Letter to the Editor

Effects of Exogenous Carbon Monoxide Releasing Molecules on the Development of Zebrafish Embryos and Larvae*

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The use of exogenous carbon monoxide releasing molecules (CORMs) provides promise for clinical application; however, the hazard potential of CORMs *in vivo* remains poorly understood. The developmental toxicity of CORM-3 was investigated by exposure to concentrations ranging from 6.25 to 400 μmol/L during 4-144 h post fertilization. Toxicity endpoints of mortality, spontaneous movement, heart rate, hatching rate, malformation, body length, and larval behavior were measured. CORM-3 disrupted the progression of zebrafish larval development at concentrations exceeding 50 μmol/L, resulting in embryonic developmental toxicity.

Carbon monoxide (CO) can influence the transport of oxygen in the body and lead to hazardous effects if 50% of human hemoglobin is occupied by CO. Despite the mentioned toxic effects, a small amount of CO exists in the body in the form of CO hemoglobin[1]. CO is increasingly recognized as a cell-signaling molecule[1]. However, the use of CO is hazardous due to difficulties in controlling the absorption and distribution of CO through inhalation. The emergence of transition metal carbonyls complexes [carbon monoxide releasing molecules (CORMs)] as 'a new class of pharmaceuticals' was initiated by mimicking the biological functions of endogenous CO[2]. To date, the toxicological profile presented *in vitro* remains poorly understood, with conflicting conclusions obtained. At a concentration of 100 μmol/L, iron-containing carbon monoxide releasing molecules (CORM-F3) did not exhibit any detectable cytotoxicity to RAW246.7 macrophages, whereas CORM-F8 caused toxicity at a concentration of 100 μmol/L to the same cell[3]. Winburn et al. revealed a narrow margin between the cytoprotective (<20 μmol/L) and cytotoxic (>100 μmol/L) concentrations of CORM-2[4]. Because the cell-based assay is a poor representation of the complimentary system *in vivo*, there remains a lack of data *in vivo* for predictive and correlative toxicity of CORMs.

The use of zebrafish provides a promising vertebrate model, and this species has found extensive application in screening chemicals for therapeutic potential owing to the abundance of information available on the genetics and developmental biology of the species[5]. CORM-3, a water soluble transitional metal carbonyls based around ruthenium, releases CO when activated by light[2]. Little is known regarding the developmental toxicity of CORM-3. In the present study, we reported for the first time on zebrafish exposure to CORM-3 (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μmol/L) over an embryonic period of 4-144 hours post fertilization (hpf), measuring the developmental endpoints of zebrafish by conducting a sequence of assessments.

$Ru(CO)_3Cl$-glycinate (CORM-3) was provided by the Pharmacy of Lanzhou University and synthesized from $[Ru(CO)_3Cl_2]_2$ as described by Clark et al.[2]. In brief, $RuCl_3 \cdot xH_2O$ (AR, Sigma) was added to a mixed solution of formic acid and hydrochloric acid, and the mixture was refluxed at 110 °C. Next, under reduced pressure, the solvent was removed and the residue was recrystallized with CH_2Cl_2-n-pentane to obtain $[Ru(CO)_3Cl_2]_2$.

$[Ru(CO)_3Cl_2]_2$ and glycine were added in methanol and sodium methoxide to react over 24 h with stirring at room temperature. The solvent in the

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reaction mixture was removed under reduced pressure, and then, the residue was redissolved in tetrahydrofuran. This solution was filtered and excess light petroleum was then added and evaporated down to obtain CORM-3. To characterize the CORM-3, infrared (IR) and mass spectra were performed (Supplementary Figure 1 see the www.besjournal.com).

AB strain zebrafish were kept in closed charcoal-filtered recirculating aquaculture systems maintained at 28±1 °C under a 14 h light/10 h dark cycle. Individuals were segregated overnight by sex in tanks with a male and female ratio of 3:2. Spawning was not induced until the beginning of the next 14 h light period. Zebrafish embryos were collected and rinsed with culture medium and then incubated in a 28.5 °C incubator to allow continuous development. Zebrafish handling and management followed the Guide for the Care and Use of Laboratory Animals, which was approved by the Institutional Animal Use and Care Committee (IAUCC) of Lanzhou University.

