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RESEARCH ARTICLE

Toothpastes for children and their detergent contents affect molecular mechanisms of odontogenesis in zebrafish embryos

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ABSTRACT

We aimed to evaluate how different types of toothpaste (TP) for children affected molecular mechanisms of odontogenesis in zebrafish embryos. Commercially available TPs were selected according to their detergent contents as the cocamidopropyl betaine (CAPB) containing TP (TP1) and sodium lauryl sulfate (SLS) containing TP (TP2). TP3 contained no detergent. Effects of SLS, and CAPB alone were also examined. TP and detergent concentrations affecting development were determined as 750 mg/L and 4 mg/L, respectively. Embryos were exposed to TP1, TP2, TP3, SLS, CAPB, and embryo medium (control) for 72 h post fertilization. Acetylcholinesterase (AChE) activity and oxidant–antioxidant parameters were analyzed spectrophotometrically. Expressions of tooth development genes were evaluated by reverse transcription PCR (RT-PCR). Intraocular distance, lower jaw, and ceratohyal cartilage length were displayed using Alcian Blue staining. *axin2* and *wnt10a* expressions increased in SLS and TP2 groups. *igf2a* and *eve1* expressions decreased in all groups except TP3. *nrOb1* expression decreased in TP1, SLS, and CAPB groups. Oxidant–antioxidant balance was disturbed in all groups except TP3, evidenced by increased lipid peroxidation, nitric oxide. SLS, and CAPB groups were more affected in terms of AChE, glutathione-S-transferase, and superoxide dismutase; perturbations were observed in cartilage structures. Altered expression of tooth development gene *axin2* correlated with *wnt10a*, and with changes in cartilage structures in SLS and TP2 groups. TP3 group presented no disruptions in oxidant–antioxidant balance. Our study shows the availability of externally developing zebrafish embryos in examining the effects of TP' contents on embryogenesis.

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Toothpaste; detergent; sodium lauryl sulfate; cocamidopropyl betaine; zebrafish embryos

Introduction

Oral health has a very important role in the quality of life and the significance of safe, effective, and cost-effective oral hygiene procedures are strongly emphasized. Toothpaste (TP) is the most effective cosmetic and therapeutic agent that is used individually to protect oral and dental health (Harris and Garcia-Godoy 2004). There are many children's TPs on the market and their ingredients and proportions vary according to different age groups. TPs generally contain abrasives, detergents, humectants, sweeteners, therapeutic agents, colorants, binders, and water (Sheen *et al.* 2001). The exact composition of a particular TP varies with each manufacturer. A typical formulation is abrasives 20–40%, water 20–40%, humectants 20–40%, detergents 1–2%, binders 2%, flavors 2%, therapeutic agents 5%, coloring agents, and preservatives 1% (Bulut *et al.* 2020).

Detergents are agents that foam up the paste, and they also have antimicrobial effects (Lippert 2013). Detergents often used in TPs are sodium lauryl sulfate (SLS), sodium lauryl sarcosinate, and cocamidopropyl betaine (CAPB).

SLS, also known as sodium dodecyl sulfate, is the most widely used detergent and has a high ability to pass into tissues. In animal studies, it has been observed that SLS causes a significant increase in epithelial thickness in the histological structure of the cheek epithelium. It also has side effects on the modification of the epithelial barrier in the human oral mucosa. TP containing SLS has been associated with more frequent inflammation and desquamation of oral mucosa (Tadin *et al.* 2019). Also, it has been found to cause changes in oral mucosal proteins and increase blood flow in the gingiva (Tabatabaei *et al.* 2019).

Betaine is a quaternary ammonium compound that does not have skin irritating properties. Betaine is also been shown to reduce the irritating properties of SLS (Cvikl *et al.* 2017). CAPB containing TP is not considered irritating and relieve dry mouth symptoms (Cvikl *et al.* 2015).

The mechanism of tooth formation, eruption, and interaction with its surrounding structures is known as tooth development or odontogenesis. A complicated chemical regulatory system that modulates tooth development has

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been discovered through research on human genetics and mutant animals. This network is mediated by transcription factors and signaling pathways that have undergone evolutionary conservation and are involved in the growth, maintenance, and regeneration of numerous other organ systems in various species (Svandova *et al.* 2020).

Igf2a (insulin-like growth factor-2a) is responsible for the growth in vertebrates and is involved in early neural and cardiovascular system development in zebrafish embryos (Hartnett *et al.* 2010). Wnt glycoproteins are involved in the signaling pathways that play a critical role in organ development and tissue regeneration. Wnt10a is associated with hair, nails, skin-associated structures, and teeth (Doolan *et al.* 2021). Defects in Wnt10a gene have been associated with non-syndromic familial tooth deficiency, non-syndromic cleft palate, crown, and root deformity in molars (Zhang *et al.* 2014, Chu *et al.* 2021). Axin2 gene functions as a negative regulator of Wnt signaling. Axin2 mutation is associated with oligodontia-colorectal cancer syndrome where a predisposition to colon polyps and/or colon cancer is seen with severe oligodontia (Lammi *et al.* 2004). Nr0b1 gene is the first marker involved in tooth development in zebrafish (Powers *et al.* 2009). Eve1 gene is expressed in the epithelial thickening of the first tooth developing in zebrafish and is involved in the differentiation of ameloblasts and enamel organ development (Laurenti *et al.* 2004).

Healthy embryo development is essential for the proper functioning of odontogenesis. The intrinsic apoptotic pathway which is activated as a response to cellular stress is involved in several developmental processes during odontogenesis (Abramyan *et al.* 2021). Reactive oxygen species function as signaling molecules that can either cause cell growth or cell death. Consequently, oxidative stress can impair the growth and development of embryos (Orozco-Hernández *et al.* 2022).

Zebrafish, a freshwater fish that lives in natural waters and rice fields, was first introduced as a model organism in developmental and embryological studies in the 1930s. Today, it has become the most powerful model organism in developmental biology studies as the zebrafish genome and the human genome show significant similarities (Ali *et al.* 2011, Karaman *et al.* 2020). External fertilization and transparent eggs provide convenience during monitoring development for toxicology studies (Karaman *et al.* 2020). Zebrafish have also been used as a model in dental research related to the biocompatibility of dental materials, periodontal diseases, fluorosis, and craniofacial diseases (Meşeli *et al.* 2021).

In our study, commercially available TPs were selected according to their detergent contents as the CAPB containing TP (TP1), SLS containing TP (TP2), and no detergent containing TP3. The effects of SLS, CAPB, and the TPs (TP1, TP2, and TP3) were investigated on development, oxidant–antioxidant status, AChE activity, and the genes related to odontogenesis in zebrafish embryos.

