

EMBRYOTOXIC AND TERATOGENIC EFFECTS OF MONOSODIUM GLUTAMATE AND SACCHARIN ON WADER (*Rasbora lateristriata*) FISH

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Abstract

The widespread utilization of food additives has garnered increasing attention owing to the safety implications. The continuous reassessment of potential toxicogenic and teratogenic risks associated with food additives is imperative. Monosodium glutamate (MSG) and saccharin, which are prevalently used in the food industry, were the focus of this study. This research aimed to evaluate the effects of MSG, saccharin, and the MSG-saccharin combination on the embryos of wader (*Rasbora lateristriata*) fish. Observations were conducted at 24, 48, and 72 h postfertilization (hpf) following substance administration. The findings indicated that the MSG- and MSG-saccharin-treated groups exhibited higher incidences of spinal abnormalities, pericardial edema, and bradycardia. Regarding safety, the survival rates of the saccharin-treated groups were higher than those of the MSG- and MSG-saccharin-treated groups. The observations further suggested variations in survival and hatching rates, body pigmentation, and blood clotting. Spinal irregularities, pericardial edema, and bradycardia were the predominant abnormalities recorded. These results establish the developmental risks associated with MSG and its combination with saccharin, emphasizing the need for further investigations to elucidate the mechanisms underlying these abnormalities.

Keywords: Abnormality; Embryo development; MSG; Saccharin; Teratogenicity; Wader

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Introduction

The association between food requirements and population dynamics has propelled the continuous evolution of the food industry, marked by a burgeoning use of food additives (Pressman *et al.*, 2017; Sambu *et al.*, 2022). Over an extended period, humans have incorporated additives into their diets to augment the flavor, employing substances such as food colorings, flavor enhancers, and sweeteners (Carocho *et al.*, 2017; Durazzo *et al.*, 2022; Vasilaki *et al.*, 2022). The industry prowess in synthesizing these chemicals for enhanced practicality, cost-effectiveness, and accessibility has contributed considerably to the pervasive use of additives (Sun *et al.*, 2021; Wu *et al.*, 2022). Nonetheless, prudent consumption is imperative, as the continued use of additives can hamper bodily growth, health, and development (Buka *et al.*, 2011; Pressman *et al.*, 2017; Sambu *et al.*, 2022).

Additives that are commonly used to improve the taste include monosodium glutamate (MSG) and saccharin (Chattopadhyay, 2017; Kazmi *et al.*, 2017; Niaz *et al.*, 2018; Zangfirescu *et al.*, 2019; More *et al.*, 2021). MSG, a water-soluble nonessential amino acid, contains sodium glutamate, which enhances the food flavor (Niaz *et al.*, 2018; Williams and Woessner, 2009; Zangfirescu *et al.*, 2019). Although MSG offers various culinary benefits, its use has been linked to adverse effects, including headaches and asthma (Stevenson, 2000; Freeman, 2006; Zangfirescu *et al.*, 2019). Moreover, MSG has been implicated in central nervous system disruption, obesity, metabolic disorders, and reproductive system irregularities (Brosnan *et al.*, 2014; Kayode *et al.*, 2020, 2023; Banerjee *et al.*, 2021). Nakanishi *et al.* (2008) observed that MSG consumption was associated with an increased incidence of metabolic syndrome and liver disease. Al-Ghamdi (2022) reported that exposure of chicken embryos to MSG led to organ development failure and growth retardation. MSG exposure in neonatal mice resulted in hypothalamic nuclei damage (Nakagawa *et al.*, 2000), whereas exposure in zebrafish induced kyphotic body malformations and pericardial edema (Abdelkader *et al.*, 2012). Noteworthy, akin to other toxic substances, MSG exerts toxic effects on the fetus, and the manifestations depend on the duration and dosage of exposure (Chakraborty, 2019).

