Effects of copper on the early development of *Xenopus laevis*: the case of $CuSO_4$ and Bordeaux mixture solutions

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Introduction

Copper is an essential metal for the organism but it can be toxic at high concentrations.[1,2] This metal is a part of several crop-protecting fungicides, algaecides and bactericides. Allowed in organic agriculture in Bordeaux mixture formulation ($CuSO_4$ and slaked lime), it is widely used to treat grapevine mildew.[3] Copper concentrations in French wine-producing regions soils and water are indeed higher. In water, copper has been detected at levels of 2.88 $\mu g.L^{-1}$ in the Riou Mort river, 1.25 $\mu g.L^{-1}$ in the Lot river and 1.4 $\mu g.L^{-1}$ in the Garonne.[4] In the soils of wine-producing Nouvelle Aquitaine region, copper concentration can be found at 508 $mg.kg^{1}$.[5] This metal is also referenced in a chemical watchlist established by the United States Environmental Protection Agency.[6] The continuous use of crop-protecting solution applications, among which copper solutions, is one of the main causes of amphibian decline.[7] As a matter of fact, their life cycle is dependent on the quality of aquatic habitats, in which phytopharmaceutical products could eventually end up.

This study aims at defining the effects of $CuSO_4$ and Bordeaux mixture expositions on the early stages of *Xenopus Iaevis* development. In this purpose, oocyte maturation (time-courses and signaling pathways), fertilization success as well as development were assessed in Cu- contaminated conditions.

Materials and Methods

Animal experiments were performed at the animal facility of the University of Lille in accordance with the European Community Council guidelines (86/609/EEC) for laboratory animal experimentation. The protocol was approved by the local institutional review board (*Comité d'Ethique en Expérimentation Animale Hauts-de-France*, CEEA 07/2010). *Xenopus laevis* oocyte maturation was investigated by different protocols described in our previous study.[8] Time-courses of the process were assessed under contaminant exposures (± progesterone) by recording the Germinal Vesicle Breakdown (GVBD) ratios every 15 minutes for 13 hours. The phosphorylation states of RSK ($p90^{RSK}$) and ERK2 from the Mitogen Activated Protein-Kinases (MAPK) cascade and Cdc2 and H3, the catalycal subunit of the M-Promoting Factor (MPF) and one of its principal target respectively, were evaluated in exposed oocytes by western blots. Then, fertilization success was determined following experimental design already conducted in previous work.[9] To assess the fertilization success (% of fertilized eggs) photographic analyses were realized after 35 minutes of exposure. At last, developmental analysis were done using an automatic biometric data recording procedure.[10] Here, distance between eyes were determined in 6-days old tadpoles exposed from the fertilization. $CuSO_4$ and Bordeaux mixture solutions were prepared weekly by dilution in ND96 (oocyte maturation) or in dechlorinated tap water (fertilization and development). Concentrations used in the experiments are environmentally relevant and were derived from the environmental quality standards defined in France in the water framework directive context.[3] The following range of concentrations has been tested: 0, 0.00399, 0.0399, 0.399 and 3.99 µM of Cu^{2+} contained in $CuSO_4$ and Bordeaux mixture solutions. Statistical analyses and graphical representation were performed with R software (Version 3.3.2; The R Foundation for Statistical Computing, 2016). The GVBD ratios were analyzed by Friedman's tests and post-hoc multiple paired comparison between conditions. Fertilization success and eye gap were assessed by Kruskal-Wallis tests and multiple paired comparison between treatments.

Results and Discussion

The maturation process was assessed by kinetics approach with measurements taken at 15 min interval during 13 hours (Figure 1). The appearance of the white spot at the animal pole was checked. GVBD occurred at approximatively 165 min in controls and in contaminated conditions when gamete maturation was stimulated by progesterone (Figure 1A-B). Same experiments were conducted without progesterone (Figure 1C-D). In presence of the hormone, neither the $CuSO_4$ nor the Bordeaux mixture affected the maturation rate. Indeed, in all exposure conditions, the final rates of maturation ranged between 75% and 90%. These results did not differ from those obtained in the controls (without Cu^{2+}) or for lead exposures in a previous work.[8] By contrast, in the absence progesterone, the highest concentration of copper in the both form was able to induce the GVBD (Figure 1C-D). These observations are called spontaneous maturations (up to 70% of oocytes undergone GVBD). Previous work demonstrated same results when oocytes were exposed to cadmium or zinc ions.[11] The time-course experiments provided also information on the beginning of maturation. Whatever the experimental conditions (with or without progesterone and with or without contaminant) the maturations began simultaneously