In light of this information, we aimed to evaluate how kinds of TP for children and their detergent contents (CAPB and SLS) affect the molecular mechanisms of odontogenesis in zebrafish embryos. For this purpose, as zebrafish has pharyngeal dentition Alcian Blue staining was performed to evaluate head cartilage (pharyngeal arch) formation, and the expressions of genes related to the molecular pathways of odontogenesis were measured. Oxidative stress parameters were determined to monitor the normal development processes in the embryo. Acetylcholinesterase (AChE) activities and the related locomotor activities of the embryos were also analyzed to assess the development progress of the embryos.

Materials and methods

Maintenance of zebrafish

Wild-type male and female AB/AB Strain zebrafish were maintained in apparently disease-free conditions. Animals were kept in an aquarium rack system (ZebTEC, Tecniplast, Buguggiate, Italy) at 27 ± 1 °C under a light/dark cycle of 14/10 h at the Zebrafish Research Laboratory located at the Department of Basic Medical Sciences, Faculty of Dentistry, Marmara University. They were fed with commercial flake fish food complemented with live Artemia twice a day. Reverse osmosis water that contains 0.018 mg L^{-1} Instant Ocean™ salt was used for all of the experiments (Avdesh *et al.* 2012).

Embryo exposure

Three different types of children's TP were selected according to their different detergent ingredients, CAPB (TP1) and SLS (TP2). TP3 which contains no detergent was selected as the negative control. TP1 and TP2 are both for children +6 years. Also, both of them have the same fluoride level (1450 ppm) as a therapeutic agent. The ingredients of TP1, TP2, and TP3 are given in Table 1.

Range-finding experiments were applied initially to find out the effective concentrations below the lethal concentration 50 (LC50) in the zebrafish embryos. The exposure concentrations for the TP and the detergents were 750 mg/L and 4 mg/L, respectively. Embryos were exposed to TP1, TP2, TP3, CAPB, and SLS solutions in well plates for 72 h post fertilization (hpf). Embryo medium (E3 medium; 5 mM NaCl, 0.17 mM KCl, 0.33 mM $CaCl_2$, and 0.33 mM $MgSO_4$) was used as the blank control. Every 24 h solutions were replaced and dead embryos were discarded. The development of embryos were monitored and documented daily under a stereomicroscope (Zeiss Discovery V8, Göttingen, Germany) (Westerfield 1995). The images of malformations were captured and the percentage of abnormal embryos was counted every 24 h during the

Table 1. Ingredients of the toothpastes.

Toothpastes	Ingredients
Toothpaste 1 (TP1)	Aqua, sorbitol, hydrated silica, glycerin, PEG-6, *cocamidopropyl betaine*, xanthan gum, flavor, sodium fluoride %0.315 w/w (1450 ppm F^-), sodium saccharin, sucralose, titanium dioxide, sodium hydroxide, limonene.
Toothpaste 2 (TP2)	Sorbitol, aqua, hydrated silica, xylitol, PEG-12, cellulose gum, benzyl alcohol, *sodium lauryl sulfate*, sodium fluoride (1450 ppm F^-), flavor.
Toothpaste 3 (TP3)	Xylitol, purified water, glycerin (coconut derived), silica, xanthan gum, potassium sorbate (naturally derived), citric acid.

exposure period. Individual malformations such as axial malformations, pericardial edema, and yolk sac edema were listed. Embryonic mortality and hatching rate were noted on each day. The hatching rate is defined as the ratio of hatching embryos to the living embryos in each well (Üstündağ *et al.* 2022). Since the embryos used were no older than five days old. The Council of Europe (1986), Directive 86/609/EEC or the Marmara University ethics committee did not require a license.

Locomotor activity

The locomotor activity of the zebrafish embryos at 72 hpf ($n = 10$, each group) was evaluated in a blinded manner as described previously (Goody *et al.* 2012). This was performed by placing a 60-mm petri dish containing embryo medium on top of the motility wheel which is on the microscope stage. The motility wheel included concentric circles with the diameters in ascending order: 5 mm, 10 mm, 15 mm, and 20 mm, to allow predetermined distances for zebrafish embryos to swim. Then, by using an embryo poker tool, the zebrafish embryo was positioned in the middle of the motility wheel, and the time it took for an embryo to swim at a predetermined distance was recorded and the average escape response was calculated.

Biochemical assays

For the biochemical assays, 100 zebrafish embryos at 72 hpf were used. They were pooled and homogenized in 1 mL PBS, followed by centrifuging briefly. The supernatant was used for the determination of LPO, NO levels, and superoxide dismutase (SOD), GST, and AChE activities.

Determination of total protein

The levels of total proteins in the samples were determined by the method of Lowry *et al.* (1951). The protein contents in the samples were expressed as mg/dL and used to express the results of the parameters per protein.

Determination of lipid peroxidation

Malondialdehyde (MDA) level, an end product of lipid peroxidation (LPO), as thiobarbituric acid reactive substances was determined by the method of Yagi (1984). The extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} was used and LPO was expressed in terms of MDA equivalents as nmol MDA/mg protein.

Determination of nitric oxide

Nitric oxide (NO) was determined by the method of Miranda *et al.*, which is based on reducing nitrate to nitrite by vanadium (III) chloride (Miranda *et al.* 2001). The colored complex was measured at 540 nm by a spectrophotometer and the results were expressed as nmol NO/mg protein.

Determination of glutathione-S-transferase

The activity of glutathione-S-transferase (GST) was determined based on the spectrophotometric evaluation of the absorbance at 340 nm of the product formed by GSH and 1-chloro-2,4-dinitro-benzenin (CDNB) conjugation (Habig *et al.* 1974).

Determination of superoxide dismutase activity

Superoxide dismutase activity was determined using the method based on the ability of SOD to increase the effect of riboflavin-sensitized photo-oxidation of o-dianisidine. The absorbance of the product was measured in 460 nm by a spectrophotometer and the net absorbance was calculated by measuring absorbances at 0 and 8th minutes of illumination. The results were expressed as U/mg protein, where 1 U of SOD was defined as the amount of SOD needed to inhibit the rate of reduction of cytochrome c by 50% (Mylroie *et al.* 1986).

Determination of acetylcholinesterase activity

Acetylcholinesterase activity in the supernatants was determined using the Ellman (1961) method. In the Ellman method, thiocholine is produced by the action of AChE, which produces a yellow color with 5,50-dithiobis (2-nitrobenzoic acid). The intensity of the yellow product color is proportional to the enzyme activity in the sample and was measured using spectrophotometer at 412 nm (Ellman *et al.* 1961).

