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Molecular Toxicity of Popular Toothpaste Formulations on Post-Juveniles of *Clarias gariepinus*

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ABSTRACT: The widespread use of toothpaste containing various chemical formulations has raised concern regarding their potential impact on aquatic ecosystems. This research examines the molecular toxicity of popular toothpaste brands on post-juveniles of C*larias gariepinus* from a commercial fish farm in Akure, Ondo State, Nigeria. Specifically assessing the impact of these toothpaste brands on the mRNA expression levels of the heat shock protein (HSP70), interleukin (IL-1β), melatonin receptors (MEL1C), and growth hormone in comparison to a control group. The results revealed a significant upregulation of HSP70, IL-1β, and MEL1C genes in the exposed group, indicating a potential stress response and immune system activation. Intriguingly, the growth hormone mRNA expression remained unaffected in the treated group compared to the control. These findings underscore the need for further exploration into the potential molecular consequences of common toothpaste ingredients on aquatic organisms, raising important questions about environmental safety and consumer product development.

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Toothpaste, a staple in daily dental hygiene, has transformed over the years into a multifunctional gel or paste, designed to support oral health by combatting bad breath, and preventing gum and dental problems (Fiorillo *et al*., 2020). With an extensive global output, the toothpaste market is projected to grow substantially, reaching an estimated value of USD 47 billion by 2028 (Mordor Intelligence, 2023). Common toothpaste constituents include a range of ingredients such as water, sodium lauryl sulphate, sodium benzoate, fluoride, triclosan, humectants, artificial colorants, and microplastics (Dusit *et al*., 2023). The composition and disposal of toothpaste products give rise to critical environmental concerns, as these compounds can infiltrate freshwater systems and soil, posing a threat to ecosystems. Despite its oral health benefits, fluoride, a common toothpaste ingredient, accumulates in natural settings, leading to ecological challenges such as apoptosis of hepatocytes, oxidative stress, mitochondrial damage and apoptosis by affecting the mitochondrial respiratory chain complex in the kidney in organisms (Zuo *et al*., 2018; Wu *et al*., 2022). Antimicrobial agents like triclosan can contribute to antibiotic resistance which can lead to ecological imbalance in microbial populations (Carey and McNamara, 2015). Furthermore, microplastics, used for their abrasive properties, have been linked to a range of toxic effects on aquatic life, from disrupted

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behavior patterns to genotoxicity (Bashirova *et al*., 2023). Notably, toothpaste usage in India alone introduced a substantial amount of microplastics into the environment amounting to around 1.4 billion grams of microplastics annually (Chidhambaram *et al*., 2022). Research has also revealed the presence of heavy metals (Orisakwe *et al*. 2016) in toothpaste, raising concerns about their impact on the ecosystem. Additionally, the leaching of chemical ingredients and preservatives from toothpaste raises worries about soil and water quality. In the aquatic environment, the principal pathways for the uptake of chemicals by organisms primarily encompass the gills, integumentary system (comprising the skin), and gut (through the consumption of contaminated food) (Tenji *et al*., 2020). Fishes in contrast to other vertebrates exhibit a remarkable ability to display flexible phenotypic adaptations in response to diverse environmental circumstances. This adaptability manifests through profound and swift alterations in gene expression patterns when exposed to external stimuli. Thus, the modulation of gene expression represents one of the foremost mechanisms employed by fish when they encounter shifts in their surrounding environmental conditions (Larsen *et al*., 2011). Thus, gene expression and its regulatory mechanisms in relation to fish health have been widely studied to determine how fish populations adjust to local environmental conditions (Tine 2017). The African sharp-toothed catfish (*Clarias gariepinus*) was selected as the subject of this study owing to its remarkable suitability for aquaculture and ecotoxicology research (Osman *et al*., 2019). Commercially, it is highly valuable (Swaleh *et al*., 2023). Although indigenous to sub-tropical Africa, it has since been domesticated worldwide. The species is raised in ponds, enclosures, and cages. The present study evaluates the influence of two popular toothpaste brands (Close up and Oral B) in Nigeria on the gene expression patterns of four proteins in fish: Growth hormone (GH), heat shock protein 70 (HSP 70), interleukin 1-beta (IL-1β), and melatonin receptors (MEL1C). GH, a multi-functional hormone, is synthesized and discharged by the pituitary gland, playing a vital role in stimulating endocrine growth during postnatal development and affecting various physiological processes such as nutrition, metabolism, reproduction, neuroprotection, immunology, osmoregulation, and social behavior (Canosa and Bertucci, 2020; Canosa and Bertucci, 2023). HSPs, a group of intracellular proteins, are highly conserved and crucial in responding to different stressors like physical, chemical, and biological stresses (Baharloei *et al*., 2021). Among the HSP families, HSP70 family, with its high conservation and substantial membership, has been extensively studied (Jing *et al*., 2012). According to Barnes *et al*. (2002), HSP70 plays a critical role in safeguarding, restoring, and purging cellular components under adverse environmental conditions. IL-1B, a pro-inflammatory cytokine, is involved in fish innate immunity, with diverse physiological roles encompassing metabolism, inflammation, and immunology (Wong *et al*., 2019; Tian *et al*., 2021), and its role in managing the inflammatory process in fish is conserved (Zou and Secombes, 2016). Melatonin, an indole derivative, regulates the circadian rhythm in vertebrates, released periodically from the pineal gland and various organs. Apart from its housekeeping effects on rhythmic changes in physiological variables, melatonin governs several body functions in aquatic animals, including antioxidation, thermoregulation, immunoregulation, neuroendocrine function, sexual maturation, seasonal reproduction regulation, and aspects of aging (Falcon *et al*., 2010; Karamian *et al*., 2016; Zhang *et al*., 2023).

