MS-222 short exposure induced developmental and behavioural alterations in zebrafish embryos

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ABSTRACT

MS-222 has been widely used as an anaesthetic in fish, thus, raising the need to infer about its toxicological safety during development. In this study, MS-222 toxicity in zebrafish embryos was evaluated after a 20-min exposure at different stages of development. Embryos exposed during the 256-cell stage displayed an increase in mortality, associated with defective early developmental pathways. Following exposure during the 50% epiboly stage, an increase in mortality and abnormal cartilage development, as well as changes in noggin expression were observed. Locomotor deficits were detected and associated with changes in early signalling pathways through the involvement of noggin. When exposed at the 1–4 somites stage, zebrafish were phenotypically normal, although presenting changes in the expression pattern of developmental genes. These findings indicate a teratogenic impact, independent of sodium channels that should be taken into consideration when MS-222 toxicity is discussed.

Keywords: MS-222, Development, Behaviour, Toxicity, Zebrafish

INTRODUCTION

The use of fish for scientific purposes as an alternative to mammalian models in a variety of medical areas has been increasing world- wide [1,2]. These organisms have convincingly shown their usefulness for improving the knowledge on the molecular and cellular mechanisms mediating several pathophysiological conditions [2]. This has been possible due to similar features with other higher vertebrates regarding physiology, morphology and histology [3]. In particular, embryonic stages of zebrafish up to 5 days post-fertilisation (dpf) are increasingly being used to address ethical and legal issues of using adult animals and higher vertebrates, as they are not protected under the current

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European Union legislation for the protection of animals used for experimental and other scientific purposes (Directive 2010/63/EU) [4,5].

Notwithstanding, during early developmental periods, most of the laboratory procedures require the sedation and/or immobilisation of the animals for manipulation and/or observation which is typically achieved by mounting specimens in agar mounts or using anaesthetic compounds [6]. MS-222 (tricaine methanesulfonate) is the only fish anaesthetic approved by the Food and Drug Administration (FDA), while in Europe, the European Medicines Agency (EMA) has licensed this anaesthetic to use in some type of fish and under specific circumstances, depending on the country's legislation [7]. It is currently the most used anaesthetic compound for anaesthesia, euthanasia and sedation of fish, both in the wild and in captivity [7,8]. MS-222 is an amino ester, structurally similar to other local anaesthetics, which has recently been suggested as being aversive for this species [9,10]. The action of MS-222 is exerted by reversible interactions with the voltage-gated sodium channels blocking the generation of action potentials [11]. Sodium channels are generally expressed as early as 10 h post-fertilisation (hpf) in zebrafish [12] but the effects of MS-222 exposure on these channels are still unclear [13]. There is evidence that anaesthetics belonging to this class may also block other channels, such as the voltage-gated calcium channels [14], although not yet been reported for MS-222. Calcium channels are important in excitable cells and its expression can be detected in zebrafish during early blastula [15].

Overall, and despite the common use of MS-222, its toxicological properties and molecular and cellular targets remain undetermined [13]. In early larval stages, the exposure to this compound has been shown to induce lethal effects [16]. However, to our knowledge, there are no reported studies assessing MS-222 effects taking advantage of the embryonic stages of zebrafish to study its mode of action and its functional and mechanistic effects on morphology and behaviour, as previously described for other anaesthetics [17]. In this sense, the objective of this study was to investigate the pharmacological impact of MS-222 on sensitive windows of time in zebrafish early developmental stages. Zebrafish embryos were exposed to MS-222 for 20-min periods during the 256-cell (2.5 hpf), 50% epiboly (5.5 hpf) and 1–4 somites (10.5 hpf) stages and several developmental parameters were evaluated.

Materials and Methods

Chemicals

Tricaine methanesulfonate (MS-222, CAS 886-86-2) was purchased from Sigma-Aldrich (Sigma-Aldrich, Steinheim, Germany). All solutions were freshly made with buffered water (28 ± 0.5 °C, 200 mg L⁻¹ Instant Ocean Salt and 100 mg L⁻¹ sodium bicarbonate; UV sterilized) prepared from City of Vila Real filtered-tap water. Instant Ocean Salt was obtained from Aquarium Systems Inc. (Sarrebouurg, France). All other chemicals were analytical or HPLC grade and obtained from Sigma-Aldrich (Steinheim, Germany).

Fish care and maintenance

Zebrafish were maintained according to established procedures at the University of Trás-os-Montes and Alto Douro [18]. Briefly, adult AB zebrafish (D. rerio) were kept at 28 ± 0.5 °C in a photoperiod of 14:10 h light:dark cycle. A circulating water system supplied with aerated, dechlorinated, charcoal-

filtered and UV-sterilized City of Vila Real tap water (pH 7.3–7.5). The fish were fed twice a day with a commercial diet (Sera, Heinsberg, Germany) supplemented with brine shrimp. Male and female fish at a proportion of 1:2 were reared separately for spawning by the onset of room lights. Embryos were collected between 1 and 2 h post-fertilisation (hpf), bleached and rinsed to remove debris as outlined before [6,19]. Fertilized embryos with normal morphology were staged under a SMZ 445 stereomicroscope (Nikon, Japan) according to standard methods [20] and maintained in buffered water. All breeding and bioassays involving zebrafish were conducted under personal and project licenses for this study approved by the Portuguese competent authority, “Direção Geral de Alimentação e Veterinária” (DGAV, Lisboa, Portugal), and in agreement with the European Directive on the protection of animals used for scientific purposes (2010/63/EU) and its transposition to the Portuguese law (Decreto-lei 113/2013) ensuring minimal animal stress and discomfort

MS-222 exposure

A MS-222 stock solution (1500 mg L⁻¹, 5.74 mM) was prepared by dissolving the MS-222 powder in buffered water, and the solution pH was adjusted to 7.4 with sodium bicarbonate in order to neutralize the acidic effects of the compound. The stock solution was stored in a dark brown bottle at 4 °C. The working solutions were prepared by dilution of the stock solution in buffered water to the desired concentrations immediately before its experimental use. Fig. 1 shows a schematic diagram of the experimental design. Static exposures of 20 min to MS-222 concentrations (50, 100 and 150 mg L⁻¹, respectively 191, 383 and 574 µM) were performed during early 256-cell (2.5 hpf), early 50% epiboly (5.5 hpf) and 1–4 somites (10.5 hpf) stages. Test concentrations were selected based on the anaesthetic profile of MS-222: 50 mg L⁻¹ was chosen as a light sedative dose, 100 mg L⁻¹ as a deep sedative level and 150 mg L⁻¹ as a surgical anaesthetic dose [21]. An additional control group in buffered water was used. Following exposure, the embryos were washed three times in buffered water and allowed to develop up to 144 hpf, with daily water renewal. Experiments were performed using at least six independent replicates containing 100 viable embryos per replicate to evaluate mortality, lethal and non-lethal parameters as well as for behavioural experiments. Additional experiments with three independent replicates of 300 and 100 viable embryos per replicate were used for gene expression studies and for cartilage and bone evaluation, respectively. HPLC MS-222 quantification was performed in three samples of 100 embryos for each concentration. Throughout all procedures, the temperature was kept at 28 ± 0.5 °C

MS-222 uptake by high-performance liquid chromatography

The accumulation of MS-222 at the different developmental stages was evaluated immediately after the end of the 20-min exposure. Additionally, in order to infer how much MS-222 was absorbed by the embryo, and assuming that the equilibrium between the external concentration and the

Figure 1: Schematic diagram showing the timing of MS-222 exposure in zebrafish early development.