Reverse transcription (cDNA synthesis) and quantitative real-time PCR

RNA was isolated from the embryos using RNeasy Mini Kit and QIAcube (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Single-stranded cDNA was synthesized from 1 mg of total RNA using RT2 Profiler PCR Arrays (Qiagen, Hilden, Germany). PCRs were performed using the DNA Master SYBR Green kit (Qiagen, Hilden, Germany). All of the real-time PCR primer products were obtained from Qiagen (Hilden, Germany). The expressions of *nr0b1*, *eve1*, *axin2*, *igf2a*, and *wnt10a* were evaluated by quantitative reverse transcription PCR (RT-PCR) using the Qiagen Rotor-Gene (Hilden, Germany). *β-Actin* was used as the housekeeping gene. Forward and reverse primers used in the study are given in Table 2. Relative transcript levels were calculated using the ΔΔCT method by normalizing the values with the housekeeping gene (Livak and Schmittgen 2001).

Head cartilage staining using Alcian Blue staining

Embryos were stained with Alcian Blue solution for the visualization of cartilage structures using the method based on the previous research (Staal *et al.* 2018). The distance between Meckel's cartilage and ceratohyal was measured as M-CH distance, the distance between the two opercles was

measured as the head wide. Ceratohyal angle was also measured. PTU exposed embryos were washed with E3 at 72 h and fixed in 4% paraformaldehyde (PFA) for 2 h at room temperature. Then, embryos were bleached with 3% H_2O_2 and 0.5% KOH. After bleaching embryos were stained overnight with 0.01% Alcian Blue in 60 mM $MgCl_2$ and 70% ethanol at 4 °C. Subsequently, stained embryos were washed with 3% H_2O_2 and 0.5% KOH for 15 min. Then, embryos were washed with 25% glycerol and 0.1% KOH and stored in 50% glycerol in 0.1% KOH. Embryos were photographed with Stereo V8/ Zeiss microscope (Göttingen, Germany).

Table 2. Forward and reverse primers used in the study.

	Primers (forward/reverse)
igf2a	5′-TACTGTGCCAAGCCGGTGAA-3′
	5′-GGGCCAACAGAATGGATGGG-3′
eve1	5′-CGAGGGAACAGCTGACTCGT-3′
	5′-CACACAGGTTCGGGTCCAGT-3′
nr0b1	5′-GGGGCCATTCTGTTCAACCC-3′
	5′-CTGTCCCCGCGGTGAATCAT-3′
axin2	5′-TTATGCTTTGCACTACGTCCCTCCA-3′
	5′-CGCAACATGGTCAACCCTCAGAC-3′
wnt10A	5′-ATTCACTCCAG-GATGAGACTTCATA-3′
	5′-GTTTCTGTTGTGGGCTTTGATTAG-3′
β actin	5′AAGCAGGAGTACGATGAGTCTG-3′
	5′-GGTAAACGCTTCTGGAATGAC-3′

Results

The representative images of the zebrafish embryos at 24, 48, and 72 hpf are given in Figure 1. Hatching can occur between 48 and 72 hpf in zebrafish embryos and at 48 hpf delayed hatching was observed in TP2 group. Yolk sac edema was observed in the SLS exposed group.

Results of locomotor activities

Locomotor activity was evaluated as average speed, total distance swam, and exploration rate. Average speed decreased in all exposure groups when compared to the control group. Although both CAPB and CAPB containing toothpaste TP1 led to decreased average speeds, average speed of the TP1 group was higher than that of the CAPB group ($p < 0.05$). However there was no significant difference between the SLS group and SLS containing toothpaste TP2 in terms of average speed (Figure 2(A)).

Exploration rate and total distance decreased significantly in all exposure groups except TP3 group (Figure 1(B,C)). There were significant decreases in the exploration rate and total distance swam of the SLS containing toothpaste TP2 when compared to the SLS group ($p < 0.001$ and $p < 0.001$, respectively) (Figure 2(B,C)).

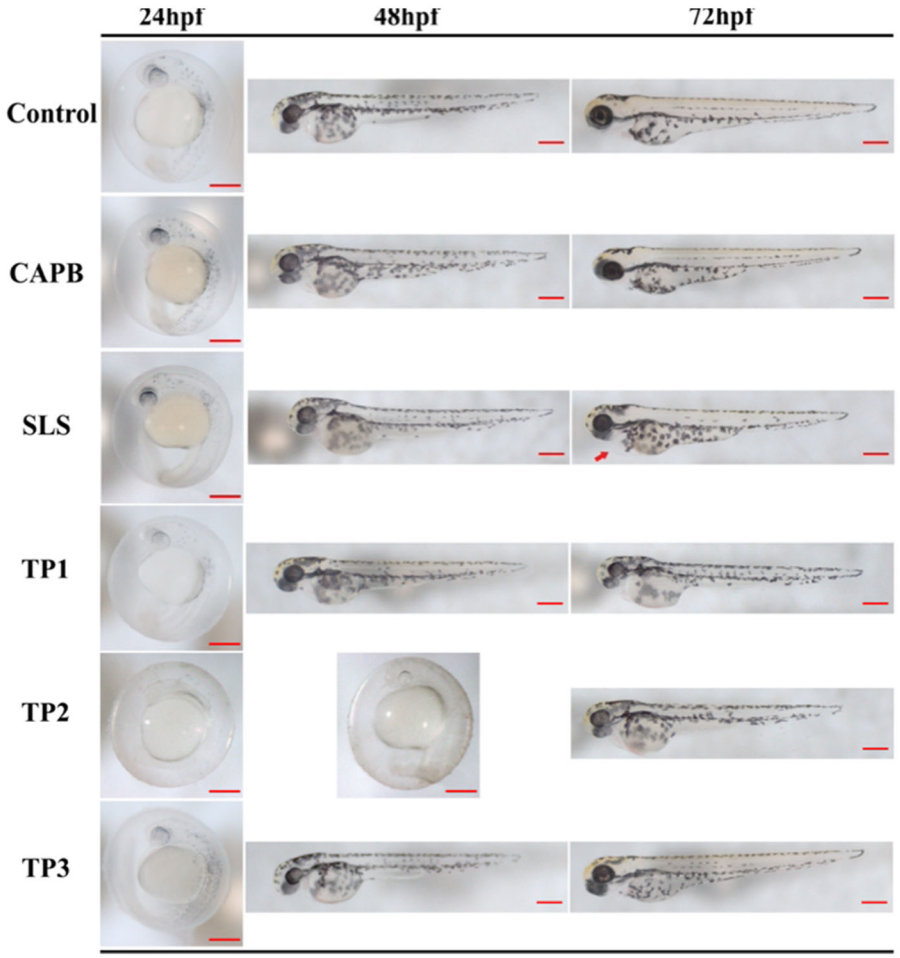

Figure 1. Representative images of the zebrafish embryos at 24, 48, and 72 hpf, given as examples of individual phenotypes observed in the control, CAPB, SLS, TP1, TP2, and TP3 groups. Delayed hatching was observed in TP2 group at 48 hpf and yolk sac edema was observed in the SLS exposed group. Scale bar: 300 μm.