The sweetness intensity of saccharin surpasses that of sucrose by a factor of 400, yet its consumption is devoid of caloric implications (Mahmood and Al-Juboori, 2020). Hence, saccharin has emerged as an alternative sweetener

for individuals afflicted with hyperglycemia and diabetes (Azeez *et al.*, 2019; Bayındır Gümüş *et al.*, 2022). However, research on the excessive intake of saccharin in both diabetic and nondiabetic cohorts has revealed a corollary induction of oxidative stress. This stress culminates in compromised glycemic control, heightened susceptibility to atherosclerosis, and hepatic and renal functional impairment (Jang *et al.*, 2011). Exposure to artificial sweeteners during embryogenesis is often linked to elevated incidences of malformations and premature birth (Cai *et al.*, 2021). While saccharin exposure during gestation has been shown to substantially reduce fetal weight in mice (Cai *et al.*, 2021), a similar exposure in zebrafish larvae has yielded inconclusive phenotypic alterations related to edema (Saputra *et al.*, 2021).

Despite the plethora of studies delineating the acute and chronic repercussions of MSG and saccharin, few studies have so far explored their impact on embryonic development. The use of wader fish (*Rasbora lateristriata*) as a model organism has gained prominence owing to its phylogenetic alignment with the family Cyprinidae and the subfamily Rasborinae. *R. lateristriata*, characterized by transparent embryos and larvae, facilitates facile observations (Mayden *et al.*, 2007; Retnoaji *et al.*, 2023). A previous study with *R. lateristriata* examined the exposure to deleterious agents such as insecticides, which resulted in developmental abnormalities (Raharjeng *et al.*, 2022). However, studies on substances deleterious to the embryonic development of *R. lateristriata*, particularly MSG and saccharin, remain scant. Therefore, this study aimed to assess the impacts of exposure to MSG and saccharin individually and in combination on the developmental trajectory of *R. lateristriata* larvae.

Materials and Methods

Chemical preparation

The salt stock solution was prepared by dissolving 35 g of noniodized NaCl (B. Braun, Indonesia) in 1 L of water. Subsequently, the salt stock solution served as the foundation for the egg water, which comprised 1.5 mL of salt stock, a drop of methylene blue (Merck, Germany), and 1 L of water. MSG (Sasa, Indonesia) was prepared at a concentration of 1,500 mg/L, and saccharin (Cap Tiga T, Indonesia) at 0.05 mM. Both solutions were freshly prepared using egg water before the experiment. These concentrations were selected

based on previous studies by Lee and Wang (2015) and Suthamnatpong and Ponpornpisit (2017).

Fish spawning and egg collection

The spawning and egg collection procedure followed the standard methods described by Raharjeng *et al.* (2022). Prior to spawning, male and female *R. lateristriata* broodstock were meticulously selected. The abdominal region was examined for sex determination, with females characterized by a larger abdomen indicative of the storage of numerous eggs vital for reproduction. The sex was confirmed via gentle massaging of the ventral region, with the release of white sperm fluid indicating males and the discharge of yellowish eggs signifying females. Fish devoid of sperms or eggs were deemed to possess immature gonads, rendering them unsuitable for broodstock purposes. Separated male and female *R. lateristriata* were then introduced into a mating chamber at a male-to-female ratio of 2:1, involving a total of 30 fish for spawning. The mating chamber was furnished with palm fiber at its base as an egg substrate to facilitate spawning. Eggs were harvested the following day, and the broodstock was relocated to the rearing pond. The water temperature was maintained at 28°C-29°C with a pH of 7.0-7.5 to provide optimal conditions for spawning and egg development.

Egg selection

The collected eggs underwent a triple cleansing process using egg water to eliminate impurities. The eggs were then examined microscopically, and embryos at the dome phase were selected based on key morphological features, such as the formation of the blastodisc and early cleavage stages.

MSG and saccharin treatments

This study encompassed four distinct treatment groups, each comprising 20 eggs with 2 replicates, resulting in 40 eggs per treatment. The groups were categorized as follows: the control group in which egg water was used as the egg-rearing medium; the MSG treatment group with a concentration of 1,500 mg/L; the saccharin treatment group with a concentration of 0.05 mM; and the MSG-saccharin treatment group combining 1,500 mg/L MSG and 0.05 mM saccharin. Embryos were placed in 24-well plates, with each well containing 1 embryo in 2 mL of the test solution. To simulate natural conditions, the experiments were conducted at a controlled temperature of 28°C ± 0.5°C under a 14:10 light/dark cycle.