At 4 hpf, embryos that developed normally were selected for subsequent experiments. These embryos were exposed to freshly prepared CORM-3 solution (0, 6.25, 12.5, 25, 50, 100, 200, and 400 μmol/L) from 4 hpf to 144 hpf for a continuing observation period. Each group contained 80 embryos. Acute endpoints including the embryonic/ larval mortality and hatching rate were evaluated. The incidence of mortality was recorded daily until 120 hpf. Embryos were considered dead when no heart beat was presented and eggs were denatured. At 72 hpf, the hatching rate was evaluated and calculated as the ratio of embryos hatched among live embryos. At 96 hpf, morphological malformation of embryos and larvae were observed with a stereoscopic dissecting microscope (Motic, China). At 144 hpf, the behavior test was performed as detailed below. A total of 12 larvae were selected from each group and transferred into a square 96 well plate with one larvae per well containing 300 μL E3. The larvae were placed in an observation chamber and allowed to acclimate to light conditions for 20 min to minimize any disturbance related to handling and transportation. Their locomotor activities were then recorded for the ensuing 5 min using a Noldus tracking device (Noldus Information Technology, Netherlands) and Media Cruiser recording software (Canopus Corporation, Japan).

The statistical analyses were conducted using SPSS 20.0 software (SPSS, USA). All data were expressed as mean±standard error (SE). One-way analysis of variance (ANOVA) followed by Tukey's test were conducted to compare the differences between the groups (P<0.05). The figures were plotted using Origin 8.0 (OriginLab, USA).

In the present study, our data showed that there was no significant increase in mortality observed during the entire exposure time at the CORM-3 concentrations <200 μmol/L, whereas a significant increase in mortality was observed at the high concentrations of 200 μmol/L and 400 μmol/L (Figure 1A). The results we obtained differed from those of previous research showing that 500 μmol/L CORM-3 had no cytotoxicity to human gingival fibroblasts[6] . We speculate that the difference is related to the complex itself and different toxicity assays. This complex contains a transition metal, such as ruthenium, iron, chromium, or tungsten, surrounded by a certain number of carbonyl groups (CO) as coordinated ligands[2,6-7]. Differences in this transition metal, structure, or substituent can affect the degree of toxicity; therefore, the overall biological activity of the complex is affected. Wang et al. found that the CORMs containing chromium or tungsten atoms showed higher toxicity than those containing ruthenium atoms[7]. Romanski et al. tested the biological activity of enzyme-triggered CO-releasing molecules (ET-CORMs) in relation to their structure and found that the structural alterations of ET-CORMs significantly affected their biological activity[8]. In addition, it has been known that the toxicity of chemicals *in vitro* differs significantly from that *in vivo* due to the poor representation of a complimentary system *in vivo*.

Apart from mortality, we determined the hatching rate of embryos at 72 hpf to evaluate the acute toxicology. In our study, the hatching rates of embryos in 50, 100, 200, and 400 μmol/L treated groups were inhibited as compared to those of the control groups (97.36%), with hatching rates of 36.06%, 32.17%, 23.04%, and 22.58%, respectively (Figure 1B). As embryos are sensitive and vulnerable to chemicals during their development, reduction in the hatching rate is a sensitive toxicological parameter and indicator for monitoring the toxicity of CORM-3 exposure. The inhibition of hatching may be related to retarded development or an inability of embryos to break the chorion. The chorion is an acellular envelope with pore canals, which is approximately 0.5-0.7 μm in diameter, and facilitates the transport of oxygen/carbon dioxide, nutrients, and excretion

products to and from the embryo[5]. Hypoxia might be the main reason for the low hatching rate observed. For each mole of CORM-3, one mole of CO was liberated[2]. CO liberated from CORM-3 could diffuse into embryos through the pore canals and might interfere with the oxygen exchange by blocking O_2 exchange with the embryo tissue. This results in the embryos abnormally secreting hatching enzyme, resulting in the digestion of the chorion at the time of hatching, and thus changing the elasticity of the chorion and leading to an inhibition of embryo hatching. We suggest that the mechanism of hatching inhibition is caused to some extent by direct or indirect interference with the digestive function of the chorionic hatching enzyme. However, further studies are required to clarify the specific mechanisms of this effect. Similar inhibition of hatching occurred as indirect nano-ZnO blocking O_2 exchange[5].