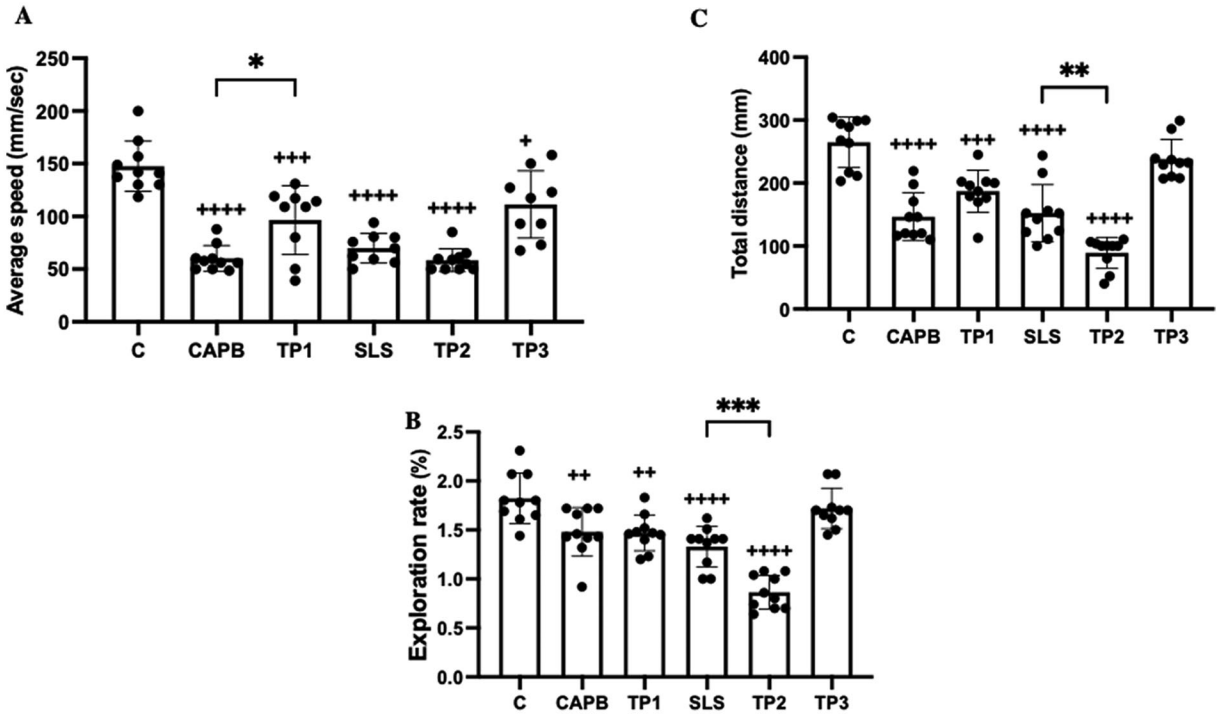

Figure 2. Locomotor activities of the groups. Data are expressed as mean ± SD ($n = 10$ embryos). *$p < 0.05$; **$p < 0.01$; ***$p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; SD: standard deviation.

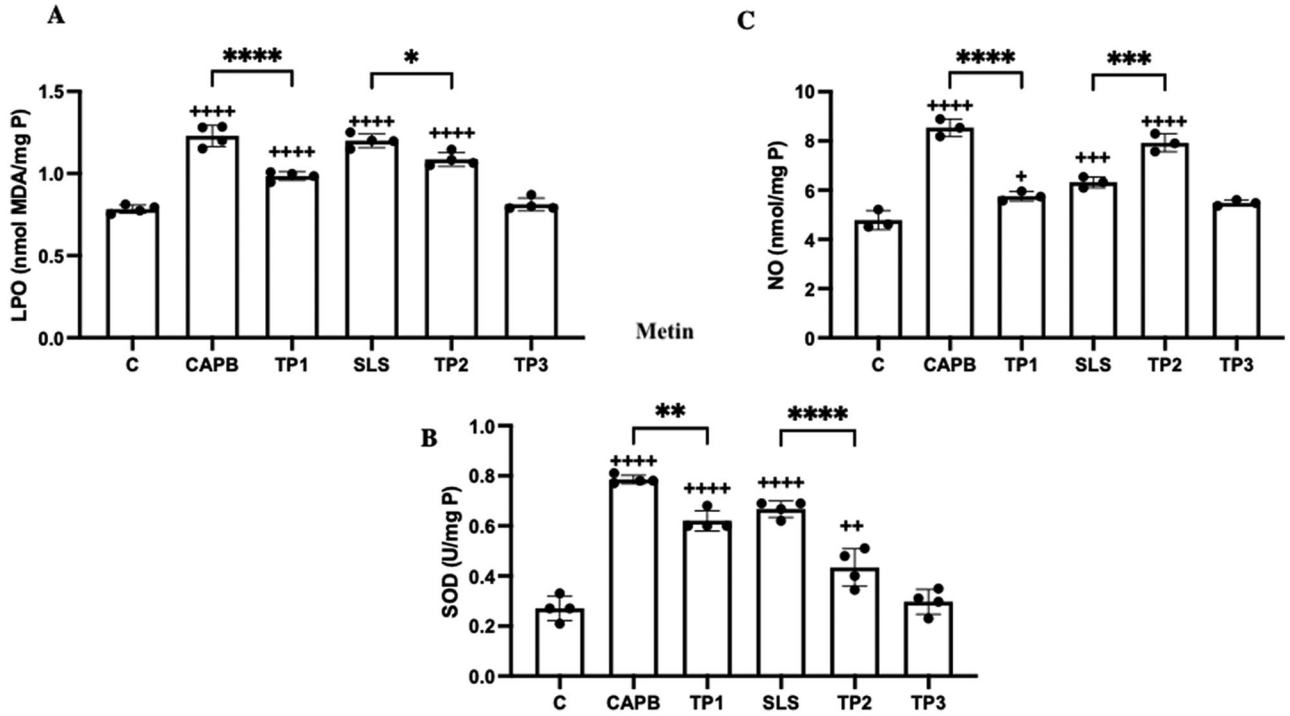

Figure 3. (A) Malondialdehyde (MDA) levels as an index of lipid peroxidation (LPO); (B) superoxide dismutase (SOD) activities; (C) nitric oxide (NO) levels of the groups. Data are expressed as mean ± SD from the three independent experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$; ****$p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; SD: standard deviation.