Independent exposures to MS-222 concentrations (0, 50, 100 or 150 mg L⁻¹) were performed in buffered water for a period of 20 min during three different developmental stages: blastula (256-cell), gastrula (50% epiboly) and segmentation (1–4 somite). At the end of each exposure, the uptake of MS-222 by the embryo was determined by HPLC-UV. Mortality was checked daily and lethal and sublethal parameters evaluated at specific time points. During the experimentation period, gene expression changes of sonic hedgehog a (*shh a*) and noggin 3 (*nog3*) were investigated by quantitative real-time PCR. At the end of the experiment, abnormal morphological characteristics were quantified using a morphological scoring and cartilage and bone were assessed using alcian blue and calcein staining, respectively. At 144 hpf (hours post-fertilisation), the effects of MS-222 exposure on some behavioural features were also evaluated.

concentration in the chorion and perivitelline (PVS) space occurs within a few minutes of exposure [22], a short exposure of 5 min was also performed to allow the MS-222 to enter into these structures, but not into the embryo. The amounts of MS-222 detected after this short exposure period were subtracted to the MS-222 detected after the 20 min exposure, resulting in the amount of MS-222 present in the embryo [22]. To quantify the anaesthetic after both exposures, samples were collected and prepared as previously described [23] with slight modifications. Briefly, surviving animals were collected to micro centrifuge tubes and the buffered water was replaced by ultrapure water after washing embryos three times. The samples were then stored at -80 °C until analysis. Thereafter, the ultrapure water was replaced by 0.5 mL of extractive solution (50:50 (v/v) methanol: McIlvaine buffer pH 4.4) and homogenised on a Tissue Lyser II (Qiagen, Hilden, Germany), at 30 Hz for 90 s. Samples were then centrifuged at 1500 xg for 3 min at room temperature. These steps were repeated twice and the combined upper layer was evaporated to dryness at 45 °C in a centrifugal evaporator (Univapo 150ECH; UniEquip, Munich, Germany). The dry residue was reconstituted in 100 µL of the mobile phase, which consisted of methanol:water (11:9). A volume of 20 µL of each sample was injected in a Dionex UltiMate 3000 HPLC system (Dionex, Olten, Switzerland) coupled with a diode array detector (Dionex PDA 100 photodiode array, Dionex, Olten, Switzerland) at 220 nm. Chromatographic separation was executed using a reversed phase ACE C18 Ultra-Inert C18 (250 × 4.6 mm, 5 µm) at 40 °C and using a flow rate of 1 mL/min. Data was acquired by the Dionex Chromeleon Software 6.70 Build 1820. MS-222 was quantified by integrating the area under the monitored peaks and comparing to known MS-222 standards (0–25 mg/L or 0–95.7 nM) prepared in methanol:water (11:9). From the linear correlation plot of the integrated areas versus standard concentrations, the amount of nanomoles (nmol) was interpolated in each sample. The amount absorbed by each embryo (nmol) was calculated analytically, by dividing the amount of MS-222 detected by the number of animals collected after 5 and 20 min of exposure.

Developmental toxicity

The embryo teratogenicity assay was conducted as previously described [17,18]. Embryos were observed daily until 144 hpf for mortality and dead embryos/larvae were removed, only being included in the mortality analysis. Somite formation, tail detachment and head development were evaluated at 24 hpf while the presence of heartbeat, edema and pigmentation was evaluated at 48 hpf. At both time points, these parameters were collected as present or absent. At 48 hpf, the heart rate (beats per minute) was calculated by counting the number of beats over a period of 15 s. At 72 hpf, the frequency of hatching rate and skeletal malformations were determined, whereas at the last day of the experiment, 144 hpf larvae were immobilised in 3% methyl-cellulose [24] and cumulative skeletal deformities evaluated. The standard body size of the larvae was assayed using a digital image analysis (Digimizer version 4.1.1.0, MedCalc Software, Mariakerke, Belgium). All these observations and imaging of phenotypes were done in 12 random zebrafish embryos from each replicate (at least four independent replicates) using a colour digital CCD camera (Color View III, Olympus, Hamburg, Germany) mounted on an inverted microscope (IX 51, Olympus, Antwerp, Belgium). Serial zebrafish images were combined, merged and processed with Adobe Photoshop CS6 (Adobe Systems, San Jose, USA) and blind-scored for anomalies including fins, heart and thorax, head, eyes, organs and tail/spine malformations using a rating scale (0 to 4, according to increasing severity) [18,25].

Whole-mount cartilage and bone staining

Alcian blue and calcein staining procedures were used for the evaluation of cartilaginous and bone structures, respectively, in 144 hpf larvae following early exposure to MS-222 as described previously [17,26,27]. Briefly, 12 larvae from each replicate were euthanised by immersion in an ice-cold bath and fixed overnight in 4% paraformaldehyde before being dehydrated and stained using 0.02% alcian blue in 70% ethanol with 60 mM MgCl₂ overnight. The samples were posteriorly bleached (3% H₂O₂ and 2% KOH) to remove excess pigmentation and larvae soft tissues were digested in 0.05% trypsin in PBS [28] then gradually cleared through a series of glycerol/KOH (20%/0.25%; 50%/0.25% and 80%/0.1% for storage). For bone structures, 12 larvae from each replicate (n = 3) were immersed in a 0.2% fluorescent calcein solution (pH 7.2 with NaOH) for 10 min in the dark and at room temperature. After staining, neurocranial (ethmoid plate, basicranial commissure, trabecula cranii, basal plate and occipital arch), pharyngeal cartilages (basihyal, ceratobranchial arches, ceratohyal, hyosymplectic, interhyal, Meckel's, palatoquadrate, basibranchial and hypobranchial) as well as vertebrae, cephalic dermal bones (parasphenoid, branchiostegal rays, operculum, entopterygoid, dentary and cleithrum), cartilage-replacement bone (CB5 ossification), occipital and notochord bones were observed under light and fluorescent microscopy using an inverted microscope (IX 51, Olympus, Antwerp, Belgium) and a colour digital CCD camera (Color View III, Olympus, Hamburg, Germany). Quantification of cartilage and bone malformations was assessed in a blinded and semi-quantitative approach according to the figures and severity rate presented in [29]: 0 as the negative control, and increasing the severity grade from 1 to 3. The head length was also measured by digital image analysis (Digimizer version 4.1.1.0, MedCalc Software, Mariakerke, Belgium) as described previously [29].

Quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA from a pool of 50 RNAlater-fixed embryos at 8 and 24 hpf was extracted using the Illustra RNAspin kit (GE Healthcare, Munich, Germany) following the manufacturer's standard protocol with slight modifications and synthesised into cDNA by reverse transcription as previously outlined [17]. Briefly, samples were homogenised on a TissueLyser II (Qiagen, Hilden, Germany), at 30 Hz for 90 s and the extracted RNA was treated with RNase-free DNase I for 1 h at room temperature. RNA was eluted into 40 µL of RNase free water and quantified by spectrophotometer readings at 260 and 280 nm (Powerwave XS2, BioTek Instruments, Inc. USA). RNA integrity and DNA contamination were evaluated by 1% agarose gel electrophoresis, using green safe premium staining (NZYTech, Ltd., Lisbon, Portugal). For each sample, 500 ng of total RNA was used as a template for reverse transcription using the iScript cDNA synthesis kit (Bio-Rad Laboratories, California, USA) following the manufacturer's instructions under the following conditions: annealing at 25 °C for 5 min, reverse transcription at 42 °C for 20 min and heat-inactivating transcriptase at 95 °C for 1 min in a Bio-Rad Mycycler personal thermal cycler (Bio-Rad Laboratories California, USA). Larvae cDNA was amplified using HOT FIREPol Eva Green qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and specific primers for sonic hedgehog a (shh) and noggin3 (nog3) signaling pathways (Table 1) in a Stratagene Mx3005 P Real-Time PCR system (Stratagene, Agilent Technologies, Santa Clara, USA). Shh was chosen due to its role in cell fate determination and embryonic patterning during early vertebrate development, as well as its role in the formation and function of a variety of tissues and organs in later developmental stages [30,31]. The nog3 was chosen, considering its association with skeleto-genic processes [32]. Changes in these pathways result in craniofacial deformations as already showed with other anaesthetics [17]. All reactions were performed in duplicate on three sets of independent samples according to the manufacturer's instructions. Briefly, the reaction mixture (20 µL) consisted of 4 µL of 5× Eva Green master mix with ROX as reference dye, 200 nM of each specific primer (STABvida, Lisbon, Portugal) and 1 µL cDNA with 40 cycles of denaturation at 95 °C for 20 s, primer annealing for 40 s and extension at 72 °C for 20 s followed by a final extension at 72 °C for 5 min. Melting curve analyses were performed to validate the specificity of PCR and to exclude the interference of primer-dimers and DNA. The β-actin gene was used as housekeeping to normalise the data as its expression levels were not significantly different between control and treated groups. Data were analysed according to the efficiency corrected calculation model of Pfaffl [33] based on standard curves of a 3 to 5-fold dilution series prepared from a mix of the used samples. Data were presented as the fold change in gene expression relative to the control group at each sampling point.

