Effect of MS222 on Hemostasis in Zebrafish

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MS222 is a compound used in anesthetizing vertebrates, including fish and frogs. Several side effects of this anesthetic have been reported, but its effect on hemostasis has not been studied. In our laboratory, we have used zebrafish for more than 2 decades as a model system to study hemostasis. During this period, we have had trouble in collecting blood from anesthetized zebrafish and observed more rapid blood clotting than in nonanesthetized counterparts. However, no systematic studies regarding the effect of MS222 on zebrafish hemostasis are available. In this study, we performed various assays such as gill bleeding, measurement of Hct, total blood cell counts, thrombocyte counts, thrombocyte aggregation, and coagulation and measured the amount of blood collected. We found that Hct values, the amount of blood collected, bleeding, and coagulation differed significantly between anesthetized and nonanesthetized fish. Our results suggest that blood collected after MS222 anesthesia of zebrafish has altered hemostasis.

Abbreviations: kPT, kinetic prothrombin time; kPTT, kinetic partial thromboplastin time; MS222, tricaine methanesulfonate

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Tricaine methanesulfonate (MS222) is a sulfonated derivative of benzocaine that is used in anesthetizing anamniotes, such as zebrafish (*Danio rerio*), *Xenopus*, and axolotl. This anesthetic is a muscle relaxant and works mainly by blocking sodium currents in the nerve membranes, thus diminishing action potentials and resulting in muscle relaxation.^{15,17} MS222 is used at doses of 0.1 to 0.15 mg/mL to anesthetize adult zebrafish. MS222 lowers the pH of water when used at therapeutic concentrations. Acidic pH can cause plasma acidosis and thus be stressful to fish. Therefore, MS222 buffered with sodium bicarbonate has been suggested for use in anesthesia.^{3,6} However, although anesthetizing catfish with high concentrations of MS222 induced stress in the fish, the pH itself does not seem to be the cause of this stress.²⁰ Other studies have shown that acidic conditions minimized the anesthetic effect of MS222.⁵ Therefore, the use of buffered MS222 for anesthesia has been recommended even though the effect of this formulation is minimal. The side effects of MS222 can vary depending on incubation time and concentration.¹⁵ For example, cardiac depression and failure and increases in glucose levels have been reported.² MS222 initially induces hypertaxia and tachyventilation and subsequently results in reduced opercular movements.²² In some studies, MS222 has been shown to cause gill bleeding and reduced heart rates and effectively helps extend to anesthesia duration and shorten recovery time.^{7,15} The MS222 dose depends on the age, size, and metabolic rate of the fish.¹⁵ Overdose of MS222 (0.2 to 0.3 mg/mL) through prolonged immersion of fish leads to death due to hypoxia.²¹

During the past 2 decades of working on zebrafish hemostasis, we noted that MS222 anesthesia led to differences in the collection of blood and bleeding between nonanesthetized and anesthetized fish. However, no systematic studies on the effects of MS222 on hemostasis are available currently. In the current study, we hypothesize that using MS222 in zebrafish can affect studies on hemostasis. Therefore, we tested whether MS222

affects parameters such as gill bleeding, Hct values, total blood cell counts, the amount of blood collected, thrombocyte aggregation, and coagulation. We found significant differences between nonanesthetized and anesthetized fish regarding various parameters, including Hct and coagulation time. These results emphasize that investigators performing studies on hemostasis in fish should consider these differences when using blood from fish exposed to MS222.

Materials and Methods

Zebrafish husbandry and anesthesia. Wildtype adult zebrafish (AB strain; age, 6 mo) were kept in 27 °C deionized water that was supplemented with Instant Ocean (Petco, Denton, TX) and maintained at pH of 7 to 7.5 in a recirculating system consisting of 6 L tanks.¹¹ Aquarium test strips (Easy Strips 6-in-1, Petco) were used to monitor the chlorine, nitrite, nitrate, alkalinity, hardness, and pH levels of the system water on a weekly basis; when test results departed from the normal allowable levels, about 1/3 of the system water was replaced. We monitored the general health of the fish daily by visual observation of their activity; sluggish or dead fish were removed immediately. The fish room was kept in a 14:10-h light:dark cycle. Zebrafish were fed with brine shrimp freshly hatched from the brine shrimp eggs (Angels Plus, Olean, NY) and fish flakes (Fish N Chirps Pet Center, Denton, TX) 3 times daily. Zebrafish were anesthetized until they lost their balance (about 2 min) in petri dishes containing 0.15 mg/mL buffered MS222 solution (pH 7.0). Anesthetized zebrafish immediately underwent evaluation (described later). All procedures were approved by the University of North Texas IACUC, and animal experiments were performed with humane care in compliance with institutional guidelines.