Results of biochemical analysis

LPO levels as indicated by thiobarbituric acid-reactive substances, increased significantly in all exposure groups except TP3. CAPB containing toothpaste TP1 led to decreased average speeds, average speed of the TP1 group was higher than that of the CAPB group ($p < 0.05$) (Figure 3(A)).

As a first line defense antioxidant SOD activities increased in all exposure groups except TP3. SOD activities of the CAPB and SLS were significantly higher than CAPB containing toothpaste TP1 and SLS containing TP ($p < 0.01$ and $p < 0.0001$, respectively) (Figure 3(B)).

NO is a multifunctional signaling molecule that functions as a neurotransmitter and inflammatory process regulator.

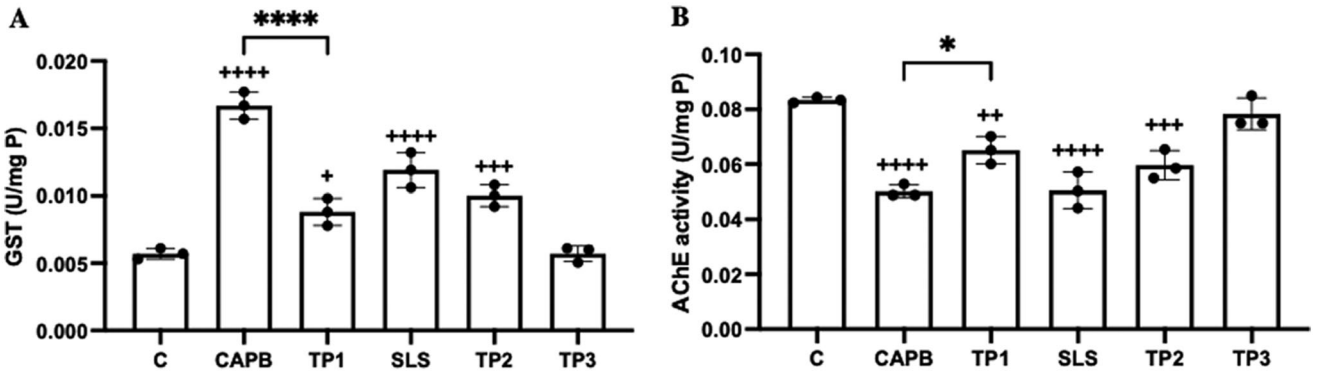

Figure 4. (A) Glutathione-S-transferase (GST) activities of the groups; (B) acetylcholine esterase (AChE) activities of the groups. Data are expressed as mean ± SD from the three independent experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). *$p < 0.05$; ****$p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; SD: standard deviation.

Both CAPB and SLS led to significant increases in NO levels ($p < 0.0001$ and $p < 0.001$, respectively). Among the TPs, both TP1 and TP2 increased NO levels compared to the control group ($p < 0.05$ and $p < 0.0001$, respectively). NO levels of the CAPB group were significantly higher than the CAPB containing toothpaste TP1 ($p < 0.0001$). On the other hand, SLS containing toothpaste TP2 had significantly higher NO levels than the SLS group ($p < 0.001$) (Figure 3(C)).

Glutathione-S-transferases (GSTs) are a type of phase II detoxification enzymes that protect cellular macromolecules from reactive electrophile attack (Townsend and Tew 2003). In our study, GST activities increased significantly in all exposure groups except TP3. CAPB containing toothpaste TP1 had significantly lower GST activity than the CAPB group ($p < 0.0001$) (Figure 4(A)).

In cholinergic synapses, AChE is known to hydrolyze the neurotransmitter acetylcholine (ACh) (Colovic *et al.* 2013). We found decreased AChE activity in all exposure groups except TP3 group. AChE activity of the CAPB group was lower than the CAPB containing toothpaste TP1 ($p < 0.05$) (Figure 4(B)).

Results of gene expression analysis

igf2a is an early gene developmental gene that is expressed highly in midbrain as well as the pharyngeal arch region at 48 hpf (Li *et al.* 2014). In our study, *igf2a* expressions decreased in all exposure groups. *igf2a* expressions of the CAPB and SLS groups were significantly lower than the CAPB and SLS containing toothpastes TP1 and TP2 ($p < 0.0001$ and $p < 0.05$, respectively) (Figure 5(A)).

In our study, *eve1* expression which is required for the initiation and morphogenesis of the first tooth in zebrafish (Laurenti *et al.* 2004), decreased in all exposure groups except TP3. In the CAPB and SLS groups, *eve1* expressions were significantly lower than the CAPB and SLS containing toothpastes TP1 and TP2 ($p < 0.0001$ and $p < 0.01$, respectively) (Figure 5(B)).

WNT pathway genes, in particular WNT10A, are needed for tooth formation in zebrafish (Yuan *et al.* 2017) and *nr0b1* is the earliest gene in the tooth development of zebrafish (Powers *et al.* 2009). Both CAPB and the CAPB containing toothpaste TP1 decreased *wnt10A* and *nr0b1* expressions ($p < 0.0001$; $p < 0.01$ and $p < 0.0001$; $p < 0.0001$, respectively). On the other hand, SLS and SLS containing toothpaste TP2 increased *wnt10A* expressions ($p < 0.0001$ and $p < 0.01$, respectively). TP3 also led to a significant decrease in *wnt10A* expression ($p < 0.01$) (Figure 6(A,C)).

The expression of *axin2,* the regulator of Wnt signaling, decreased significantly in the CAPB and TP3 groups ($p < 0.0001$ and $p < 0.01$, respectively); however, no significant decrease was observed in the CAPB containing toothpaste TP1 group. On the other hand, both SLS and SLS containing toothpaste TP2 increased *axin2* expressions significantly ($p < 0.0001$) (Figure 6(B)).

Although SLS led to a significant decrease in *nr0b1* expression ($p < 0.01$), the SLS containing toothpaste TP2 and TP3 did not change *nr0b1* expressions significantly compared to the control group.

Results of head cartilage staining

When compared with the control group, M-CH length decreased significantly in the CAPB, SLS, and TP1 groups ($p < 0.01$, $p < 0.05$, and $p < 0.0001$, respectively) (Figure 7(A)). CH angle decreased significantly in the CAPB group ($p < 0.05$) and increased significantly in the TP1 and TP2 groups compared to the Control group ($p < 0.01$) (Figure 7(B)). The head wide is the distance between the two opercles, and it was found to be decreased in the TP1 group compared to the control group ($p < 0.0001$) (Figure 7(C)).