Table 1 - Information of specific primers used for amplification in real-time PCR with GenBank accession numbers shown in parentheses

Gene	Primer Sequence (5'-3')	Fragment size (bp)	Annealing temperature (°C)	R ²	Efficiency (%)
<i>β-actin</i> [86] (NM_181601.4)	Fwd - ACT GTA TTG TCT GGT GGT AC	197	60	0.993	94.0
	Rev - TAC TCC TGC TTG CTA ATC C				
<i>shh a</i> [87] (NM_131063.2)	Fwd – GCA AGA TAA CGC GCA ATT CGG AGA	117	60	0.993	105.0
	Rev – TGC ATC TCT GTG TCA TGA GCC TGT				
<i>nog3</i> [88] (NM_130982.1)	Fwd – ACC GTG CTC ATT TTC TCC CTC GGG	389	60	0.969	125.4
	Rev – GAC CAG AGC CAC AGC TGA AGC C				

Videotracking-based behavioural procedures

The behaviour of exposed larvae was evaluated at 144 hpf during the light period at 28 ° C using an automated video-tracking system as previously described [34,35] and an inverted 15.6" laptop LCD screen (1366 × 768 pixel resolution, an average brightness of 173.6 cd m⁻² and a contrast of 208:1

with a black level of 0.83 cd m⁻²). The behavioural tests consisted of randomly placing one morphologically non-malformed larva in each well of a 6-well F-bottom multiwell plate (Greiner Bio-one item-No. 657102, 35 mm diameter) with 3 mL of buffered water. Each well had a circular swimming area of 27 mm created by cutting 5 mL of melted 0.5% agarose in a ring [35]. The videos were recorded at maximum resolution of 1920 × 1080 (30 fps) from the top (50 cm) with a Sony Nex-5 digital camera (14.2 megapixel APS-C CMOS sensor, Sony International, Europe), equipped with a zoom lens (Sony SEL1855, E 18–55 mm, F3.5–5.6 OSS zoom). Experiments were performed in such a way that all groups were equally present in each well plate to minimise differences in experimental timing. The quantification of behaviour parameters was achieved using TheRealFishTracker software [36] after calibration by inputting well dimensions. After 5 min of acclimation, the following behaviour parameters were quantified during the 10 min of video recording: mean speed, total distance moved, mean distance to the centre zone (i.e. distance to a 5 mm radius circle drawn in the centre of the well) to assess thigmotaxis related to anxiety-like behaviours, percentage of time active, and mean absolute turn angle. The mean absolute turn angle is equivalent to the positive/absolute mean difference between changes in direction of the centre point of the animal between consecutive frames during the recorded interval.

Statistical analysis

To generate information regarding power and biological sample sizes in the context of the analysis performed in this study, power analysis was performed in G*Power 3 (version 3.1.9.2, University of Düsseldorf, Germany). The sample sizes used for each parameter were calculated to detect a potentially significant effect (at least with 25% difference between groups) with a power of 95% and with an alpha error of 0.05 based on data from previous studies in which similar approaches were used [17,18,37]. Normality of data (Shapiro–Wilk's test) and homogeneity of variance (Levene's test) were tested prior to hypothesis testing. When data were not normally distributed, Kruskal-Wallis' test followed by Dunn's pairwise comparison tests was applied to investigate statistical differences and data expressed as median and interquartile range. When the data was normally distributed one-way analysis of variance (ANOVA) was applied followed by Tukey's pairwise comparison tests and data expressed as mean ± standard deviation. When different groups have data following both parametric and non-parametric distributions, both are expressed as median and interquartile range as it is a more conservative approach. Categorical data, expressed as a percentage, was analysed on log-transformed data using the one-way ANOVA followed by multiple comparisons with Bonferroni correction and then presented as back-transformed mean of the log transformed variable and upper and lower limits from the 95% confidence interval. Non-detected values were assigned a zero value for statistical purposes. For each parameter, significant differences ($p < 0.05$) between group means were calculated using SPSS for Windows (Version 22.0; Chicago, IL, USA).

RESULTS

MS-222 absorption

The relationship between MS-222 tested concentrations and their absorption by the embryos immediately after exposure in each developmental phase is depicted in Table 2. In control samples,

no MS-222 was detected. Under the chromatographic conditions described in the previous section, the retention time of MS-222 was 5.83 ± 0.15 min and the method was found to be linear over the concentration range tested ($r^2 = 0.999$). After 20 min of incubation during the 256-cell phase, the accumulation of MS-222 in the embryo was 47.2 ± 38.9 , 27.7 ± 4.65 and 96.7 ± 64.4 pmol for the lowest, middle and highest concentration tested, respectively. The embryos exposed during 50% epiboly stage ($F(2,8) = 1.735$, $p = 0.316$) presented mean levels of MS-222 reaching the embryo of 22.6 ± 4.94 , 80.4 ± 34.5 and 125.3 ± 89.2 pmol in the 50, 100 and 150 mg L⁻¹ groups, respectively. The intermediate and high concentration exposed during 1–4 somites stage presented mean amounts of MS-222 in the embryo of 19.8 ± 11.1 and 152.9 ± 91.6 pmol, respectively, while the low MS-222 concentration was not detected in the embryo. The absorption of MS-222 was not statistically different between groups in all the stages of development studied.

Table 2 - Concentrations of MS-222 quantified from zebrafish eggs after 5 min (corresponding to the concentration in the chorion and in the PVS) and 20 min (total concentration) exposure to concentrations of 50, 100 and 150 mg L⁻¹ (respectively 191, 383 and 574 μ M) at each developmental stage. Concentration in embryo was calculated from the difference between the amount of MS-222 detected after the 20 min exposure and the 5 min exposure assays.

Developmental phase	Concentration (μ M)	5 min exposure (pmol/embryo)	20 min exposure (pmol/embryo)	Concentration in embryo (pmol)
Blastula (256-cell)	191	43.8 ± 3.51^a	90.0 ± 42.4^a	47.2 ± 39.0
	383	42.2 ± 0.59^a	69.9 ± 5.23^a	27.7 ± 4.65
	574	130.0 ± 5.93^b	226.6 ± 68.0^b	96.6 ± 64.4
Statistical test		$F(3,8)=374.9$	$F(3,8)=7.717$	$F(3,8)=1.453$
<i>p</i>		<0.0001	0.030	0.318
50% Epiboly	191	50.2 ± 6.02^a	72.8 ± 11.0^a	22.6 ± 4.93
	383	101.8 ± 5.33^b	182.2 ± 29.1^{ab}	80.4 ± 34.5

	574	168.8 ± 19.5 ^c	294.1 ± 69.8 ^b	125.3 ± 89.2
Statistical test		F(3,8)=47.85	F(3,8)=12.58	F(3,8)=1.735
p		0.005	0.035	0.316
1-4 somites	191	80.9 ± 5.28 ^a	59.0 ± 12.8 ^a	ND
	383	141.8 ± 8.94 ^{ab}	161.6 ± 2.19 ^a	19.8 ± 11.1
	574	183.4 ± 44.2 ^b	336.2 ± 67.7 ^b	152.9 ± 91.6
Statistical test		F(3,8)=9.825	F(3,8)=30.82	F(3,8)=5.894
p		0.019	0.002	0.052

Data from 3 independent replicates expressed as mean ± SD. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison. Different lowercase letters indicate significant differences between groups ($p < 0.05$). ND: not detected.

Mortality after exposures at the 256-cell and 50% epiboly stages

Cumulative mortality for each MS-222 exposure is presented in Fig.2. The mortality of the control group was found not to exceed 20% during the experimental period. Increases in embryo mortality were observed after exposure to MS-222 during the 256-cell stage comparatively to the control group. Although data had a high variability, significant differences ($X^2(3) = 8.262$, $p = 0.041$) were observed at 48 hpf between MS-222 exposed embryos and the control group ($p = 0.026$, 0.013 and 0.038, respectively to 50, 100 and 150 mg L⁻¹ groups). No significant differences between MS-222 groups were observed. At later time points, the statistical differences remained ($X^2(3) = 9.390$ and $p = 0.025$ for both time points) between MS-222 groups and the control group ($p = 0.014$, 0.012 and 0.021, respectively to 50, 100 and 150 mg L⁻¹ groups). In the following developmental stage, 50% epiboly, a significant difference was found at 48 hpf ($F(3,19) = 3.800$, $p = 0.031$) between the highest MS-222 concentration and the control group ($p = 0.023$). However, this difference was not observed at later time points, as the mortality of the control group had a slight increase. Relative to the 1-4 somites stage exposure, the mortality in MS-222 exposed groups was similar to the one observed in the control group ($p > 0.05$).