Bleeding assays. For chemically induced gill bleeding assays, individual size- and sex-matched nonanesthetized and anesthetized zebrafish were placed in cuvettes containing 3 mL of 20 mM NaOH solution to induce gill bleeding.¹² Bleeding was recorded by using a 16-megapixel cellphone camera to photograph these fish 1 min after they were exposed to NaOH. For bleeding by mechanical injury, each zebrafish was laid laterally on a dry paper towel. The fish body was wiped with a

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Figure 1. Effect of MS222 on bleeding from the caudal vessel. (A) The caudal vessel of nonanesthetized (top 2 fish) and anesthetized (bottom 2 fish) zebrafish was clipped to initiate bleeding. (B) The total red pixel intensity (mean ± SEM; $n = 12$) from the photograph in panel A. †, Values differ ($P < 0.01$) between control and anesthetized fish.

Figure 2. Effect of MS222 on gill bleeding. (A) Gill bleeding in nonanesthetized (top 2 fish) and anesthetized (bottom 2 fish) zebrafish. (B) The total red pixel intensity (arbitrary units; mean ± SEM; $n = 8$) from the photograph in panel A. $n = 8$; *, Values differ ($P < 0.05$) between control and anesthetized zebrafish.

laboratory tissue to remove any moisture. The caudal artery of the fish was then clipped by using a pair of dissecting scissors as described previously.¹⁰ To induce uniform injury, we used a marker to draw a line at approximately 2 mm above the scissors' tips. The scissors' tips were then inserted into the fish body until the marker line touched the skin between the ventral and dorsal fins and between the second and fourth black stripes from the dorsal end, and an incision was made. After 1 min, bleeding was recorded by taking photographs as described. To quantify bleeding, we used ImageJ software (<https://imagej.nih.gov/ij/>). The red pixels were counted as an index of the area and

were multiplied by the mean pixel intensity to yield the total red intensity representing the extent of bleeding.

Blood collection. For blood collection, zebrafish were laid on a fresh paper towel and, the investigator held the head by using fingers, and the caudal artery was clipped by using a pair of dissecting scissors as described earlier. The blood that welled out ($2 \mu\text{L}$) was collected by using a pipette tip, placed in a microfuge tube containing $0.5 \mu\text{L}$ 3.8% sodium citrate, and mixed quickly. Because blood collection is a terminal procedure, the fish were euthanized immediately afterward by MS222 overdose (0.3 mg/mL).

Figure 3. Effect of MS222 on Hct and total volume of blood collected. (A) Photographs of the Hct capillary tubes for nonanesthetized (top) and anesthetized (bottom) zebrafish. (B) Comparison of total length (mean $\pm$ SEM; $n = 24$) of RBC plus plasma, representing the total volume of blood collected from nonanesthetized and anesthetized fish. †, Values differ ($P < 0.01$) between nonanesthetized and anesthetized fish. (C) Comparison of the percentage of RBC according to volume (represented as the length of the RBC layer; mean $\pm$ SEM; $n = 24$) between nonanesthetized and anesthetized fish. †, Values differ ($P < 0.01$) between nonanesthetized and anesthetized fish.

Hct assay. The caudal artery of nonanesthetized and anesthetized zebrafish was clipped as described earlier. A microhematocrit capillary tube (Fisher Scientific, Hampton, NH) was placed on the blood welling from the blood vessel and allowed to flow into the tube by capillary action until all of the blood available prior to clotting was collected. Then the end of the capillary tube containing blood was placed vertically on the sealer (Critoseal, Oxford Labware, St Louis, MO) such that the blood faced it. The tube was then pressed gently so that it sealed, placed into a microcentrifuge (MicroMB, International Medical Equipment, North Andover, MA) and centrifuged for

5 min to separate the plasma from RBC. The capillary tubes were photographed as described and photographs loaded into a PowerPoint (Microsoft, Redmond, WA) file. The pictures were magnified such that the width of each tube was identical. Then straight lines were drawn on boundaries of plasma, and the lengths of the RBC layer only and of the plasma layer plus the RBC layer were measured by clicking on the line properties in the PowerPoint program. From these values, the RBC percentages were calculated.

Flow cytometry. Thrombocytes and total blood cells were counted by using flow cytometry. To this end, 0.5 μ L of blood

Figure 4. Comparison of the total number of blood cells between nonanesthetized and anesthetized fish. Histogram plots show the separation of RBC and WBC from thrombocytes in (A) nonanesthetized and (B) anesthetized zebrafish blood. (C) Total blood cell counts (that is, RBC + WBC) and (D) thrombocyte counts of nonanesthetized and anesthetized fish. Data are shown as mean $\pm$ SEM; $n = 10$. *, Values differ ($P < 0.05$) between nonanesthetized and anesthetized fish.