Discussion

The toxicity of all compounds used in the oral cavity must be assessed. Organizations responsible for testing dental materials are The American Dental Association (ADA), the Food and Drug Administration (FDA), and the International Organization for Standardization (ISO) (Tabatabaei *et al.* 2019). Although cell culture is one of the key assays used to detect the cytotoxic effects of these compounds, zebrafish embryo toxicity evaluation *in vivo* is an alternative assay to substitute for traditional animal experiments (Ha *et al.* 2021). In our study, zebrafish embryos were used to evaluate the effects of commercially available types of TP for children on

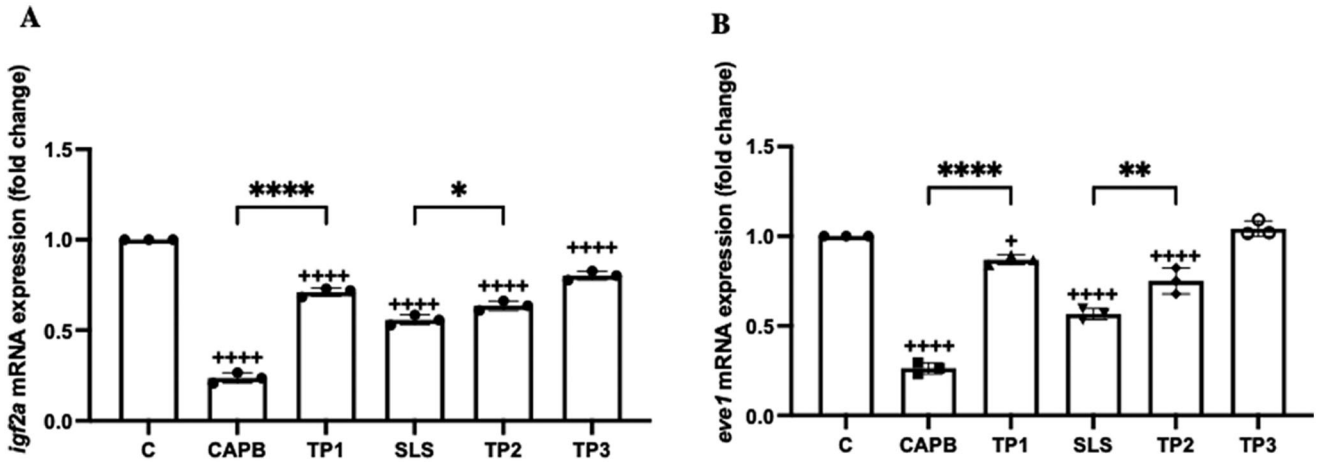

Figure 5. Bar graph presentation of the fold change of (A) *igf2a* and (B) *eve1* (transcripts quantified by RT-PCR). All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). Data presented are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{****}p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; SD: standard deviation.

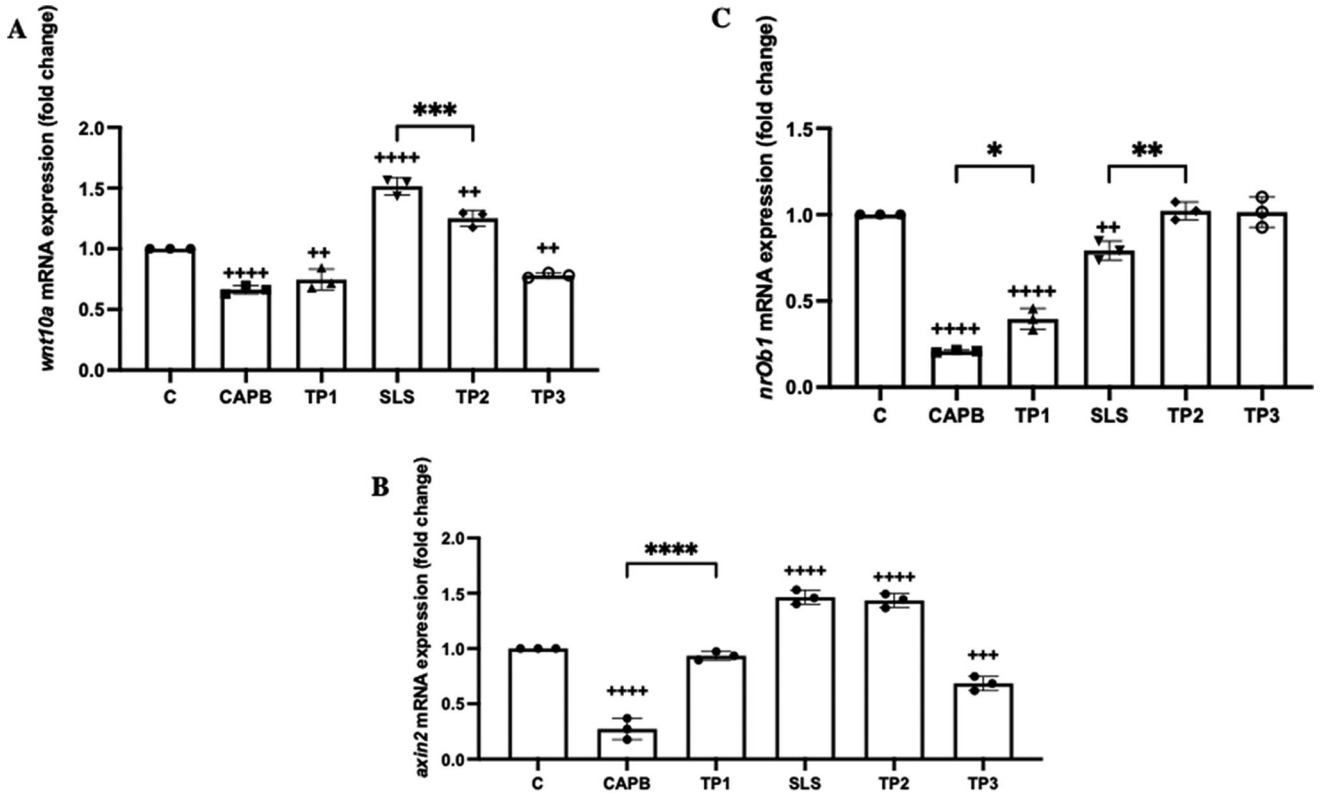

Figure 6. Bar graph presentation of the fold change of (A) *wnt10a*, (B) *axin2*, and (C) *nrOb1* transcripts quantified by RT-PCR. All RT-PCR results are normalized to β-actin, the housekeeping gene, and expressed as change from their respective controls. The average values were obtained from three experiments ($n = 3$, three biological replicates for each group, 50 embryos/pool). Data presented are mean ± SD. $^{*}p < 0.05$; $^{**}p < 0.01$; $^{***}p < 0.001$; $^{****}p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; SD: standard deviation.

the molecular mechanisms of odontogenesis. The individual effects of CAPB and SLS were also determined.

