet normally shaped teeth (Fig. 3 D). It is contradicting, however, that the tooth length and width have previously been observed to be increasingly less than the control at all time points.

These parameters were observed to be consistently decreased in Alcohol + WNT-C59 treatment groups. Contrary to the dominant effect of Alcohol in Alcohol + LiCl samples, Alcohol combined with WNT-C59 appears to produce a synergistic inhibitory effect on zebrafish teeth and developing tooth germs, causing more developmental defects than their individual parts. We observed severe hypodontia and hypomineralization at all time points (Fig. 2, Fig 1 U-X). Histologically, an irregular epithelial organization (Fig. 4 J) and mineralization pattern (Fig. 4 L) can be seen. EM analysis revealed not only a consistently thin predentin around the pulp, but also a low-density mineralization front with a hypomineralized enameloid (Fig. 4 L), further indicating that the ameloblast secretion is suppressed. This may provide insight into the possibility that the inhibition resulting from the Alcohol and Wnt signaling pathway interaction affects both dentin and enameloid formation. In fact, a study stated that Wnt10b was mainly detected in the inner dental epithelium at the late bell stage[60]. Furthermore, upon histological examination of the morphology, functional teeth presented with distorted cusps and pulps (Fig. 3 F) as well as reduced cusp length to tooth length ratios. Indeed, misshapen teeth have been observed as one of the adverse effects of PAE[61].

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CONCLUSION

From our research findings and correlating them with previous studies, we can conclude that Alcohol interacts with the Wnt signaling pathway to develop the dental anomalies as seen in PAE. Interestingly, Alcohol has a strong inhibitory role that can neutralize the activating effects of LiCl, and Alcohol and WNT-C59 act together to synergistically disrupt development. Our EM analyses were limited by low magnification; thus, future assessment at a higher magnification would allow for better visualization of odontogenic processes and the collagen matrix, accounting for the temporal and spatial process of tooth development and its gene expression.

Key findings

1. Tooth development, such as tooth number and mineralization pattern, was stimulated by Wnt agonist LiCl and inhibited by Alcohol, Wnt antagonist WNT-C59, and combined treatments.
2. Alcohol and Wnt modulators significantly altered the shapes and sizes of functional teeth and developing tooth germs.
3. Alcohol neutralized the activating effects of LiCl on Wnt expression and synergistically enhanced the inhibitory effects of WNT-C59 on Wnt expression.

Author contributions

DA planned the study, assisted with interpreting the results, and revised and finalized the manuscript. PA conducted the chemical treatment and analysis the bone stained and insitu hybridization data. SC conducted the histological analysis, EM and immunofluorescence drafted and revised the manuscript.

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Conflicting interest

The authors have no conflicting interests.

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