Figure 2: Cumulative mortality rate of zebrafish embryos after exposure to MS-222 at 256-cell, 50% epiboly and 1–4 somite stage. The mortality of the embryos increased in a dose-, time- and stage-dependent manner. Overall, mortality of embryos is reduced as exposure is performed in more developed embryonic stages. Data are expressed as median and interquartile range (blastula) or as mean and standard deviation from at least five independent experiments. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p < 0.05$) at each developmental stage.

Interference of MS-222 in zebrafish embryo testing

Lethal and sub-lethal responses elicited by MS-222 exposures are presented in Supplementary Tables 1 and 2 and morphological analyses results are shown in Supplementary Tables 3–5. Overall, lethal and sub-lethal endpoints were not significantly induced by exposure to MS-222. Regarding morphological alterations, only embryos that were exposed to the highest concentration of MS-222 at the 256-cell stage showed a lower incidence of stunted ($X^2(3) = 9.476$, $p = 0.024$) and malformed ($X^2(3) = 9.130$, $p = 0.028$) fin abnormalities compared to the other exposed groups (malformed fins: $p = 0.045$ and $p = 0.003$; stunted fins: $p = 0.037$ and $p = 0.004$, compared with the lowest and middle dose of MS-222, respectively).

MS-222 exposure at 50% epiboly promotes abnormal cartilage development

The effects of MS-222 exposures on skeletal, bone and cartilage development are summarised in Supplementary Tables 6–8, while representative images of craniofacial abnormalities are presented in Fig. 3. The 20 min exposure during the 256-cell and 1–4 somites stages did not cause significant changes in skeletal structures. However, when exposure was performed during the 50% epiboly stage, changes in pharyngeal cartilages were observed. The palatoquadrate cartilage, a mandibular structure, was shortened and flat or presented irregular lengths ($X^2(3) = 7.940$, $p = 0.047$) in the groups exposed to 100 and 150 mg L⁻¹ compared to the control group ($p = 0.012$ and $p = 0.020$, respectively). Likewise, cartilaginous structures such as hypobranchial and basihyal arches were also affected in the same way ($X^2(3) = 7.940$, $p = 0.047$ in both cases), showing reduced size or damages with irregular shapes in the groups exposed to the lowest ($p = 0.012$) and highest ($p = 0.020$) concentration of MS-222 compared to the control group. No other statistical differences were observed.

Figure 3: **MS-222 exposure during 50% epiboly cause malformations of cartilage and bone in zebrafish.** A lateral view (A) shows that in control animals all cartilages and bones appear normally developed. Cartilage malformations are observed after exposure to higher concentrations of MS-222 dose, namely in the palatoquadrate (pq), basihyal (bh), and hypobranchial (hb) arches with different positions and different angles between the structures. A ventral view (B) of exposed animals also shows the same malformations during this developmental stage. Scale bar represents 500 μm

MS-222 up-regulates developmental-related genes at 24 hpf

After MS-222 exposure, sonic hedgehog a (*shh a*) and noggin 3 (*nog3*) gene expression were determined at 4 time-points and data are presented in Fig. 4. There was no significant effect on the *shh a* gene expression following exposure at the 256-cell stage. Similarly, exposure during the 50% epiboly stage did not significantly affect the expression of *shh a* despite a three-fold increase of this gene expression at 24 hpf ($X^2(3) = 7.205$, $p = 0.066$). However, MS-222 exposure during 1–4 somites stage induced significant changes ($F(3,11) = 15.393$, $p = 0.01$) at 24 hpf ($p = 0.026$) which were subsequently abolished at later time points. In comparison to the control group, at 24 hpf, *shh a* levels increased 3.97- ($p = 0.07$) and 3.57-fold ($p = 0.031$), respectively for 100 and 150 mg L^{-1} . The qRT-PCR results showed that there were no statistical differences between groups in *nog3* transcripts when embryos were acutely exposed during the 256-cell stage. However, significant changes were observed when exposures were performed during the 50% epiboly and 1–4 somites stage at 24 hpf ($F(3,11) = 7.360$, $p = 0.011$ and $F(3,11) = 8.181$, $p = 0.008$, respectively). Therefore, when exposure was performed during the 50% epiboly stage, a 3.52-fold increase in *nog3* transcripts was observed for the highest MS-222 concentration relative to the control group ($p = 0.007$). In the 1–4 somites stage, it was the exposure to 100 mg L^{-1} that induced a 3.34-fold increase in the expression of this gene relatively to the control group ($p = 0.005$).

Figure 4: RNA transcript levels of developmental-related genes (*shh a* and *nog3*) following exposure to MS-222. Gene expression changes were analysed at 8, 24, 72 and 144 hpf and normalized to β -actin levels. Data resultant of three independent samples ($n = 50$ /each) and expressed as mean $\pm$ standard deviation. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test. Different lowercase letters indicate significant differences between groups ($p < 0.05$) at each developmental stage.

Larvae locomotor activity reduced after exposure at 50% epiboly

The analysis of video-tracking regarding behavioural parameters with significant differences is shown in Fig. 5, while the remaining data are presented in Supplementary Fig. 1. The 20 min exposures during the 256-cell and 1–4 somites stages did not induce significant changes in behavioural responses compared to the control group. However, when embryos were exposed during the 50% epiboly stage, larvae exhibited different behavioural responses compared to the control group. Dose-dependent decreases were observed for the mean speed ($F_{(3,19)} = 3.998$, $p = 0.027$), for the total distance moved ($F_{(3,19)} = 4.856$, $p = 0.014$) and for the percentage of time larvae were active ($F_{(3,19)} = 7.466$, $p = 0.002$). This reduction of locomotor activity was only significantly different from the control group in the group exposed to 150 mg L⁻¹ ($p = 0.028$ for mean speed, $p = 0.027$ for total distance moved and $p = 0.003$ for percentage of time active). Statistical differences between the groups with the lowest and highest dose were also observed for the total distance moved ($p = 0.017$) and for the percentage of time larvae were active ($p = 0.009$). Furthermore, the exposure to the middle and highest MS-222 concentrations during the 50% epiboly also resulted in a significant reduction in the absolute turn angle compared to the control group ($p = 0.037$ and $p = 0.002$, respectively). Also, the larvae exposed to the lowest dose of MS-222 had a higher absolute turn angle compared with the ones exposed to the highest MS-222 dose ($p = 0.016$).

Figure 5: **MS-222 early exposure effects on exploratory behaviour at 144 hpf larvae.** (A) Swim speed, (B) total distance moved, (C) percentage of time larvae were active, and (D) mean absolute turn angle were decreased when exposure to the highest MS-222 concentration was performed at 50% epiboly stage. Values are expressed as mean ± standard deviation. Data from at least five independent replicate exposures (n = 5 replicates with six animals each per group). Different lowercase letters indicate significant differences between groups (p < 0.05, one-way ANOVA followed by Tukey's multiple-comparison test) at each developmental stage

DISCUSSION

The present study was designed to evaluate the developmental toxicity of MS-222 during zebrafish embryogenesis. To our knowledge, this is the first report that describes the toxicological effects of MS-222 in the early stages of zebrafish development, showing that zebrafish embryos exposed for a short period are vulnerable to anaesthetic concentrations of MS-222 with more pronounced effects when exposure occurs during the 256-cell or 50% epiboly stages. Embryos exposed during the 256-cell stage only showed changes in survival, while embryos exposed during 50% epiboly stage had increased mortality, cartilage and bone malformations, up-regulation of developmental genes and alterations on long-term behaviour, denoting the existence of a sensitive window to MS-222 during zebrafish early developmental period. While there were alterations on several parameters in embryos exposed during 50% epiboly stage, the mortality was higher when embryos were exposed during 256-cell stage. This may have eliminated potentially malformed animals, therefore, no difference was detected on the other parameters in the earliest stage of exposure.