The teeth of zebrafish is restricted to the pharyngeal region. Tooth development starts in the ventral row from 2 days post fertilization (dpf) to 16 dpf, which is followed by the mediodorsal (from 14 dpf to 24 dpf) and dorsal rows (from 24 dpf to 28 dpf) and the dentition completes in 26 days. As our study was composed in zebrafish embryos up to 72 hpf, we performed Alcian Blue staining to evaluate head cartilage (pharyngeal arch) formation (Karaman *et al.*,2020). Decreased M-CH length in the CAPB, SLS, and TP1 groups together with the decreased head width in the TP1 group indicated the impaired development of the pharyngeal arch in these groups.

When evaluating the morphological alterations of zebrafish embryos by exposure to the TPs and their detergent

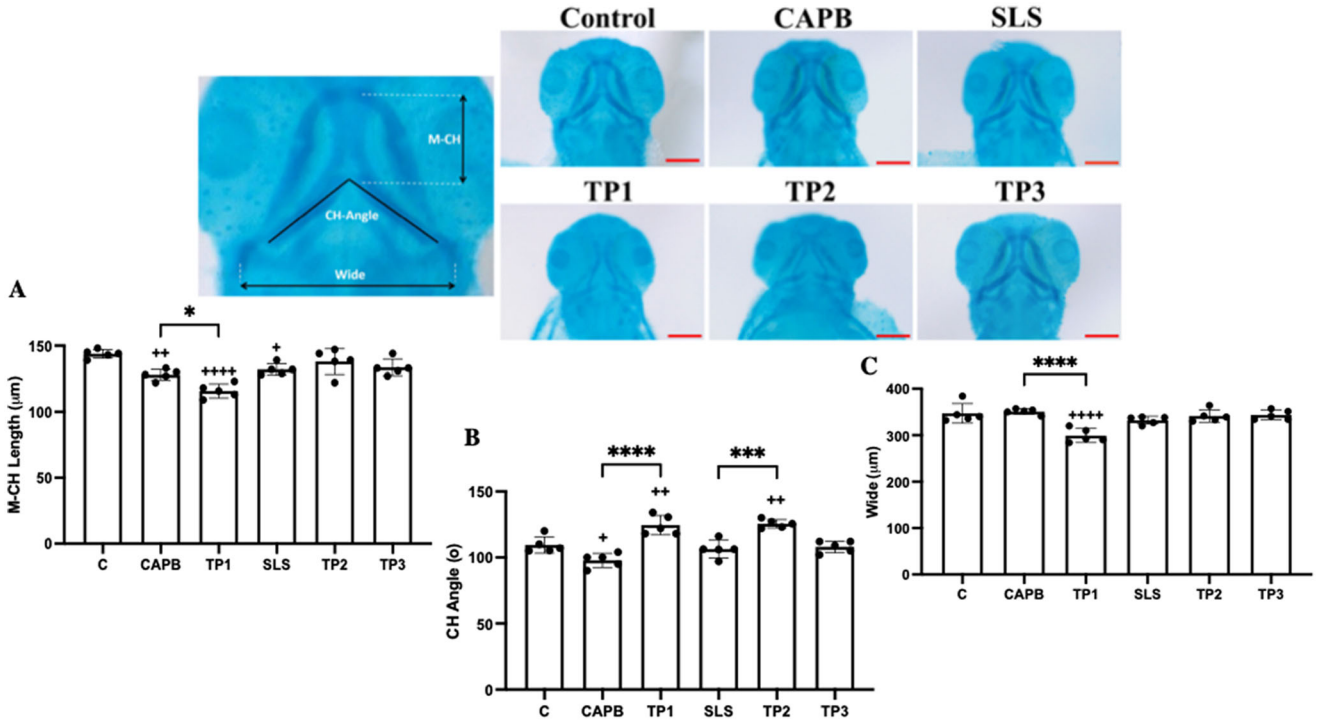

Figure 7. M-CH length, wide, and CH angle measured on the Alcian Blue stained zebrafish embryos. The black lines in the figures represent the lengths and the angle measured on the stained embryos. (A) M-CH distance (µm), (B) CH angle (°) (C) distance between the two opercles, measured as the head wide (µm), $^{*}p < 0.05$; $^{***}p < 0.001$; $^{****}p < 0.0001$; C: control; CAPB: cocamidopropyl betaine; SLS: sodium lauryl sulfate; TP: toothpaste; CH: ceratohyal cartilage; SD: standard deviation; scale bar: 150 µm.

contents, we observed yolk sac edema in the SLS exposed group and delayed hatching was evident in the SLS containing TP2. As an anionic surfactant, SLS interacts with cell membranes, increasing intracellular Ca^{2+} influx and increasing ROS production (Mizutani 2016). Accordingly, damage to the cell membrane, which causes an osmotic change and the resulting hyperaccumulation of liquids, can be used to explain yolk sac edema in the SLS exposed group (Orozco-Hernández *et al.* 2022). The absence of embryonic movements that would facilitate the exit of the embryo could explain the hatching delay caused by TP2 in our study. Decreased AChE activity in the TP2 group may be suggested to be related to hatching retardation causing gradual skeletal muscle myopathy and, as a result, impairment of embryo mobility (Orozco-Hernández *et al.* 2022).

Our results showed that locomotor activities were affected both by CAPB, SLS as well the CAPB and SLS containing toothpaste TP1 and TP2. SLS also known as SDS, is an anionic surfactant commonly used as an emulsifying cleaning agent (Tadin *et al.* 2019). Wang *et al.* (2015) evaluated the toxicity of three surfactants as the anionic SLS, cationic dodecyl dimethyl benzyl ammonium chloride (1227), and nonionic fatty alcohol polyoxyethylene ether (AEO) surfactants by investigating their effects on early development and behavioral activity of zebrafish. They reported that three types of surfactants induced retardation of embryonic development in a concentration-dependent pattern. Different from our study, the highest concentration of SLS (1 µg/mL) used in their study, did not effect the behavior of zebrafish embryos. In our study, SLS treatment inhibited locomotor activity as evidenced by decreased average speed, total distance, and exploration rate. This is likely because the SLS concentration applied in our study (4 mg/L) was much higher than in this study. To our knowledge, the effects of CAPB which is a zwitterionic ammonium compound on behavior and locomotor activity have not been evaluated before. We are the first to report that at the concentration used, CAPB treatment inhibited the locomotor activity in zebrafish embryos. It was observed that locomotor activity was similarly inhibited in CAPB and SLS containing TP applied groups, TP1, and TP3.