Embryo observation

The embryos were observed at 24, 48, and 72 h postfertilization (hpf). The survival rate, hatching rate, eye pigmentation, pericardial edema, body pigmentation, spinal abnormalities, blood clots, and heartbeat were the parameters scrutinized, which were selected based on a previous report by Dewanti *et al.* (2023). The survival and hatching rates were recorded via accumulation counting at 24, 48, and 72 h postfertilization. Eye and body pigmentations were quantitatively evaluated by capturing images and analyzing the pigmentation intensity using ImageJ version 1.53 (National Institutes of Health, Bethesda, MD, USA). The recorded observations were documented and analyzed using SPSS Statistics version 24.0 (IBM Corporation, Armonk, NY, USA).

Results and Discussion

The wader fish (*R. lateristriata*) was selected for this study because of its distinctive advantages over other model organisms. For instance, embryo transparency permits direct, noninvasive visualization of developmental anomalies, such as spinal deformities and cardiovascular abnormalities, enabling real-time evaluation of teratogenic effects (Retnoaji *et al.*, 2023). Owing to rapid embryonic development and short generation time, *R. lateristriata* is an efficient and practical model for toxicity assessment (Retnoaji *et al.*, 2023). Moreover, as a species native to Southeast Asia and phylogenetically related to the zebrafish (*Danio rerio*), *R. lateristriata* serves as a novel yet analogous model system. This analogy enhances the contextual relevance of the findings to regional aquatic ecosystems.

MSG and saccharin are additives with discernible effects on the hatching and survival rates of *R. lateristriata*. Specifically, at 72 hpf, embryos exposed to MSG or saccharin exhibited a lower hatching rate than the control (Figure 1a). Comparative assessment with other experimental groups revealed that both the control group and the MSG-saccharin combination achieved a 100% hatching rate. Hence, there was no hatching delay. Significant differences in survival rates were not observed among the treatment groups (Figure 1b).

The MSG- or saccharin-exposed embryos demonstrated significantly lower hatching rates than the control group at 72 hpf, which suggested a potential adverse impact on the developmental processes of *R. lateristriata* embryos. The reduced

hatching rates could be attributed to the specific influence of MSG or saccharin, which affected factors crucial for embryonic viability and proper hatching. Interestingly, the MSG-saccharin combination group showed a 100% hatching rate, which implied that the combined exposure to both additives did not hinder or delay the normal hatching process. The lack of hatching delay in the MSG-saccharin combination group suggests potential interactions between the two compounds. Nonetheless, the specific mechanisms of these interactions have not been adequately elucidated and warrant further research.

In addition, the survival rates did not differ significantly among the treatment groups (Figure 1b). This lack of difference indicates that exposure to MSG, saccharin, or their combination did not exert noticeable adverse effects on the overall survival of *R. lateristriata* larvae. The robust survival across all treatment groups implies a degree of resilience in the embryonic development of *R. lateristriata* when exposed to these concentrations of MSG and saccharin. This finding suggests a degree of tolerance or the lack of acute toxicity at the tested concentration ranges. Thus, further studies are required into the specific mechanisms and factors contributing to the observed survival outcomes.

Fish embryos are enveloped by a chorion layer, which serves as a protective barrier against environmental factors, such as exposure to toxins (Lubzens *et al.*, 2010; Valdebenito *et al.*, 2021; Shuliakovich *et al.*, 2022). The chorion of *R. lateristriata* displays a protective mechanism (Retnoaji *et al.*, 2023) and contains pores similar to those found in zebrafish (Hamm, 2019; Chen *et al.*, 2020). Because of this structural similarity, certain compounds can penetrate the embryos, causing hatching delays and ultimately resulting in embryo mortality. The findings from this study indicate that exposure to MSG and saccharin adversely affects the developmental trajectory of *R. lateristriata* embryos. This deleterious effect could be ascribed to the comparatively smaller molecular size of MSG and saccharin than the chorion pore size of *R. lateristriata* embryos, facilitating their entry.