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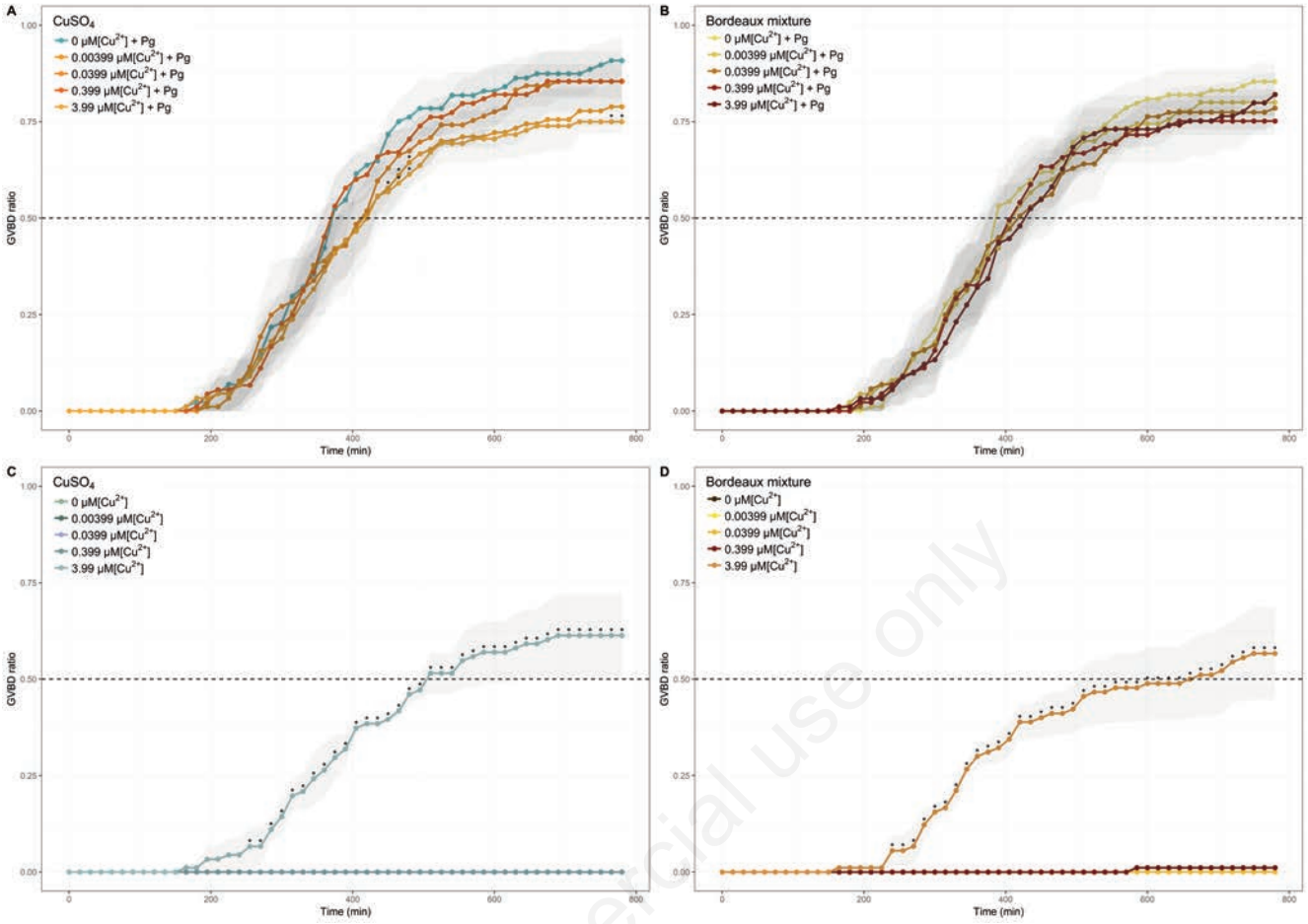

Figure 1. Effects of $CuSO_4$ and Bordeaux mixture exposures on X. laevis oocyte's maturation. Oocytes were exposed to increasing concentrations of $CuSO_4$ (A, C) or Bordeaux mixture (B, D) in presence (A, B) or not (C, D) of progesterone (Pg) for 13 h. Every 15 minutes, the maturation was assessed according to the white spot appearance. Results are expressed as mean ± SEM (grey areas) and compared to other treatments using Friedman rank sum test (P<0.05).