Figure 5. Plate tilt assays of blood from nonanesthetized and anesthetized zebrafish. (A) The photograph shows the migration of blood from the top of the well (indicated by the white star) in the presence of ADP. (B) The photograph shows the migration of blood from the top of the well (indicated by the white star), in the presence of collagen agonist. Data are given as mean $\pm$ SEM; $n = 6$. Values did not differ between nonanesthetized and anesthetized zebrafish.

was collected from zebrafish by gently poking the caudal artery of the fish and collecting with the pipette tip as described above. The blood sample was added to a 1.5-mL microfuge tube containing 500 μ L PBS; then the tube was placed into a flow cytometer (BD ACCURI C6, Beckton Dickinson, San Jose, CA), and the cells counted.¹⁹

Plate tilt assay. In a microwell plate (Nunc, Penfield, NY) in which the capacity of each conical well is 10 μ L, 8 μ L PBS and 1 μ L of either 0.2 mM ADP or 1 mg/mL collagen were added to each well; 0.5 μ L of citrated blood was then added from the top such that blood went through the agonist solution to the bottom of the well. At 3 to 5 min later, the plate was tilted at 45° for 10 s. The migration of the blood down the sloped well wall was photographed as described earlier. The distance of blood migration from the top of the well down the wall was measured from the photograph. To normalize between pictures, we resized them such that the diameter of the well was the same in all pictures. The lack of or decreased mobility of blood down the slope indicated a positive aggregation in response to agonists.¹⁰

Clotting parameters. In a 96-well flat-bottomed (VWR, Radnor, PA) microtiter dish which was kept on ice, 10 μ L of fibrinogen (10 mg/mL) and 1 μ L of zebrafish plasma were mixed in each well. Then 20 μ L of zebrafish muscle thromboplastin for kinetic prothrombin time (kPT) or 10 μ L of ALEXIN (Sigma, St Louis, MO) for kinetic partial thromboplastin time (kPTT) were added to each well. Each reaction was brought to 94 μ L, and then 6 μ L of 100 mmol/L CaCl_2 was added. The plate was removed from the ice, wiped thoroughly by using a laboratory tissue, incubated for 4 min at room temperature, and then loaded into the Synergy H1 hybrid reader (BioTek Instruments, Winooski, VT). After loading, the plate underwent double orbital shaking for 1 min. The absorbance at 405 nm was recorded every 1 min for 15 to 20 min. In the negative controls, the clotting reagent was replaced with PBS.⁸

Statistical analysis. Statistical analysis was performed by using one-way ANOVA with Sigma Plot (Systat Software, San Jose, CA) using posthoc Tukey Honestly Significant Difference tests. Differences at a P value of less than 0.05 were considered significant.

Results

To test whether MS222 affects bleeding, we created a mechanical injury by clipping the caudal artery and initiated bleeding in both nonanesthetized and anesthetized fish. After cessation of bleeding, the total intensity of red pixels was quantified by ImageJ. The total intensity of red pixels representing the bleeding was greater ($P < 0.01$) in nonanesthetized fish than anesthetized fish (Figure 1). Even though the results showed significant differences in bleeding and despite creating an injury that was approximately the same between the 2 groups of fishes—similar to the mechanical injury we have reported in zebrafish larvae—it was challenging to create a uniform injury among these fishes, thus requiring quantitation by statistics.⁹ Therefore, to support the mechanical injury results, we used a more uniform, chemical injury induced by sodium hydroxide and repeated the gill bleeding assay. The results again showed that nonanesthetized fish bled significantly more than anesthetized fish (Figure 2).

In the Hct assay, the total length of RBC plus plasma (representing the total volume of blood) was measured. The results indicated that the total length of the plasma plus RBC was greater ($P < 0.01$) in nonanesthetized fish compared with anesthetized fish (Figure 3). In addition, the percentage of the volume of RBC represented by the length of the packed RBC (that is, Hct) was calculated. We found that the Hct values of the anesthetized were greater ($P < 0.01$) than those obtained from the nonanesthetized fish (Figure 3).

Figure 6. Kinetic curves of fibrin formation in nonanesthetized and anesthetized fish. The rate curves were generated in the presence of zebrafish muscle thromboplastin (kPT) and ALEXIN (kPTT). (A) kPT rate curves generated from nonanesthetized and anesthetized fish plasma respectively. The graph beneath the rate curves shows the clotting times at a half-maximal fibrin formation. Values did not differ ($P = 0.156$) between nonanesthetized and anesthetized fish. (B) kPTT rate curves generated from nonanesthetized and anesthetized fish plasma respectively. The graph beneath the rate curves shows the clotting times at half-maximal fibrin formation; ‡, Values differ ($P < 0.001$) between nonanesthetized and anesthetized fish. Data are given as mean $\pm$ SEM; $n = 6$.