CORM-3 may potentially exert an adverse effect on zebrafish. To address this issue, we observed the morphology of zebrafish at 96 hpf. Larvae in the control group showed no obvious defects and exhibited normal development, whereas larvae treated with 100, 200, and 400 μmol/L CORM-3 showed two types of abnormalities, including yolk sac and pericardial edema, which have often been found in developing embryos exposed to toxic agents[5]. These abnormalities were observed not only in the hatched larvae but also in some unhatched embryos (Figure 2).

Although the mortality and hatching rate of zebrafish embryos and larvae exposed to CORM-3 in the present study provided critical and informative data, further studies that comprehensively evaluate the effect of CORM-3 on the development of zebrafish are warranted. Spontaneous movement and heart rate of zebrafish embryos/larvae exposure to CORM-3 were recorded at 24 hpf and 48 hpf, respectively (Supplementary Figure 2 see the www.besjournal.com). At 120 hpf, the growth in terms of body length between the anterior-most part of the head and the tip of the tail was calculated (Supplementary Figure 3 see the www.besjournal.com). The behavior test of 6 days post fertilization (dpf) larvae on the ability of locomotor activity was investigated to determine the influence on the nervous system by comparing the

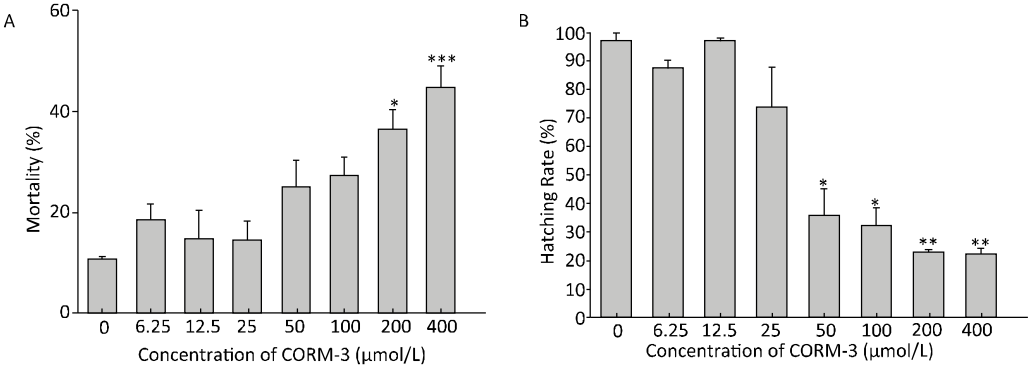

Figure 1. The mortality rate (A) and hatching rate (B) of zebrafish embryos induced by different concentrations of carbon monoxide releasing molecules (CORM-3) solution at 120 h post fertilization (hpf) and 72 hpf, respectively (80 embryos). Data are expressed asmeans±standard error (SE) from three independent experiments (N=3, $^{*}P$<0.05, $^{**}P$<0.01, $^{***}P$<0.001).