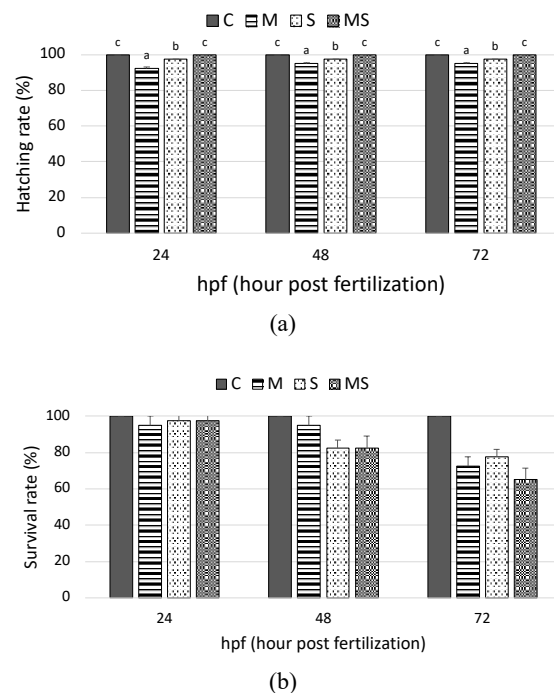
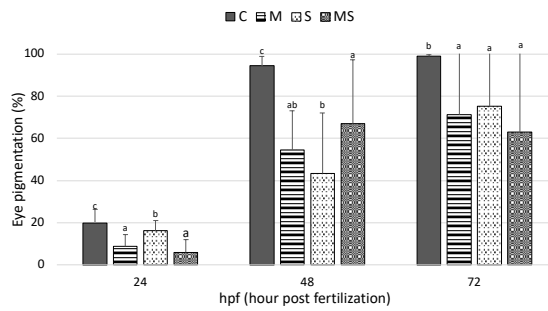


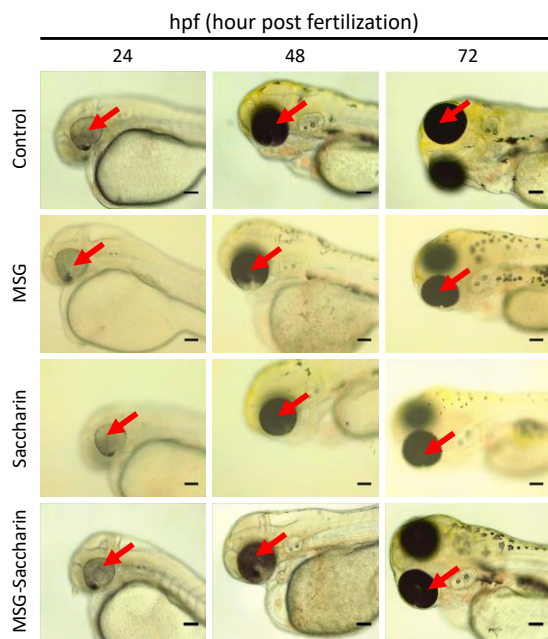
Figure 1. Hatching rate (a) and survival rate (b) of *R. lateristriata* exposed to additives. C: control; M: MSG; S: Saccharin; MS: MSG and Saccharin. Hatching and survival rates at 48 and 72 hpf are cumulative. Different letters indicate statistically significant differences ($P \leq 0.05$).

Furthermore, this study quantified the eye pigmentation in *R. lateristriata* embryos, which revealed aberrations induced by exposure to MSG, saccharin, and the MSG-saccharin combination. Statistical analyses indicated significant differences ($p < 0.05$) across all treatment groups. According to the findings depicted in Figure 2a, the pigmentation intensity in *R. lateristriata* exposed to MSG and MSG-saccharin treatments did not differ significantly at the 24 hpf time point. However, by the 72 hpf interval, discernible distinctions surfaced. When the effect on eye pigmentation was assessed, MSG-saccharin emerged as the most influential (65%), followed by MSG (72.5%) and

saccharin (77.5%). The variations in eye pigmentation are visually illustrated in Figure 2b. The MSG-saccharin combination was associated with the lowest percentage of eye pigmentation intensity.