This study addresses the gap in understanding the cumulative impact of complete toothpaste formulations on aquatic organisms, unlike the predominant focus on isolated toothpaste components in existing studies.

MATERIALS AND METHODS

Test Chemical: Two 400g packs of Close-up (CLP) and Oral B (ORB) toothpaste brands were purchased from local stores in Akure, metropolis, Ondo State. Nigeria.

Experimental exposure: One hundred and fifty post-juveniles of *Clarias gariepinus* were obtained from a commercial fish farm in Akure, Ondo State, Nigeria, with a mean weight of 103-117 g and a total length of 21.50 ± 0.3 cm. To alleviate stress, the organisms were conveyed to the laboratory in well-aerated glass tanks filled with water from the point of collection. They were acclimatized for 10 days and kept in a laboratory setting (temperature 30 ± 2 °C) with a 12-hour light/dark cycle. Throughout the experiment, the fish were fed twice daily with a Coppens designed diet (44% crude protein), and the water in the fish's holding tank was changed every 24 hours to eliminate the accumulation of waste metabolites. A static renewal acute toxicity bioassay was performed in accordance with the Organization for Economic Cooperation and Development's (OECD) criteria for fish toxicity testing (OECD, 2010), and was authorized by the local ethics commission (CERAD/REC/16/02/023). The test organisms were randomly divided into five exposure groups and a control group of 30 fish each, with no regard for sex. Each group was then randomly assigned to three repeat tests of ten fish each in 10-L glass aquaria. A predetermined concentration of CLP

(0.7, 0.8, 0.9, 1.0, 1.1 mg/L), and ORB (0.6, 0.7, 0.8, 0.9, 1.0 mg/L) was measured from the stock solution and added to the bioassay tanks; the mixture was carefully stirred with a glass rod to ensure even distribution of the chemical and allowed to stand for 30 minutes before randomly introducing the test organisms. The sublethal experiment was based on environmentally relevant values extrapolated from the $1/10^{th}$ and $1/100^{th}$ of the 96 hr lethal concentration (LC_{50}) for CLP (0.772 mg/L) and ORB (0.692 mg/L). The test material was renewed with the same concentration and untreated control every 24 hours. At the end of the exposure period on day 15, test organisms were recovered and anaesthetized with tricaine methanesulfonate (MS-222) and dissected to get liver organs required for molecular studies.

Table 1: Forward and Reverse Primer Sequence used in the Study

S/N	Gene name	Forward primer sequence	Reverse primer sequence
1	B-ACTIN	CATCGGCAATGAGCGTTTC	GATGGAGTTGAAGGTGGTCTC
2	GH	CCCTGTCATTCTGCAACTCT	CCATGACTCGATCAGACGATAAG
3	HSP70	TGGCCTTTCAAGGTCATCAG	CAGCAACCATGGAGGAGATTT
4	IL-1β	CAGTGAATCCAAGCGCTACA	AAGCGAGCAGAAAGAGGAAAC
5	MEL1C	GATTTGGGCGACAGCAATTC	CCAGCATACAGCAAACAACAC