When exposure was performed during the 256-cell stage, the intermediate concentration had the lowest MS-222 absorption to the embryo. In this early developmental stage, both the chorion and the aqueous PVS are assumed to play a protective role against toxicants and can influence the determination and interpretation of internal concentration profiles [22,38]. In this regard, and although MS-222 is a lipid-soluble compound with a rapid diffusion across lipid membranes [21], the

reduced flow limit may be explained by a hampered passage of MS-222 through the pore channels of the chorion as the penetration forces are higher at 256-cell and 50% epiboly stage [39]. Indeed, the embryos exposed to 150 mg/L had higher MS222 concentrations quantified at the later stages than in the 256-cell stage, which may be related with the chorion softening in later stages, allowing MS-222 absorption according to the concentration provided. Yet, when the 1–4 somites stage was exposed to the lowest concentration, MS-222 was accumulated in the chorion and PVS rather than in the embryo. These observations could have resulted from toxicodynamic or toxicokinetic changes that influence diffusion and accumulation of MS-222 in the embryo. In this regard, further detailed studies would unravel how chorionic structures and age influence the uptake of MS-222. In agreement, and although longer exposure times may increase internal concentrations and reduce effect concentration as observed in another anaesthetic compound [40,41], it has been described that the toxicological outcomes following exposure to compounds during these early life stages are rather related to the toxicity of the compound and to differences in the chorion permeability, rather than to differences in the internal concentrations [38,42]. In fact, in the current study, a dose-dependent increase in mortality was observed following exposure in the 256-cell stage, without showing the highest internal concentration of the drug. In addition, a previous study with other anaesthetic compound has shown stage-dependent teratogenicity with highest sensitivity when embryos were exposed for 20-min at the 256-cell stage [17]. The early embryonic development of zebrafish is a highly complex regulated and coordinated process [43]. At these early stages and until the onset of the transcription at the zygotic genome activation, which occurs at approximately 3 hpf [44], embryo development is dependent upon maternal factors [45]. However, several transcripts are detected as early as the 64-cell stage (around 2 hpf) [46,47]; for instance, expression of *pou5f1/pou2*, a homolog of mammalian Oct4 [48], has been detected during the 256-cell stage [46]. *Pou5f1* is a key regulator for the control of the zygotic gene activation and is critically required for the correct early embryonic development [48–50]. Without *Pou5f1* expression, embryos die due to a disorganised development involving a reduced bone morphogenetic protein (BMP) signaling [51] and changes in cell adhesion and motility [52,53]. In zebrafish embryos, BMP activity is evident during early blastula [54], being this early stage also marked by the development of the calcium signaling [55], which represents the beginning of the coordinated developmental networks [56]. Increases in calcium levels have been shown to relate with an inhibition in BMP [57]. It has also been reported that the calcium signaling pathway can be modulated by several secreted factors belonging to the Wnt family [58], that are thought to play a role in a variety of developmental events [59]. Blastula cells are responsive to Wnt signaling [60]; furthermore, evidence is emerging that anaesthetics induce changes in Wnt signaling response during development [61]. In general, different signaling pathways act cooperatively and redundantly throughout early development in order to guarantee the correct development of the embryo. Therefore, it is possible that exposure to MS-222 during this early zebrafish phase promotes defective changes in some factors involved in some of these early signaling pathways which may not be compatible with cell survival. Indeed, defective early developmental pathways and/or protein modulators have been shown to significantly reduce the number of cells at late blastula stages, as well as induce various morphological defects later on development [62,63]. Still, little is known about the molecular events and mechanisms regarding these signals during early development after exposure to chemical compounds, and, in particular, after exposure to MS-222. Thus, further molecular evidences are required to support this hypothesis.

During early gastrula (50% epiboly), survival was also affected, although the mortality was not as high as in the previous stage. In addition, abnormal cartilage and behavioural defects, as well as changes at the transcript level of *nog3* at 24 hpf, were evident in MS-222 treated animals. Thus, this

period revealed to be the most sensitive early developmental stage to MS-222. The zebrafish gastrula comprises a set of critical morphogenetic movements that ultimately organise the internal and external animal [64,65]. In particular, during 50% epiboly, as a consequence of zygotic genome activation, and rearrangement into three distinct germ layers, a series of movements of gastrulation begins simultaneously, which will determine cell fates [66]. These movements are mediated by signaling pathways such as the Wnt/planar cell polarity (PCP) pathway [65,67]. Changes in this pathway, emerging from abnormal gastrulation movements, have been related with organogenesis defects [68]. In this regard, the protein Noggin₄ has been shown to modulate this pathway, inducing gastrulation impairment and defective phenotypes in *Xenopus laevis* through the activation of the non-canonical proteins Wnt11 and Wnt5a [69]. Changes in the expression of non-canonical proteins (down and up-regulation) inhibit morphogenetic movements during gastrulation [70]. In addition, Wnt signals control the expression of myogenic regulatory factors such as myf5 [71], a myogenic regulatory factor required for the correct craniofacial cartilage development in zebrafish embryos [72]. The expression of myf5 can be blocked by inhibiting Wnt signaling [73], thus, leading to the disruption of cranial cartilage development with several defective cranial cartilages [72] similar to those observed in this study. Moreover, the observed behavioural phenotypes are consistent with the craniofacial defects, such as the difference on the absolute turn angle, which performance reflects issues on morphological development [74]. Although this has not yet been described in zebrafish, and knowing that the Wnt signaling pathway is under complex regulation during development, these findings support the view of a central interaction of MS-222 with noggin transcripts resulting in the inhibition of Wnt signaling and the consequent lasting effects. Moreover, evidence supports a direct crosstalk between noggin, Wnt and Shh pathway for the induction of myogenesis [71,75], and amino anaesthetics as MS-222 have been shown to affect myogenesis [76], further supporting this hypothesis. Notwithstanding, it is worth noting that according to the literature, transcripts of noggin₃ are first detected at 48 hpf [32]; therefore, a more detailed molecular analysis is required to elucidate the role of MS-222 during this early stage of development. Thus, MS-222 may still be influencing a maternal transcript when an increase is observed on nog₃ expression at 24 hpf. The decrease on larvae activity induced by the MS-222 is in line with reports of local anaesthetics inducing hypo-activity when exposed during mid-pregnancy in rats [77]. Also, the observed defective morphological development of cartilaginous structures may physically impair a proper locomotion.

While the above data clearly indicates an effect of MS-222 at gastrula stage, the same was not observed for the exposure during segmentation (1–4 somites), where only changes at the transcriptional levels of shh and nog₃ were observed at 24 hpf. These alterations were not reflected in any morphological or behavioural defects. It is worth noting that, during this period, the molecular targets of MS-222 (sodium channels) are already detected in the embryo [12] but the factors that regulate these channels are still unclear [13]. During this stage, several developmental processes are evident such as the development of somites [20,78] based on the activation of several signaling pathways that create oscillations in gene expression [78]. Perturbation of these pathway results in abnormal somitogenesis and, at later stages, in spinal deformities or truncations [79,80]. Yet, no structural malformations were observed in zebrafish embryos exposed to MS-222. However, the overexpression of shh mRNA has been related with flattened somites [81], as well as the expansion of slow muscle [78], while the overexpression of noggin genes causes dorsalisation of the embryo [82,83]. The alteration of gene expression in this study may be related to changes in some early molecular signaling factor that may induce defective somite phenotypes after exposure of MS-222 that were not explored in this study. Evidence has emerged that noggin and Shh function synergistically to stimulate the expression of other genes during embryogenesis [84,85]. However,

further experimental evidence is required in order to help to explain the possible effects mediated by these two signals following exposure to MS-222 during early somitogenesis.

Although this study was not proposed to explain the presumed mechanism of action of MS-222 during early development of zebrafish, the data obtained provides relevant toxicological information that can be helpful for further studies and supports a differential sensitivity in the early embryonic stages of zebrafish. As such, MS-222 interferes with early embryonic development of zebrafish, causing dose- and stage-specific effects during the blastula and especially, during the gastrula stage. Overall, the results obtained may have functional implications in zebrafish and in other aquatic models used in research, in which MS-222 is used as an anaesthetic agent for routine procedures. Even though longer exposure times still need to be tested to assess MS-222 overall sensitivity and toxicity in zebrafish early life stages, the data obtained allowed to identify sensitive windows of exposure and support a concept in which MS-222 interacts with other signaling pathways and cellular elements, rather than only with voltage-gated sodium and calcium channels during embryogenesis.