To test whether the MS222 affects total blood cell counts and thrombocyte counts, we collected 0.5 μ L of blood from nonanesthetized and anesthetized fish and performed flow cytometry (thrombocytes are 5 μ m in diameter, whereas RBC and WBC are about 8 μ m).¹⁰ There was a significant ($P < 0.05$) increase in total blood cell number in anesthetized fish compared with nonanesthetized fish. However, the number of thrombocytes did not differ between groups (Figure 4).

To test whether MS222 anesthesia had an effect on thrombocyte aggregation, we performed plate tilt assays using ADP and collagen as agonists. Whole blood aggregation did not differ significantly between nonanesthetized and anesthetized fish (Figure 5). We also tested whether MS222 affects blood clotting; to this end, we evaluated assaying extrinsic and intrinsic coagulation (that is, kPT and kPTT, respectively) by monitoring fibrin formation (indicated by the increase in absorbance at 405 nm) in plasma samples obtained from nonanesthetized and anesthetized fish. We found that the kPT curves were essentially the same between the 2 groups. However, the kPTT curve of the

anesthetized fish plasma was shifted to the left and was more linear compared with the sigmoidal curve that was observed for nonanesthetized fish (Figure 6). We then repeated the kPT and kPTT assays and calculated the clotting times at half-maximal fibrin formation. Whereas the results of kPT showed no difference in clotting time between anesthetized plasma and nonanesthetized plasma, the kPTT results showed significant ($P < 0.001$) differences between groups (Figure 6).

Discussion

In this research, we found that MS222 affects the extent to which blood wells up after caudal injury of zebrafish. The fact that blood flow after an incision is made in an anesthetized fish is retarded compared with the free flow seen in nonanesthetized fish suggests that vasoconstriction may occur during anesthesia.¹³ Similar results with gill bleeding assay also suggested that MS222 enhances vasoconstriction. Interestingly, we found that the total blood cell counts were increased in anesthetized fish compared

with nonanesthetized fish. Several studies showed that various hematologic parameters change after anesthesia.^{4,23} However, in the studies on fish, the fish were kept under anesthesia for several hours, in contrast to our study, where we collected blood immediately after the anesthesia-induced loss of balance in the fish.²³ Because we collected blood immediately after anesthesia, there was not sufficient time for hematopoiesis to increase the circulating blood cells. We do not know the mechanism through which the blood cell counts increase. A plausible explanation is that splenic contraction may occur during anesthesia, thus increasing the number of cells in the bloodstream,¹⁴ as has been suggested elsewhere.¹⁸ Our interpretation is consistent with these reports.

Surprisingly, thrombocyte counts did not increase after anesthesia. If splenic contraction is indeed responsible for the increase in blood cell counts after anesthesia, we do not know why RBC might be expelled selectively from spleen, so that they—but not thrombocytes—increase in number. It is interesting that an earlier report indicated that the mobilization of leukocytes proceeds more slowly than that of RBC and is consistent with the lack of increase in circulating thrombocyte numbers.¹⁸ The morphology of the cells may play a role in this selective splenic effect. More work is needed to understand this mechanism. The lack of change in thrombocyte numbers suggests that thrombocyte aggregation may not differ between nonanesthetized and anesthetized fish. Indeed, our results show that thrombocyte aggregation did not differ between groups and was consistent with our findings regarding thrombocyte numbers.

According to our results, kPTT—but not kPT—differed between groups, suggesting that MS222 might affect the intrinsic blood coagulation system. Interestingly, even though thrombocytes appeared to aggregate similarly between anesthetized and nonanesthetized fish, coagulation in plasma is altered. Although our observations are preliminary, investigators should be aware of these effects.

Our blood collection method involved clipping the caudal vessels.¹⁰ Even though a minimal amount of tissue factor is released during blood collection, the method is reproducible for measuring clotting times. After we developed the first blood collection method for zebrafish, other laboratories have reported various blood collection methods.^{1,16,24} One of the other techniques, a modified version of the tail-clipping method we developed, enables larger volumes of blood to be collected.¹ However, the pronounced wounding and increased time needed to obtain the sample may lead to the generation of tissue factor at the site of injury that will activate coagulation. In another method, nonlethal repeated blood collection was performed.²⁴ However, with that method, serial blood samples were used for hematologic studies other than coagulation research. At present, our current blood collection method appears to be the most suitable for zebrafish coagulation studies because collection is performed in 5 to 10 s or less.¹⁰

In summary, we found that MS222 affected blood collection characteristics, total blood cell counts, and coagulation parameters in zebrafish. Investigators using zebrafish should be aware of the effects of MS222 on blood coagulation.

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