Quantitative real-time PCR: Using TRI reagent (Zymo Research, USA), total RNA was extracted from fish liver tissues. Following DNAse I (ThermoFisher Scientific) treatment, DNA impurities were removed by following the manufacturer's methodology. The extracted RNA was then reconstituted in nuclease-free water and quantified with a Hitachi-U1900 spectrophotometer at 260 nm by measuring the absorbance (Elekofehinti *et al*., 2018). 1µg of the extracted RNA was used for the reverse transcription reaction to synthesize complementary DNA (cDNA) with the ProtoScript ll First Strand cDNA synthesis kit (BioLabs, New England) under the following conditions: 65 ^{0}C for 5 minutes, 42 ^{0}C for one hour, and 70 ^{0}C for five minutes. OneTaq® 2X Master Mix (BioLabs, New England) was used to perform PCR amplification, which was subsequently run on a Labgene thermocycler. The primers were personally designed, then sent for synthesis and purchase from Inqaba Biotec (Hatfeild, South Africa). PCR was conditioned as follows: 1 cycle at 95 ^{0}C for 5 minutes, 30 cycles at 95 ^{0}C for 30 seconds, 30 cycles at 55 ^{0}C for 30 seconds, 30 cycles at 72 ^{0}C for 1 minute and a final extension step at 72 ^{0}C for 5 minutes. The relative amount of cDNA was subsequently quantified using ImageJ software and the gene expression normalized with β-actin gene as the housekeeping gene. The forward and backward primer sequences of the following genes, namely, growth hormone (GH), heat shock protein 70 (HSP 70), interleukin 1-beta (IL-1β), and melatonin receptors (MEL1C) are presented in Table 1.

Data analysis: Acute toxicity data involving quantal response mortality was analyzed using the probit analysis. ImageJ software was used to quantify the intensities of the band from agarose gel electrophoresis densitometrically. The densitometric analysis was subjected to an analysis of variance (ANOVA) using Graphpad Prism version 7 at a 5% ($P < 0.05$) level of significance, and the Tukey post hoc test was applied to further separate the means.

RESULTS AND DISCUSSION

Relative Acute Toxicity of Closeup and Oral-b Exposed to *C. gariepinus:* On the basis of the computed 96 hr LC_{50} values, of Oral-b was 1.12 times (0.692 mg/L) more toxic to fish than Closeup (0.772 mg/L). The distinct formulations of the two brands of toothpaste may be the reason for the contrasting sensitivity of the fish to the test compounds. Consequently, these factors affected the fish's vulnerability to the noxious substance. In addition, the presence of the microbeads in the Oral-b formulations may likely exert more negative impact than the closeup brand that had none of it.

Gene Expression Studies: The result of the densitometric analysis of the reference genes is shown in Figure 1 - 4. Data are expressed as mean ± SEM (n = 6); ****$p < 0.05$; denotes the significance of the data of the exposed groups when compared with the control group. The mRNA genes of heat shock protein (HSP70), interleukin (IL-1β), and the melatonin receptors (MEL1C), in the exposed group were significantly expressed when compared to the control group. In contrast, the mRNA genes of the growth hormone were not significantly expressed in the treated group when compared to the control group.

HSP, IL-1β, and MEL1C are often upregulated in cells exposed to environmental stresses (Cengiz *et al*., 2012; Kleszczynski *et al*., 2014). This work examines these biomarkers to assess the ecotoxicity of oral cleaning solutions. The growth hormone in fish has a significant impact on metabolism and can alter fish behavior. This includes stimulating hunger, increasing swimming activity, promoting aggression, and lowering anti-predatory behavior (Hayuningtyas *et al*., 2022). Based on this study, toothpaste formulations

had no effect on GH, contrary to that of Stachurski *et al*. (2023).

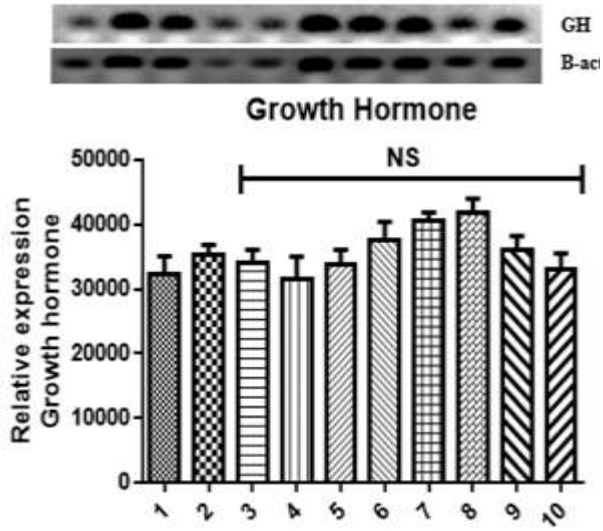

Fig 1: mRNA Expression in the Fish Following Chronic Exposures to Close-up and Oral B Toothpaste formulation on Growth Hormone.