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Table S1 - Frequency (%) of lethal endpoints identified at 24 and 48 hpf in zebrafish embryos after acute exposure to MS-222.

Developmental phase	Dose (mg L ⁻¹)	24 hpf			48 hpf
		No somites formed	No tail detachment	No head developed	No heartbeat
Blastula (256-cell)	0	0.44 (0.00-2.70)	0.44 (0.00-1.45)	0.44 (0.00-2.70)	ND
	50	0.60 (0.00-4.40)	2.08 (0.00-2.11)	0.44 (0.00-2.70)	2.00 (0.00-9.61)
	100	ND	2.34 (0.00-2.82)	ND	ND
	150	0.44 (0.00-2.70)	0.44 (0.00-1.45)	ND	0.44 (0.00-2.70)
	Statistical test	$F(3,20)=0.349$	$F(3,20)=0.811$	$F(3,20)=0.667$	$F(3,20)=2.857$
<i>p</i>		0.790	0.503	0.582	0.063
Gastrula (50% epiboly)	0	ND	ND	ND	1.08 (0.00-2.56)
	50	ND	ND	0.55 (0.00-1.67)	1.48 (0.00-6.59)
	100	1.41 (0.00-2.33)	2.74 (0.00-2.74)	1.41 (0.00-2.33)	1.48 (0.00-6.59)
	150	ND	1.74 (0.00-3.04)	ND	3.43 (0.00-3.06)
	Statistical test	$F(3,20)=2.247$	$F(3,20)=2.444$	$F(3,20)=1.410$	$F(3,20)=0.318$
<i>p</i>		0.128	0.094	0.269	0.812
Segmentation (1-4 somites)	0	ND	0.44 (0.00-2.70)	ND	ND
	50	0.44 (0.00-2.70)	ND	0.44 (0.00-2.70)	0.44 (0.00-2.70)
	100	ND	ND	2.34 (0.00-9.61)	ND
	150	ND	ND	ND	1.08 (0.00-5.84)
	Statistical test	$F(3,20)=1.000$	$F(3,20)=1.000$	$F(3,20)=2.857$	$F(3,20)=1.410$
<i>p</i>		0.413	0.413	0.063	0.269

Categorical parameters are presented as back-transformed mean of the log transformed variable and 95% confidence interval. At least 4 independent replica of 12 animals each were used and statistical test was one-way ANOVA followed by Tukey's multiple-comparison test ($p < 0.05$). ND: malformation not detected.

Table S2 - Frequency (%) of sublethal endpoints identified at 48, 72 and 144 hpf in zebrafish embryos/larvae after acute exposure to MS-222.

Developmental phase	Dose (mg L ⁻¹)	48 hpf			72 hpf		144 hpf	
		Edema presence	Pigmentation not visible	Heart Rate (bpm)	Hatching rate	Skeletal Deformities	Skeletal Deformities (cumulative)	Body Length (mms)
Blastula (256-cell)	0	0.48 (0.00-1.32)	ND	159±18	77.8 (33.3-100)	0.00 (0.00-2.08)	12.5±4.17	4.32±0.22
	50	0.53 (0.00-1.51)	ND	157±22	100 (41.7-100)	12.5 (0.00-25.0)	14.2±6.97	4.26±0.30
	100	0.77 (0.01-1.84)	0.44 (0.00-2.70)	160±22	100 (31.9-100)	16.7 (8.33-25.0)	18.3±6.32	4.15±0.19
	150	1.31 (1.10-1.54)	ND	158±20	92.3 (43.4-100)	16.7 (0.00-25.0)	15.8±7.45	4.39±0.35
Statistical test		$F(3,20)=1.582$	$F(3,20)=1.000$	$F(3,23)=0.022$	$X^2(3)=3.198$	$X^2(3)=7.347$	$F(3,19)=0.767$	$F(3,23)=0.881$
p		0.225	0.413	0.995	0.362	0.062	0.529	0.467
Gastrula (50% epiboly)	0	0.31 (0.00-1.04)	ND	164±22	100	ND	10.0±6.97	4.19±0.16
	50	0.77 (0.10-1.84)	ND	150±13	100	16.7 (0.00-29.2)	18.3±8.64	4.39±0.23
	100	0.70 (0.10-1.63)	ND	153±16	100	8.33 (4.17-12.5)	16.7±9.31	4.22±0.31
	150	0.49 (0.00-1.38)	ND	166±12	100	8.33 (0.00-25.0)	14.2±8.64	4.38±0.20
Statistical test		$F(3,18)=0.607$	N/A	$F(3,17)=1.209$	N/A	$X^2(3)=5.641$	$F(3,19)=0.923$	$F(3,22)=1.121$
p		0.618	N/A	0.343	N/A	0.130	0.452	0.365
Segmentation (1-4 somites)	0	0.71 (0.10-1.68)	ND	172 (158-173)	100	8.33 (8.33-12.5)	8.33 (8.33-12.5)	4.40±0.13
	50	1.00 (0.38-1.89)	ND	165 (152-165)	100	16.7 (4.17-25.0)	12.5 (8.33-22.9)	4.46±0.29
	100	1.27 (1.06-1.51)	ND	157 (157-181)	100 (94.0-100)	16.7 (4.17-41.7)	20.8 (14.6-31.3)	4.39±0.33
	150	1.26 (1.00-1.55)	ND	174 (162-181)	100 (87.0-100)	16.7 (4.17-37.5)	16.7 (12.5-27.1)	4.34±0.30

Statistical test	$F(3,18)=1.162$	N/A	$X^2(3)=2.899$	$X^2(3)=0.714$	$X^2(3)=0.640$	$X^2(3)=4.368$	$F(3,17)=0.151$
<i>p</i>	0.349	N/A	0.407	0.870	0.887	0.224	0.928

Data from at least 4 independent replicates of 12 animals each are expressed as mean $\pm$ SD for parametric data distribution or median and interquartile range for non-parametric data. Categorical parameters (edema and pigmentation) are quoted as back-transformed mean of the log transformed variable and upper lower limits from the 95% confidence interval. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or Kruskal-Wallis followed by Dunn's test ($p < 0.05$). ND: malformation not detected; N/A: not applicable.

Table S3 - General outcomes of morphological analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during blastula (256-cell).

Group Dose (mg L ⁻¹)		MS222				Statistical test	p
		0	50	100	150		
Fins	Stunted	0.08 (0.04-0.22) ^{ab}	0.18 (0.09-0.32) ^a	0.33 (0.15-0.42) ^a	0.00 (0.00-0.08) ^b	X ² (3)=9.476	0.024
	Malformed	0.17 (0.13-0.25) ^{ab}	0.18 (0.09-0.27) ^a	0.33 (0.15-0.42) ^a	0.00 (0.00-0.08) ^b	X ² (3)=9.130	0.028
	Missing	ND	ND	ND	ND	N/A	N/A
Heart/thorax	Cardiac enlarg.	0.08 (0.00-0.21)	0.00 (0.00-0.18)	0.00 (0.00-0.13)	0.25	X ² (3)=3.726	0.293
	Dist. Thoracic region	0.25 (0.04-0.25)	0.00 (0.00-0.18)	0.22 (0.00-0.28)	ND	X ² (3)=7.126	0.068
	Brachycephalic	0.25±0.09	0.20±0.07	0.21±0.21	0.19±0.19	F(3,19)=0.100	0.959
Head	Undershot jaw	0.24±0.16	0.27±0.07	0.35±0.25	0.19±0.19	F(3,19)=0.727	0.551
	Enlarged otoliths	ND	ND	ND	ND	N/A	N/A
	Dolichocephalic	0.08 (0.00-0.08)	0.00 (0.00-0.09)	0.00 (0.00-0.17)	ND	X ² (3)=3.591	0.309
Eyes	Microcephaly	0.00 (0.00-0.13)	0.18 (0.08-0.23)	0.10 (0.05-0.37)	0.09 (0.04-0.18)	X ² (3)=4.564	0.207
	Microphthalmia	0.23±0.31	0.27±0.11	0.18±0.19	0.07±0.07	F(3,19)=1.015	0.412
	Ocular edema	0.17 (0.00-0.22)	0.00 (0.00-0.09)	ND	ND	X ² (3)=6.798	0.079
Organs	Enlarged organs	0.08 (0.00-0.08)	0.00(0.00-0.14)	0.13 (0.00-0.26)	ND	X ² (3)=5.548	0.136
Tail / Spine	Kink in tail	0.21±0.10	0.22±0.12	0.27±0.18	0.16±0.13	F(3,19)=0.555	0.652
	Lordosis / kyphosis	0.27 (0.13-0.42)	0.18 (0.18-0.27)	0.22 (0.19-0.46)	0.13 (0.08-0.23)	X ² (3)=4.694	0.196
TOTAL		2.33 (1.64-2.33)	2.09 (1.22-2.68)	2.00 (1.25-3.95)	0.82 (0.33-1.58)	X ² (3)=6.700	0.082

Parametric data presented as mean $\pm$ SD and non-parametric data presented as median and interquartile range of at least four independent replicates of 12 animals per replicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or using the Kruskal–Wallis test followed by Dunn's test ($p < 0.05$). ND: malformation not detected; N/A: not applicable.