Embryo development must function properly for healthy odontogenesis. Apoptosis, the most prevalent form of programmed cell death throughout embryonic development, serves several important roles during odontogenesis, from the commencement of tooth development to the later morphogenesis of tooth germs and the elimination of the enamel knot signaling core. During odontogenesis, the intrinsic apoptotic pathway, which is activated in response to cellular stress, participates in several developmental processes (Abramyan *et al.* 2021). The signaling molecules known as reactive oxygen species can either promote cell division or trigger cell death. Therefore, oxidative stress has the potential to hinder embryonic growth and development (Orozco-Hernández *et al.* 2022). In our study, the oxidant–antioxidant balance was disrupted as evidenced by increased LPO and NO levels in all exposure groups except the TP3 group containing no detergent. Our group has previously shown increased LPO in SLS exposed zebrafish embryos (Meşeli *et al.* 2021). Inconsistent with this finding in our current study, similar to SLS, CAPB also led to a significant increase

in LPO levels. Moreover, when compared with the SLS group, CAPB caused a significant increase in NO levels. In our study, administration of CAPB and SLS alone increased LPO more than TP containing them. This can be attributed to the fact that the applied CAPB and SLS concentrations are below the lethal but effective doses for zebrafish embryos and higher than the detergent concentrations in the applied TP.

SOD enzyme plays a critical role in the antioxidant defense against oxidative stress in an organism (Landis and Tower 2005). Increased SOD activities observed in all exposure groups except TP3 are consistent with increased oxidative stress in these groups as evidenced by increased LPO and NO. The highest activity of GST was observed in the CAPB group in support of the highest levels of oxidative stress markers LPO and NO detected in the same group and the TP containing CAPB, TP1 was found to give a similar response.

The increases in GST activity and NO levels caused by SLS were not as much as in the CAPB group, but yet statistically significant. Although SLS containing toothpaste TP2 led to increased NO and LPO levels and reduced SOD activity compared to the CAPB containing toothpaste TP1, no significant difference was observed between GST activities.

SLS has been demonstrated to have considerable toxicity. As an anionic detergent, SLS, attaches to positively charged groups of proteins, leading the protein to denaturation. Due to its denaturing nature on proteins, SLS (5%) has been shown to have a degenerative effect on epithelial cell membrane (Healy *et al.* 2000). SLS has also been to change oral mucosal proteins and increase gingival blood flow (Herlofson and Barkvoll 1996).

When the effects of different ingredients of TPs were determined on cell viability, SLS and amine fluoride were found to inhibit cell viability whereas TPs containing CAPB had less effect (Cvikl *et al.* 2015). In another study, SLS and CAPB were found to be highly cytotoxic, even at quantities below the acceptable threshold for use in TPs and exerted maximum cytotoxicity shortly after exposure. The cytotoxicity of CAPB was reported to be lower than SLS (Tabatabaei *et al.* 2019).

The cholinergic system's main neurotransmitter is ACh. The presynaptic nerve terminal secretes ACh, which binds to Acetylcholine receptors (AChRs) concentrated in the postsynaptic membrane. ACh is degraded by AChE after it is released into the synaptic cleft (Massoulié *et al.* 1993). The central nervous system and muscle are among the tissues that express AChE. At the six-somite stage in zebrafish embryos, the expression of AChE starts as an anterior-to-posterior wave in the presomitic mesoderm, far earlier than the start of body movement. Behra *et al.* (2002) defined a non-classical function of AChE during vertebrate development and suggested that AChE activity is essential for the formation and maintenance of the axial muscle, as well as the survival of primary sensory neurons, and reported that AChE mutation resulted in significant motility impairment in zebrafish embryos. In accordance with this report, in our study, TP1 and TP2 and their detergent contents caused decreased AChE activity and locomotor activity in zebrafish embryos. The interaction of different detergents and amphiphilic drugs with AChE was reported to cause altered activity of the enzyme (Zimmermann *et al.* 2009). In our study, decreased AChE activity in parallel with the decreased locomotor activity in the TP1, TP2, and detergent groups, is notable for pointing out the inhibitory effect of TP and their detergent ingredients on neuromuscular development suggesting a non-classical function of AChE during in the embryonal period.

Igf2a signaling is needed for the early neural development and embryonic growth in zebrafish embryos (Li *et al.* 2014). In our study, *igf2a* expressions decreased in all exposure groups, but the most dramatic decrease was seen in the CAPB group. Our finding regarding SLS supports the finding of our previous study showing that SLS delays the hatching rate which is an important developmental parameter in zebrafish embryos (Meşeli *et al.* 2021). On the other hand, it is noteworthy that CAPB caused more severe inhibition than SLS and TPs.

The inhibitory effect of CAPB was also observed in the expression of *eve1*. Eve1 expression in the pharyngeal region is related to the initiation and morphogenesis of the first tooth as well as the ameloblast differentiation of all developing teeth (Laurenti *et al.* 2004). Although *eve1* expressions decreased in all groups except TP3, the highest decrease was seen in CAPB. CAPB and CAPB containing toothpaste TP1 also caused significant decrease in *nr0b1* expression which the earliest gene in the tooth development of zebrafish (Powers *et al.* 2009). The decreased M-CH, head wide length, and increased CH angle indicating alterations in cartilage structures correlate with the decreased *nr0b1* expression in the TP1 group. Wnt10a is expressed in the craniofacial area during important time points for tooth formation. In zebrafish embryos, and disruptions of wnt10a expression affected normal tooth development and terminated tooth development at 5 dpf (Yuan *et al.* 2017). Decreased *wnt10a* expressions were observed in the CAPB and the CAPB-containing TP groups whereas increased *wnt10a* expressions were observed in the SLS and SLS-containing TP groups. Similar results were observed in the *axin2* which is the regulator of Wnt signaling. Accordingly, altered expression of tooth development gene *axin2* correlated with *wnt10a*, and with changes in cartilage structures in the SLS group.

According to the results of our study, CAPB and SLS, which are the main detergent ingredients of children's TP, significantly inhibited developmental parameters and expressions of genes related to tooth development in zebrafish embryos. Similar effects were seen in embryos exposed to TP containing these detergents, although not as much as exposure to the detergents themselves. This may be because the concentrations of CAPB and SLS were chosen as effective concentrations in zebrafish embryos, which is much lower than the detergent contents in TP.

It is essential to acknowledge several limitations of our study. We evaluated the effects of the TPs and their detergent contents on odontogenesis through the changes analyzed at the transcriptional level. Functional analysis of Eve1, Dax1 (the protein product of *nr0b1*) Wnt10A, and Axin2 would support the identified changes. Another limitation of our study was the lack of ROS determination that would

support the oxidative condition revealed through increased LPO and NO. Based on the findings of our study, we may suggest that TPs for children and their detergent contents SLS and CAPB disrupted oxidant–antioxidant balance and affected the molecular mechanisms of odontogenesis at the transcriptional level in zebrafish embryos. We also suggest the availability of externally developing zebrafish embryos in examining the effects of TP' contents on embryogenesis.

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