(a)



(b)

Figure 2. Eye pigmentation of *R. lateristriata* exposed to MSG and saccharin. (a) Percentage of eye pigmentation at 24, 48, and 72 hpf. (b) Representative images of *R. lateristriata* eye pigmentation exposed to MSG and saccharin. C: Control; M: MSG; S: Saccharin; MS: MSG and Saccharin. Different letters indicate statistically significant differences ($P \leq 0.05$).

The embryonic phase is critical for both ocular and overall physiological development. During fish embryogenesis, the progression of the eyes exhibits notable responsiveness. Prominently sized eyes adorned with black spots emerge on the increasingly pigmented epithelium. This visual responsiveness persists until the 72 hpf mark (Richardson *et al.*, 2017). In this study, at 24 hpf, the pigmentation intensity in *R. lateristriata* under MSG and MSG-saccharin treatment showed no significant differences. However, by 72 hpf, MSG-saccharin had the greatest impact on eye pigmentation, followed by MSG and saccharin. These variations in eye pigmentation could be ascribed to the specific effects of MSG, saccharin, and their combination on the development of *R. lateristriata* embryos. The effect of MSG-saccharin on eye pigmentation, as evidenced by the lowest percentage (65%), implies a potential synergistic or antagonistic interaction between the two additives. Therefore, the combined exposure to both compounds might have a greater impact on pigmentation development than individual exposures. Moreover, the specific biochemical pathways and mechanisms involved in pigmentation development during embryogenesis should be considered. The differences observed in pigmentation at the 72 hpf interval could signify altered melanin synthesis, pigment cell migration, or other processes influenced by the additives. Molecular and histological analyses must be conducted, and appropriate positive controls should be included to discern the underlying reasons for eye pigmentation variations among the different treatment groups.

According to the results depicted in Figure 3, the body exhibited hypopigmentation patterns similar to those observed in the eyes. However, statistical analysis indicated the absence of significant differences among the treatment groups. Inherent natural variability in body pigmentation, the potential for threshold levels beyond the concentrations tested, compensatory mechanisms in embryonic development maintaining consistent pigmentation, or the specificity of MSG's and saccharin's impacts on other developmental pathways rather than pigmentation are the probable reasons for this lack of significance. Additional studies are needed to explore other concentrations and exposure durations to improve our understanding of the potential effects on body pigmentation.

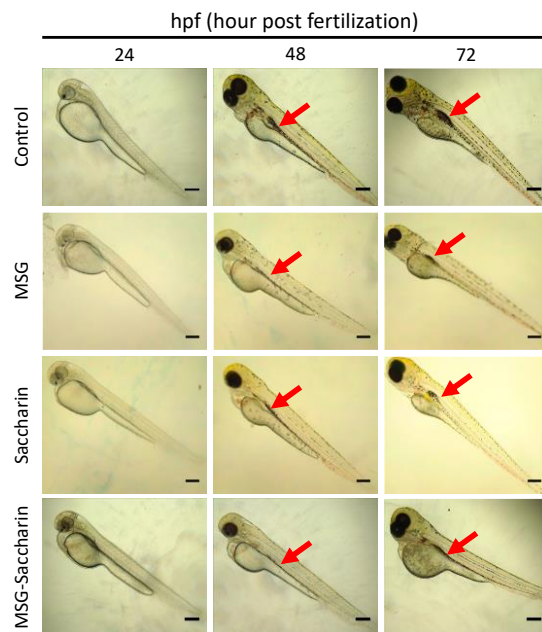


Figure 3. Body pigmentation of *R. lateristriata* exposed to MSG and saccharin at 24, 48, and 72 hpf.