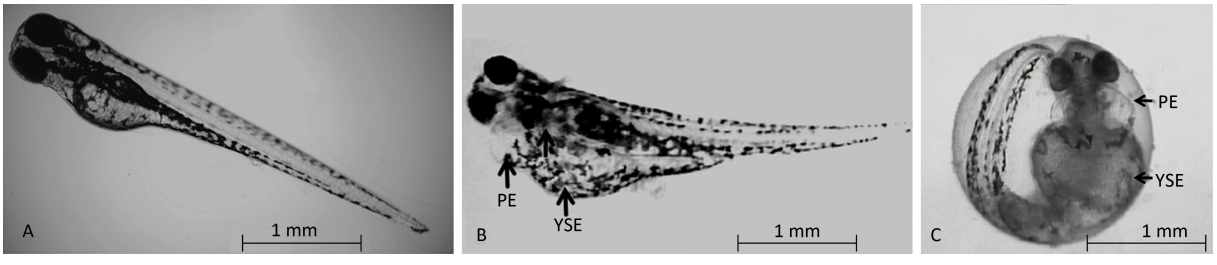

Figure 2. Malformation of zebrafish embryos exposed to 400 μmol/L carbon monoxide releasing molecules (CORM-3). (A) normal larvae; (B, C) abnormal larvae. Malformations are indicated by black arrows. PE, pericardial edema; YSE, yolk sac edema.

distance moved during the light period, because larvae at 6 dpf show a richer behavioral response and robust response to light stimulation compared with larvae of other ages[9].

In our study, the lower exposure concentrations (6.25 and 12.5 μmol/L CORM-3) did not show any apparent difference compared to the control group, whereas the higher concentration (400 μmol/L) led to a significantly active retardation during light periods (Figure 3). These data suggest that CORM-3 has a significant impact on zebrafish locomotor activity. Chemicals such as gold nanoparticles have been tested using the same method and have also exhibited a similar change[10]. The present study demonstrated that permanent derailment of development may occur during embryonic development if any molecular pathways are perturbed during this period[10].

In conclusion, our study demonstrated that CORM-3 exerts toxicity on the development of zebrafish embryos, leading to malformation in some larvae and unhatched embryos, and causing inhibition of hatching and inducing change in spontaneous movement, heart rate, and locomotor behavior of zebrafish embryos and larvae. Thus, our findings suggest that CORM-3 could be a potential hazardous factor for pharmaceutical agents. Further studies of adverse effects and biological mechanisms are required for evaluating the safety of CORM-3 exposure.

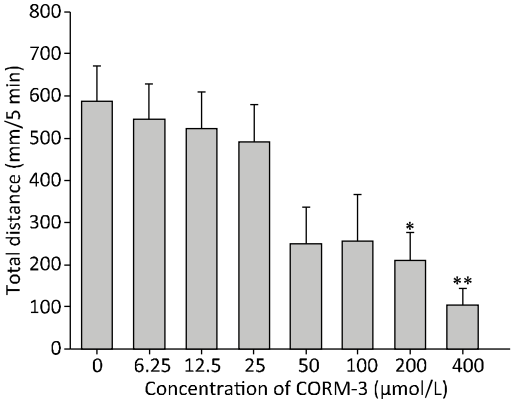

Figure 3. Total distance of larvae in the visible light test after 144 hpf exposure to carbon monoxide releasing molecules (CORM-3) (12 larvae). Data are expressed as means±standard error (SE) from three independent experiments (N=3, $^{*}P$<0.05, $^{**}P$<0.01).