Table S4 – General outcomes of morphological analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during gastrula (50% epiboly).

Group Dose (mg L ⁻¹)		MS222				Statistical test	p
		0	50	100	150		
Fins	Stunted	0.14 (0.06-0.29)	0.13 (0.00-0.23)	0.15 (0.00-0.22)	0.00 (0.00-0.10)	X ² (3)=4.151	0.246
	Malformed	0.14 (0.00-0.29)	0.13 (0.00-0.23)	0.15 (0.00-0.22)	0.00 (0.00-0.10)	X ² (3)=3.107	0.375
	Missing	ND	ND	ND	ND	N/A	N/A
Heart/thorax	Cardiac enlarg.	0.00 (0.00-0.04)	0.08 (0.00-0.22)	0.05 (0.00-0.11)	ND	X ² (3)=5.617	0.132
	Dist. Thoracic region	0.11 (0.00-0.16)	0.08 (0.00-0.34)	0.06 (0.00-0.13)	0.05 (0.00-0.11)	X ² (3)=1.193	0.755
	Brachycephalic	0.18±0.11	0.18±0.21	0.11±0.12	0.09±0.11	F(3,22)=0.902	0.458
Head	Undershot jaw	0.22±0.09	0.22±0.19	0.14±0.17	0.09±0.11	F(3,22)=1.036	0.399
	Enlarged otoliths	ND	ND	ND	ND	N/A	N/A
	Dolichocephalic	0.00 (0.00-0.15)	0.08 (0.00-0.10)	0.00 (0.00-0.11)	0.04 (0.00-0.10)	X ² (3)=0.482	0.923
Eyes	Microcephaly	0.23±0.13	0.23±0.19	0.15±0.14	0.10±0.11	F(3,22)=1.178	0.345
	Microphthalmia	0.11 (0.11-0.33)	0.13 (0.00-0.35)	0.10 (0.00-0.25)	0.05 (0.00-0.19)	X ² (3)=2.152	0.542
	Ocular edema	ND	0.00 (0.00-0.02)	ND	ND	X ² (3)=2.833	0.418
Organs	Enlarged organs	0.07 (0.00-0.15)	0.08 (0.00-0.12)	0.00 (0.00-0.03)	0.00 (0.00-0.11)	X ² (3)=2.692	0.442
Tail / Spine	Kink in tail	0.35±0.14	0.27±0.16	0.36±0.15	0.28±0.17	F(3,22)=0.516	0.676
	Lordosis / kyphosis	0.29±0.11	0.26±0.21	0.19±0.20	0.26±0.19	F(3,22)=0.399	0.755
TOTAL		2.02±1.12	2.04±1.70	1.61±0.99	1.05±0.87	F(3,22)=0.843	0.487

Parametric data presented as mean $\pm$ SD and non-parametric data presented as median and interquartile range of at least four independent replicates of 12 animals per replicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or using the Kruskal–Wallis test followed by Dunn's test ($p < 0.05$). ND: malformation not detected; N/A: not applicable.

Table S5 – General outcomes of morphological analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during segmentation (1-4 somites).

Group Dose (mg L ⁻¹)		MS222				Statistical test	p
		0	50	100	150		
Fins	Stunted	0.00 (0.00-0.10)	0.00 (0.00-0.04)	0.08 (0.00-0.09)	0.08 (0.00-0.21)	X ² (3)=2.740	0.433
	Malformed	0.00 (0.00-0.08)	0.00 (0.00-0.04)	0.08 (0.00-0.09)	0.08 (0.00-0.21)	X ² (3)=3.182	0.364
	Missing	ND	ND	ND	ND	N/A	N/A
Heart/thorax	Cardiac enlarg.	0.00 (0.00-0.08)	ND	0.00 (0.00-0.05)	0.00 (0.00-0.13)	X ² (3)=2.243	0.524
	Dist. thoracic region	0.00 (0.00-0.17)	0.09 (0.05-0.13)	0.17 (0.08-0.18)	0.08 (0.04-0.18)	X ² (3)=2.774	0.428
	Brachycephalic	0.08 (0.04-0.17)	0.00 (0.00-0.25)	0.18 (0.08-0.35)	0.08 (0.00-0.25)	X ² (3)=2.185	0.535
Head	Undershot jaw	0.08 (0.08-0.10)	0.09 (0.00-0.25)	0.25 (0.09-0.39)	0.08 (0.00-0.25)	X ² (3)=2.727	0.436
	Enlarged otoliths	ND	ND	ND	ND	N/A	N/A
	Dolichocephalic	ND	0.00 (0.00-0.05)	0.00 (0.00-0.14)	ND	X ² (3)=2.116	0.549
Eyes	Microcephaly	0.08 (0.08-0.10)	0.00 (0.00-0.21)	0.08 (0.00-0.25)	0.08 (0.00-0.21)	X ² (3)=0.494	0.920
	Microphthalmia	0.08 (0.11-0.43)	0.00 (0.00-0.17)	0.08 (0.00-0.30)	0.08 (0.00-0.17)	X ² (3)=2.173	0.537
	Ocular edema	ND	ND	ND	ND	N/A	N/A
Organs	Enlarged organs	0.00 (0.00-0.04)	0.00 (0.00-0.04)	0.00 (0.00-0.04)	0.00 (0.00-0.08)	X ² (3)=0.605	0.895
Tail / Spine	Kink in tail	0.23±0.11	0.23±0.16	0.22±0.22	0.17±0.15	F(3,19)=0.138	0.936
	Lordosis / kyphosis	0.16±0.13	0.17±0.13	0.24±0.25	0.20±0.18	F(3,19)=0.186	0.904
TOTAL		1.09±0.72	0.93±0.79	1.46±1.23	1.18±1.08	F(3,19)=0.255	0.857

Parametric data presented as mean±SD and non-parametric data presented as median and interquartile range of at least four independent replicates of 12 animals per replicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or using the Kruskal–Wallis test followed by Dunn's test (p<0.05). ND: malformation not detected; N/A: not applicable.

Table S6 - General outcomes of cartilage and bone analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during blastula (256-cell).

	Group Dose (mg L ⁻¹)	MS222				Statistical test	p
		0	50	100	150		
Cartilage	Meckel's cartilage	0.17 (0.17-0.18)	0.17 (0.17-1.08)	0.55 (0.18-1.31)	0.17 (0.00-0.90)	X ² (3)=3.252	0.354
	Palatoquadrate	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=2.783	0.426
	Hyosymplectic	ND	ND	ND	ND	N/A	N/A
	Hypobranchial	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=2.783	0.426
	Interhyal	ND	ND	ND	ND	N/A	N/A
	Ceratohyal	0.18 (0.08-0.22)	0.17 (0.08-1.08)	0.55 (0.09-1.31)	0.17 (0.00-0.90)	X ² (3)=2.493	0.476
	Basihyal	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=2.783	0.426
	Ceratobranchial 1	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=1.853	0.603
	Ceratobranchial 2	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=1.853	0.603
	Ceratobranchial 3	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=1.853	0.603
	Ceratobranchial 4	0.08 (0.08-0.09)	0.08 (0.08-0.54)	0.46 (0.09-0.77)	0.17 (0.00-0.58)	X ² (3)=1.853	0.603
	Basibranchial	ND	ND	ND	ND	N/A	N/A
	Ethmoid plate	ND	ND	ND	ND	N/A	N/A
	Trabeculae cranii	ND	ND	ND	ND	N/A	N/A
	Anterior basicranial commissure	ND	ND	ND	ND	N/A	N/A
Bone	Basal plate	ND	ND	ND	ND	N/A	N/A
	Occipital arch	ND	ND	ND	ND	N/A	N/A
	Notochord	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Parasphenoid	ND	0.00 (0.00-0.30)	0.00 (0.00-0.10)	0.00 (0.00-0.20)	X ² (3)=1.281	0.734
	Branchiostegal rays	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Operculum	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Entopterygoid	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	CB5 ossification	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Dentary	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Occipital	ND	0.00 (0.00-0.30)	ND	ND	X ² (3)=3.000	0.392
	Vertebrae	ND	0.00 (0.00-0.30)	0.00 (0.00-0.10)	0.00 (0.00-0.20)	X ² (3)=3.000	0.734
	Cleithrum	ND	0.00 (0.00-0.30)	0.00 (0.00-0.10)	0.00 (0.00-0.20)	X ² (3)=3.000	0.734