(after 165 min of treatment). This suggests that the same molecular mechanisms or signaling pathways are involved in hormone-dependent maturation and spontaneous maturation induced by $CuSO_4$ or Bordeaux mixture exposures at the higher concentrations compare to what we observed after cadmium exposures ($CdCl_2$) where spontaneous maturation occurred much later than hormone-dependent ones[8]

Two major regulation pathways involved in the maturation process were also studied by immunoblotting: MAPK (ERK2 & RSK) and MPF (Cdc2 & H3) signaling pathways. No anomaly of phosphorylation patterns was detected after the exposures, whether the maturation was hormone-dependent or spontaneous (data not shown). These results differ from other studies concerning oocyte maturation of *Xenopus laevis*. Cadmium was shown to disrupt numerous phosphorylation steps in the MAPK pathway, in the activation of MPF and its activity.[8]

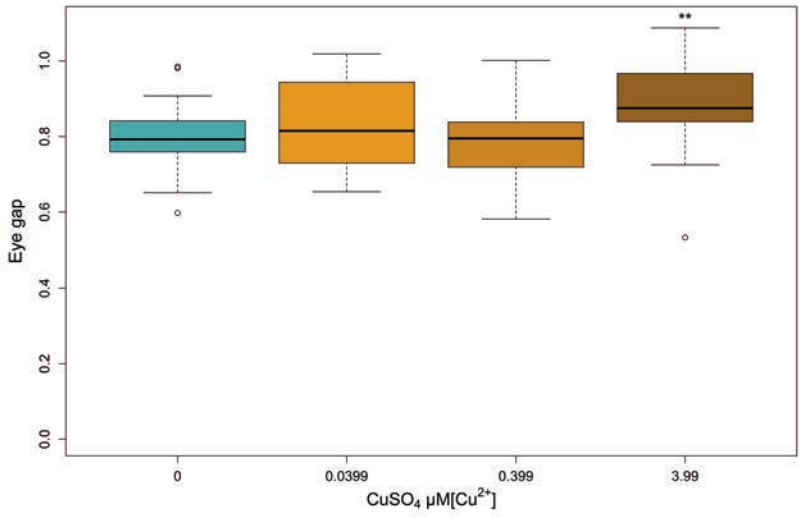

Figure 2. Effects of $CuSO_4$ exposures on eye gap in 6-days old tadpoles. Tadpoles were exposed increasing concentrations of $CuSO_4$ from the fertilization. After alcian blue staining, automatic image analysis was performed under Image J software (see Slaby *et al.*, 2016, for details). Results are expressed as boxplots and compared to others using Kruskal-Wallis rank sum test (P<0.01).

In a second set of experiments, *in vitro* fertilizations were performed in increasing concentrations of $CuSO_4$ or Bordeaux mixture. After 35 minutes of exposure, the number of fertilized eggs was measured. The data revealed no effect of the both forms of Cu^{2+} on fertilization success (data not shown). Similar results have already been observed with lead. Indeed, $PbCl_2$ did not modify fertilization success.[9]

In order to study the effects of $CuSO_4$ on the tadpoles' growth, after 6 days of exposure from the fertilization, tadpoles were fixed and stained in Alcian blue.[10] Data for distance between the eyes (eye gap) are shown in Figure 2 in embryos exposed to $CuSO_4$. At the highest concentration of $CuSO_4$ an increase in the ocular distance which suggest that the tadpoles became larger (or at least that the individual will have a larger head) was observed.

Conclusions

Although progesterone-stimulated maturation and fertilization ratio were not affected by copper exposures, these results showed that both forms of Cu^{2+} induced spontaneous maturation at the highest concentration and affected tadpoles' biometry. This study shows the importance of early development stages in amphibian toxicology because of their high sensitivity to contaminant at this early stage in life.

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