In this study, the transparent nature of *R. lateristriata* larvae at 24, 48, and 72 hpf enabled the direct observation of spinal abnormalities. Exposure to MSG, saccharin, and their combination resulted in bone bending, a vital indicator of spinal deformities (Figure 4). This conclusion is supported by statistical analyses that revealed significant differences within each treatment group ($p < 0.05$). Spinal abnormalities were absent in the control and saccharin groups, implying that exposure to these substances did not cause bone bending. In contrast, both MSG and MSG-saccharin clearly exhibited spinal abnormalities at 72 hpf. This observation implies that exposure to MSG, either alone or in combination with saccharin, had a distinct and adverse impact on the development of the vertebral column. The bent bones observed in these groups suggest that the spinal development process is particularly vulnerable or sensitive to the presence of MSG. The combination of MSG and saccharin did not mitigate the observed effect, as evidenced by the persistence of spinal abnormalities in the MSG-saccharin group. Hence, caution should be exercised, and the potential developmental effects of exposure to MSG and its combination with saccharin should be investigated further.

The findings from this study indicate that both MSG and saccharin potentially impact the genesis and progression of the central nervous system via

spinal abnormalities (Niaz *et al.*, 2018). An earlier study has also documented that exposure to MSG can lead to lateral sclerosis and multiple sclerosis (Kazmi *et al.*, 2017). In addition, MSG treatment has been established to elicit neurotoxicity, neurodegeneration, and structural damage to brain tissues (Zanfirescu *et al.*, 2019). Moreover, artificial sweeteners, such as saccharin, are metabolized to phenylalanine, and elevated doses are correlated with increased phenylalanine levels and are linked to neurological and spinal disorders (Kim *et al.*, 2011).

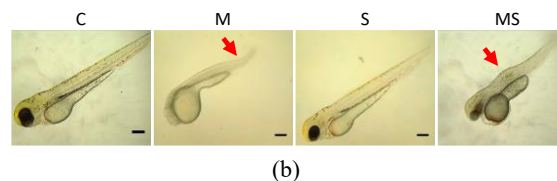
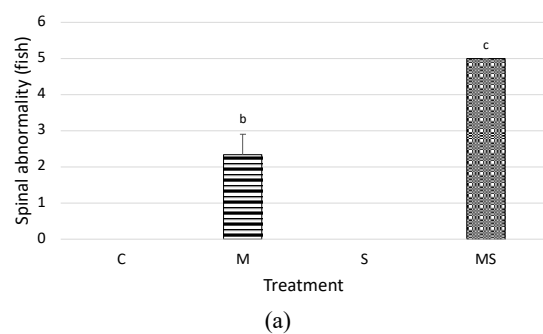


Figure 4. Spinal abnormalities of *R. lateristriata* exposed to MSG and saccharin. (a) Percentage of spinal abnormality at 72 hpf. (b) Representative images of *R. lateristriata* spinal abnormalities. C: Control; M: MSG; S: Saccharin; MS: MSG and Saccharin. Different letters indicate statistically significant differences ($P \leq 0.05$).

This study further highlights that exposure to both MSG and saccharin can lead to cardiac complications, resulting in abnormalities in the heartbeat patterns in *R. lateristriata* (Figure 5). Statistical analysis of the obtained data revealed significant differences within each treatment group, emphasizing the impact of these additives on heart function ($p < 0.05$). Heart rate assessments performed between 48 and 72 hpf showed a consistent decline across all exposure treatments. The MSG-saccharin treatment group exhibited the lowest recorded heart rate. Interestingly, although significant differences were not observed between the MSG and MSG-saccharin groups at 48 hpf,

a subsequent examination at 72 hpf revealed significant variations across all treatment groups. This temporal difference in the observed effects suggests that the impact of MSG and saccharin on heart rates may evolve over time, potentially indicating a delayed or cumulative effect.