TOTAL	1.08±0.22	6.28±5.07	5.87±5.26	4.32±5.32	F(3,11)=0.817	0.520
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Parametric data presented as mean±SD and non-parametric data presented as median and interquartile range of three independent replicates of 12 animals per replicate. Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple-comparison test or using the Kruskal–Wallis test followed by Dunn's test ($p<0.05$). ND: malformation not detected; N/A: not applicable.

Table S7 - General outcomes of cartilage and bone analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during gastrula (50% epiboly).

	Group Dose (mg L ⁻¹)	MS222				Statistical test	p
		0	50	100	150		
Cartilage	Meckel's cartilage	0.17 (0.17-0.18)	0.25 (0.10-0.29)	0.46 (0.20-0.56)	0.27 (0.25-0.46)	X ² (3)=5.461	0.141
	Palatoquadrate	0.08 (0.08-0.09) ^a	0.17 (0.10-0.21) ^{ab}	0.36 (0.20-0.40) ^b	0.27 (0.17-0.36) ^b	X ² (3)=7.940	0.047
	Hyosymplectic	ND	ND	ND	ND	N/A	N/A
	Hypobranchial	0.08 (0.08-0.09) ^a	0.17 (0.10-0.21) ^{ab}	0.36 (0.20-0.40) ^b	0.27 (0.17-0.36) ^b	X ² (3)=7.940	0.047
	Interhyal	ND	ND	ND	ND	N/A	N/A
	Ceratohyal	0.20±0.04	0.23±0.12	0.44±0.23	0.39±0.22	F(3,11)=1.423	0.306
	Basihyal	0.08 (0.08-0.09) ^a	0.17 (0.10-0.21) ^{ab}	0.36 (0.20-0.40) ^b	0.27 (0.17-0.36) ^b	X ² (3)=7.940	0.047
	Ceratobranchial 1	0.11±0.05	0.17±0.08	0.34±0.12	0.30±0.15	F(3,11)=2.946	0.099
	Ceratobranchial 2	0.11±0.05	0.17±0.08	0.34±0.12	0.30±0.15	F(3,11)=2.946	0.099
	Ceratobranchial 3	0.11±0.05	0.17±0.08	0.34±0.12	0.30±0.15	F(3,11)=2.946	0.099
	Ceratobranchial 4	0.11±0.05	0.17±0.08	0.34±0.12	0.30±0.15	F(3,11)=2.946	0.099
	Basibranchial	ND	ND	ND	ND	N/A	N/A
	Ethmoid plate	ND	ND	ND	ND	N/A	N/A
	Trabeculae cranii	ND	ND	ND	ND	N/A	N/A
	Anterior basicranial commissure	ND	ND	ND	ND	N/A	N/A
	Basal plate	ND	ND	ND	ND	N/A	N/A
	Occipital arch	ND	ND	ND	ND	N/A	N/A
Bone	Notochord	ND	ND	ND	ND	N/A	N/A
	Parasphenoid	ND	0.00 (0.00-0.10)	ND	0.00 (0.00-0.10)	X ² (3)=2.200	0.532
	Branchiostegal rays	ND	ND	ND	ND	N/A	N/A
	Operculum	ND	ND	ND	ND	N/A	N/A
	Entopterygoid	ND	ND	ND	ND	N/A	N/A
	CB5 ossification	ND	ND	ND	ND	N/A	N/A
	Dentary	ND	ND	ND	ND	N/A	N/A
	Occipital	ND	ND	ND	ND	N/A	N/A
	Vertebrae	ND	0.00 (0.00-0.10)	ND	0.00 (0.00-0.10)	X ² (3)=2.200	0.532

Cleithrum	ND	0.00 (0.00-0.10)	ND	0.00 (0.00-0.10)	$X^2(3)=2.200$	0.532
TOTAL	1.08±0.22	1.86±0.84	3.23±1.34	3.06±1.39	F(3,11)=2.788	0.109

Non-parametric data presented as median and interquartile range of three independent replicates of 12 animals per replicate. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p < 0.05$). ND: malformation not detected; N/A: not applicable.

Table S8 - General outcomes of cartilage and bone analyses of zebrafish larvae at 144 hpf after acute exposure to MS-222 during segmentation (1-4 somites).

	Group Dose (mg L ⁻¹)	MS222				Statistical test	p
		0	50	100	150		
Cartilage	Meckel's cartilage	0.17 (0.17-0.18)	0.42 (0.33-0.43)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=4.849	0.183
	Palatoquadrate	0.08 (0.08-0.09)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.440	0.486
	Hyosymplectic	ND	ND	ND	ND	N/A	N/A
	Hypobranchial	0.08 (0.08-0.09)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.440	0.486
	Interhyal	ND	ND	ND	ND	N/A	N/A
	Ceratohyal	0.18 (0.17-0.22)	0.42 (0.33-0.43)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=4.832	0.185
	Basihyal	0.08 (0.08-0.09)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.444	0.486
	Ceratobranchial 1	0.09 (0.08-0.13)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.300	0.513
	Ceratobranchial 2	0.09 (0.08-0.13)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.300	0.513
	Ceratobranchial 3	0.09 (0.08-0.13)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.300	0.513
	Ceratobranchial 4	0.09 (0.08-0.13)	0.25 (0.25-0.29)	0.00 (0.00-0.21)	0.09 (0.00-0.21)	X ² (3)=2.300	0.513
	Basibranchial	ND	ND	ND	ND	N/A	N/A
	Ethmoid plate	ND	ND	ND	ND	N/A	N/A
	Trabeculae cranii	ND	ND	ND	ND	N/A	N/A
	Anterior basicranial commissure	ND	ND	ND	ND	N/A	N/A
Bone	Basal plate	ND	ND	ND	ND	N/A	N/A
	Occipital arch	ND	ND	ND	ND	N/A	N/A
	Notochord	ND	ND	ND	ND	N/A	N/A
	Parasphenoid	ND	0.00 (0.00-0.20)	ND	0.00 (0.00-0.10)	X ² (3)=2.212	0.530
	Branchiostegal rays	ND	ND	ND	ND	N/A	N/A
	Operculum	ND	ND	ND	ND	N/A	N/A
	Entopterygoid	ND	ND	ND	ND	N/A	N/A
	CB5 ossification	ND	ND	ND	ND	N/A	N/A
	Dentary	ND	ND	ND	ND	N/A	N/A
	Occipital	ND	ND	ND	ND	N/A	N/A
	Vertebrae	ND	0.00 (0.00-0.20)	ND	0.00 (0.00-0.10)	X ² (3)=2.212	0.530

Cleithrum	ND	0.00 (0.00-0.20)	ND	0.00 (0.00-0.10)	$X^2(3)=2.212$	0.530
TOTAL	1.00 (0.91-1.17)	2.58 (2.42-3.50)	0.00 (0.00-1.88)	0.82 (0.00-2.21)	$X^2(3)=3.251$	0.355

Non-parametric data presented as median and interquartile range of three independent replicates of 12 animals. Statistical analysis was performed using the Kruskal–Wallis test followed by Dunn's test. Different lowercase letters indicate significant differences between groups ($p < 0.05$). ND: malformation not detected; N/A: not applicable.

Figure S1: Effects on the distance to the centre of the arena of zebrafish larvae at 144 hpf after exposure to MS-222 at 256-cell, 50% epiboly and 1-4 somite stage. Values are expressed as mean $\pm$ standard deviation. Data from at least five independent replicate exposures ($n = 5$ replicates with six animals each per group).