Key: 1 & 2 = Control replicates; 3 & 4 = 1/10th of 96-hour Closeup LC_{50}; 5 & 6 = 1/100th of 96-hour Closeup LC_{50}; 7 & 8 = 1/10th of 96-hour Oral B LC_{50}; 9 & 10 = 1/100th of 96-hour Oral B LC_{50}

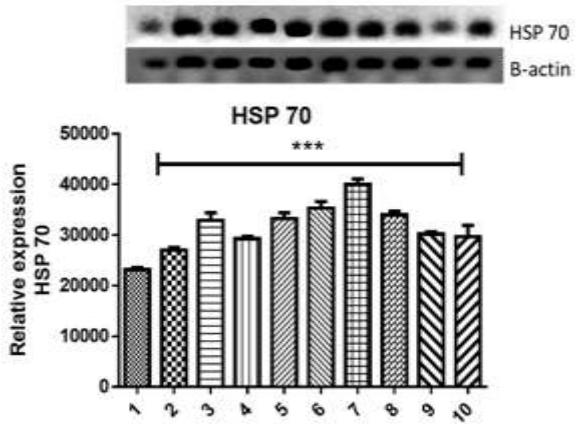

Fig2: Figure 1: mRNA Expression in the Fish Following Chronic Exposures to Close-up and Oral B Toothpaste formulation on Heat Shock Protein 70.

Key: 1 & 2 = Control replicates; 3 & 4 = 1/10th of 96-hour Closeup LC_{50}; 5 & 6 = 1/100th of 96-hour Closeup LC_{50}; 7 & 8 = 1/10th of 96-hour Oral B LC_{50}; 9 & 10 = 1/100th of 96-hour Oral B LC_{50}

The researchers found that toothpaste formulations inhibited growth factors in zebrafish models. Heat shock proteins (HSPs) are a diverse group of chaperones that are highly conserved and play key roles in responding to environmental stresses (Eissa *et al*., 2017). The regulation of HSP expression mostly occurs through the interaction of HSF1 with HSEs located in the promoter region of the HSP genes (Sun *et al* 2021).

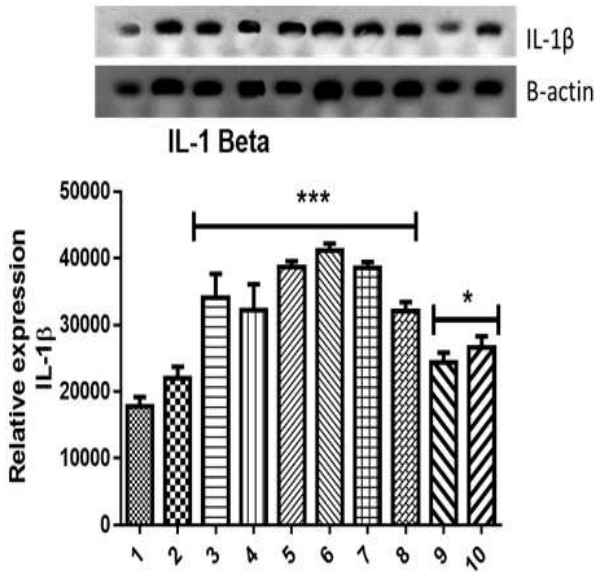

Fig 3: *mRNA Expression in the Fish Following Chronic Exposures to Close-up and Oral B Toothpaste formulation on Interleukin 1β Gene.*

Key: 1 & 2 = Control replicates; 3 & 4 = 1/10th of 96-hour Closeup LC50; 5 & 6 = 1/100th of 96-hour Closeup LC_{50}; 7 & 8 = 1/10th of 96-hour Oral B LC_{50}; 9 & 10 = 1/100th of 96-hour Oral B LC_{50}