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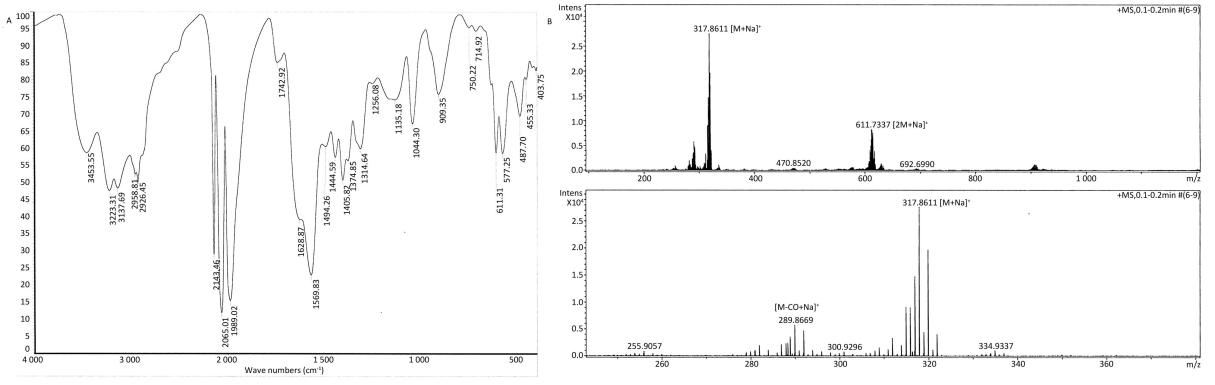
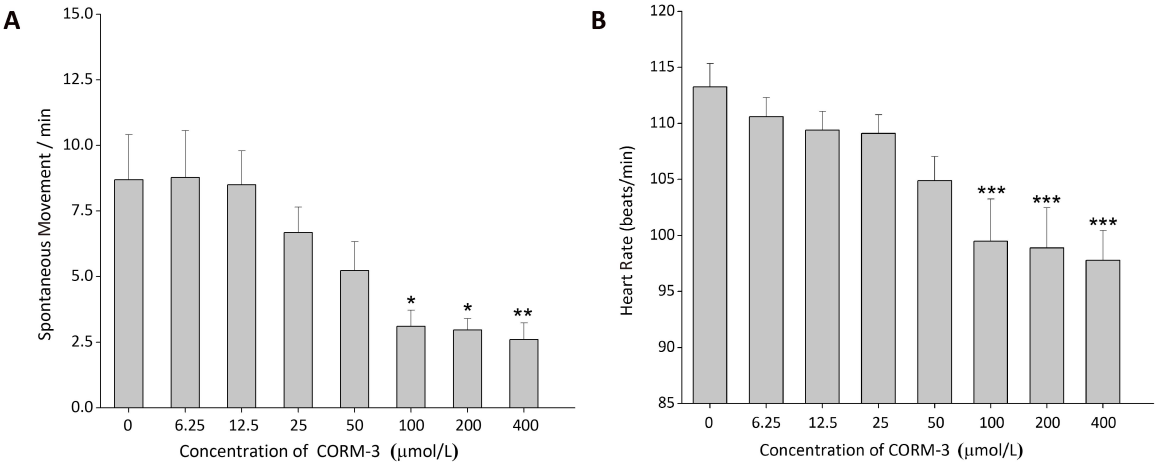
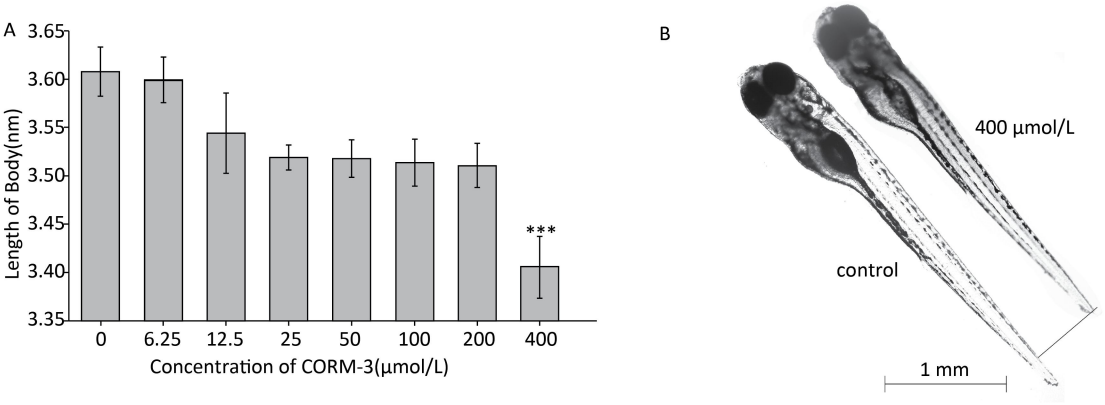


Supplementary Figure 1.



Supplementary Figure 2.



Supplementary Figure 3.