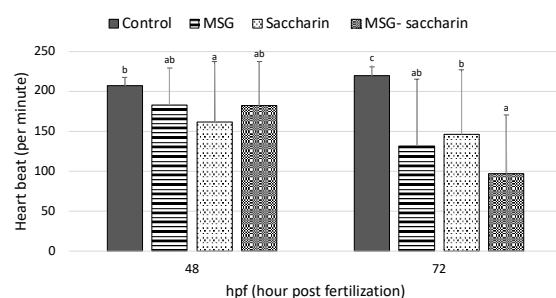


Figure 5. Heartbeat rate in *R. lateristriata* exposed to MSG and saccharin at 48 and 72 hpf. Different letters indicate statistically significant differences ($P \leq 0.05$)

The MSG-induced deceleration of the heart rate is aligned with the existing literature and could be attributed to diminished baroreflex sensitivity. This phenomenon is often associated with reduced vagal nerve activity (Konrad *et al.*, 2012). The vagal nerve plays a crucial role in regulating the heart rate, and any alteration in its activity can alter cardiac functioning. These findings provide a potential mechanistic explanation for the observed cardiac effects of MSG in the context of *R. lateristriata* embryonic development. However, the results of this study differ from those reported by Saputra *et al.* (2021), who observed a significant increase in zebrafish heart rates following exposure to pure artificial sweeteners such as acesulfame K, neotame, saccharin, and sucralose. This discrepancy indicates that the effects are compound-specific, emphasizing the need for a detailed understanding of the impact of various additives on cardiovascular function. The specific mechanisms via which MSG and saccharin influence heart rates in *R. lateristriata* may involve complex physiological pathways. Further research is warranted to elucidate these mechanisms and their potential implications in broader contexts, including human health.

Pericardial edema and blood clot formation were observed specifically at the 48 hpf mark in the groups exposed to MSG and the MSG-saccharin combination (Figure 6). In contrast, blood clots were not seen within the same time frame in the control and saccharin-only groups. These

observations could be explained by the potential adverse effects of MSG and MSG-saccharin combination on the developmental trajectory of *R. lateristriata* larvae. Pericardial edema, characterized by an abnormal fluid accumulation around the heart, and blood clot formation, indicate disturbances in the circulatory system and suggest compromised cardiovascular health in the affected larvae.

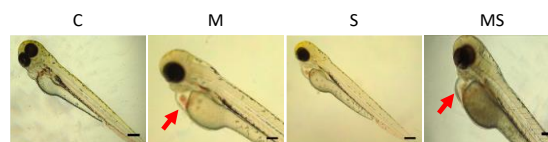


Figure 6. Pericardial edema and blood clots in *R. lateristriata* after exposure to MSG and saccharin at 48 hpf. C: Control; M: MSG; S: Saccharin; MS: MSG and Saccharin. Arrows indicate pericardial edema and blood clots

The MSG and saccharin concentrations used in this study were determined based on established toxicological parameters in aquatic models and do not reflect typical human consumption levels. These elevated concentrations were selected to simulate high-exposure conditions that facilitate the assessment of acute embryotoxic and teratogenic effects in aquatic species. Although the administered doses exceeded the average human dietary exposure, the findings provide valuable insights into the potential developmental hazards associated with excessive exposure. The results highlight the need for a more profound understanding of the dose-response dynamics across different species.

Conclusions

The findings from this study have delineated the distinct effects of MSG and saccharin on *R. lateristriata* embryos. While MSG or saccharin alone led to a decreased hatching rate at 72 hpf, the MSG-saccharin combination exhibited a normal hatching rate, signifying an absence of delay. Survival rates remained consistent across all treatments, signifying that *R. lateristriata* larvae were resilient to these concentrations. However, differences were evident in the eye pigmentation, particularly at 72 hpf, with MSG-saccharin displaying the lowest intensity. Body pigmentation exhibited hypopigmentation patterns without statistical significance. In addition, spinal

abnormalities were noted in both MSG and MSG-saccharin groups, indicating the vulnerability of vertebral development to these additives. Lastly, both MSG and saccharin led to cardiac complications, altering the heartbeat patterns and causing pericardial edema and blood clots, especially in the combined treatment group.

Future studies should explore the molecular mechanisms underlying the observed effects and consider diverse concentrations and exposure durations to enhance our understanding. Moreover, the role of chorion in substance permeability and its impact on delayed hatching should be investigated. In addition, comparative studies with other Kim longitudinal studies beyond the embryonic stages may unravel delayed or cumulative impacts. Finally, strategies to mitigate adverse effects must be examined to augment our understanding of additive safety.

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