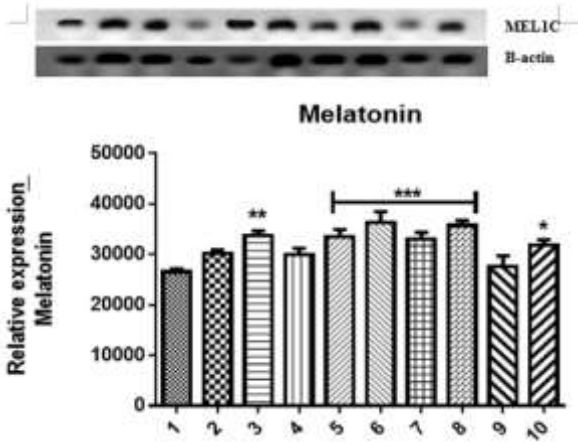

Fig 4: Figure 1: mRNA Expression in the Fish Following Chronic Exposures to Close-up and Oral B Toothpaste formulation on Melatonin receptors.

Key: 1 & 2 = Control replicates; 3 & 4 = 1/10th of 96-hour Closeup LC_{50}; 5 & 6 = 1/100th of 96-hour Closeup LC_{50}; 7 & 8 = 1/10th of 96-hour Oral B LC_{50}; 9 & 10 = 1/100th of 96-hour Oral B LC_{50}

HSP molecules, which are generated in reaction to stressful circumstances, serve a dual purpose: they not only facilitate the initial reaction to stressors but also strengthen the host's defenses against chronic pathogens and neoplastic growth (Chellapandian *et al*., 2023). The study observed that the toothpaste

formulation caused sub-lethal toxicity, which was shown by a significant increase in the expression of HSP70 genes. Consequently, the pollutant may cause tissue damage, decrease the activities of several detoxifying enzymes, induce oxidative stress, and ultimately lead to increased DNA damage and degradation (Dutta *et al*., 2017). Multiple studies have demonstrated that environmental toxicants, such as heat, heavy metals, and endocrine disrupting chemicals (EDCs), can cause an increase in the production of HSPs in organisms (Rhee *et al*., 2009; Kim *et al*., 2014).

Similar to other cytokines, interleukin 1β is a crucial component of the innate immune response in fish. They participate in an extensive array of physiological and immunological processes, as well as metabolic and inflammatory processes (Wong *et al*., 2019; Tian *et al*., 2021). The significant expression of IL-1β in this study may be a classical sign of inflammation, which is associated with cells and tissues responding to pathological cell injury caused by internal stimuli, including damage-associated products and metabolites, as well as external stimuli (Kaneko *et al*., 2019). The findings of this study contradict the results of Varghese *et al*. (2019), who reported that using guava leaf extract as a mouth rinse decreased the levels of inflammatory cytokines in human gingival epithelial keratinocytes (HGEK-16). Emphasizing the advantages of using oral cleaning solutions that are entirely derived from plants. MEL1C regulates various bodily processes in aquatic species, in addition to its housekeeping effects on rhythmic variations in several physiological variables (Maitra, 2011; Acharyya *et al*., 2021). Melatonin secretion influences a variety of physiological functions, including antioxidation, thermoregulation, immunoregulation, neuroendocrine function, sexual maturation, seasonal reproduction regulation, and some aspects of aging (Karamian *et al*., 2016; Gao *et al*., 2022). The significant increase in melatonin levels could potentially be attributed to its ability to scavenge free radicals. MELIC exhibits a wide range of antioxidant activities, including an indirect impact that involves the downregulation of pro-oxidant enzymes and the upregulation of antioxidative enzymes. Specifically, it inhibits the activity of glutathione peroxidase (GPx), glucose-6-phospahte dehydogenase, superoxide dismutase (SOD), catalase (CAT), and NO synthases (Olcese, 2020). The immunoregulatory properties of MELIC have been documented to be linked with the enhanced synthesis of interleukin cytokines (Bromage *et al*., 2001; Acharyya *et al*., 2021). This correlation was also seen in this research.

Conclusion: The findings of this research demonstrated that toothpaste formulations have the capacity to induce molecular stress responses in fish. Significant upregulation of HSP, IL-1β, and MEL1C was observed in the treated groups, highlighting the urgent requirement for the development of safer alternatives that are free from detrimental side effects. Additionally, the biomarkers that were sampled, namely HSP, IL-1β, and MEL1C, have the potential to function as significant indicators in evaluating the consequences of oral hygiene products on aquatic ecosystems. Thus, contributing to the broader understanding of environmental health.

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