

LC50 and Effect of Sublethal Concentration of K₂Cr₂O₇ on Different Developmental Stages of *Osteochilus vittatus*

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LC₅₀ and Effect of Sublethal Concentration of K₂Cr₂O₇ on Different Developmental Stages of *Osteochilus vittatus*

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ABSTRACT

Chromium in form of hexavalent had been reported to be genotoxic and carcinogenic, affects some physiological, and reproductive features. The ability of aquatic animals including fish to tolerate chromium may vary according to different developmental stages. Therefore, this research was conducted to (1) determine the LC₅₀ of K₂Cr₂O₇ for embryo, larvae, and juvenile of *Osteochilus vittatus*, and (2) evaluate the chronic exposure of sublethal concentration of K₂Cr₂O₇ in embryo and juvenile. The LC₅₀ 96h was determined using Probit analysis. Two experimental studies were conducted using a completely randomized design. In experiment-1: 5 groups of 2-cell stage embryos were exposed to 0, 20, 40, 60, and 80 ppm of K₂Cr₂O₇, 8 units replicate each containing 200 embryos were provided, the parameters were hatching rate (HR) and survival rate (SR) of 4dph larvae. In experiment-2: three developmental stages i.e. 30dph, 60 dph, and 90 dph juveniles were exposed to 0 ppm, 2.5 ppm, and 5.00ppm of K₂Cr₂O₇, for 30 days; the observed parameters were body length, body weight, and SR. The results showed that LC₅₀ for embryos was 100ppm, for larvae was 14.37ppm and for juveniles was 8.1ppm. The results of experiment-1 showed that the hatching rate of embryos treated with 0, 20, 40, 60, and 80 ppm of K₂Cr₂O₇ were 90.61±2.52%, 81.13±0.70%, 87.21±4.62%, 76.30±3.20%, and 77.30±4.12% respectively. The survival rate of 4 dph larvae of the treated embryos were 100%, 99.66±0.29%, 98.71±0.84%, 98.31±1.79, and 94.61±1.75 respectively. The results of experiment-2 showed that 30dph juvenile treated with 0ppm, 2.5ppm, and 5.00ppm of K₂Cr₂O₇ have a body length of 14.67±1.52mm, 12.33±5.51mm and 10.00±0.81 respectively; the treated 60dph juvenile have a body length of 26.75±3.88mm, 20.25±3.84mm, 20.62±3.85mm respectively; the treated 90dph fries have body length of 8.00±1.7mm, 35.00±2.16mm, and 33.33±1.52mm respectively. The body weights of juveniles exposed to K₂Cr₂O₇ were significantly lower ($p<0.01$) compare to the control group. The SR of the fries exposed to K₂Cr₂O₇ were was significantly lower ($p<0.01$) compared to the control group. Based on all parameter it can be concluded that the LC₅₀ of K₂Cr₂O₇ in *O. vittatus* was varied according to its developmental stages and its effects was detrimental. Attention needs to be taken in regulating the release of effluent-containing chromium to the environment.

Keywords: Chromium, Embryo, Larvae, LC₅₀, *Osteochilus vittatus*.

1. INTRODUCTION

Chromium is one of the heavy metal presences in effluent originated from tanneries, mining, and stainless steels assembling and elastic assembling factory [1] Chromium is also present in batik waster waste effluent [2]. Studies at two batik-producing Centres showed that water of Rowojembangan Creek, Kulon Progo contained total chromium of 0.18 – 0.303 ppm (0.123±0.090 ppm), and Cibentar River, Salem Brebes Regency contained total chromium of 0.013 – 0.219

ppm (0.084±0.060ppm) [2]. Chromium present in the water enters the cells via the sulfate-anion channel [3] and can be accumulated in various tissue of fish. Chromium had been reported to be accumulated in the fin, gill, and muscle of *Oreochromis aureus* [4], *Hypophthalmichthys molitrix*, *Oreochromis nilotica*, *L. rohita*, *O. mossambica*, *Channa marulius* [5], and *Catla catla* [5, 6], *Labeo bata* and *Puntius sarana* [6].

Chromium in the form of hexavalent had been reported to be genotoxic and carcinogenic [7, 8], affects some physiological [9], and reproductive features [10].

Most studies on chromium toxicity are only conducted at a particular stage of development. In the aquatic environment, fishes are exposed to chromium throughout their life span. There is a lack of information on the effects of long-term exposure of chromium at the sublethal concentration on the development, health, and survival of aquatic animals. In addition, the ability of aquatic animals including fish to tolerate chromium may vary according to different developmental stages. Therefore, this research was conducted to (1) determine the LC₅₀ of K₂Cr₂O₇ for embryo, larvae, and juvenile of *Osteochilus vittatus*, and (2) evaluate the chronic exposure of sublethal concentration of K₂Cr₂O₇ in embryo and fry. *Osteochilus vittatus* was chosen as an experimental animal since this species is widely cultured for food needs. Thus, information from this research will be useful for the fish farmers as well as for decision-makers in designing policy to regulate the limit of chromium concentration allowed to be released to the environment.

2. METHODS

2.1. Animals

The *O. vittatus* embryos and larvae were obtained from induced spawning at our laboratory. Pairs of brooders were induced to spawn using 0.5ml.kg⁻¹ body weight salmon GnRH analogue with domperidone (Syndel Laboratory, Vancouver Canada). As the spawning sign was observed, the brooders were removed from the aquaria then were gently stripped to obtain the oocytes and sperm. The oocytes and sperm were mixed to facilitate fertilization for 3 minutes, rinsed in slow running water then were incubated in the hatching aquaria. The juveniles age of 30 dph, 60 dph, and 90 dph were obtained from Pandak Freshwater fish breeding Centre in Banyumas Regency. The juveniles were acclimated for one week under laboratory conditions prior to the treatment. The juveniles were reared in 60x40x30cm aquaria filled with earth water and fully aerated for a continuous supply of Oxygen. The juveniles were fed with commercial fish pellets containing 41% protein, 5% lipid, 5% fibre, and 10% water. The food was given daily as much as 5% of total body mass. The water in the aquaria was syphoned and refreshed every 3 days. The fish were monitored daily at 7.00 – 8.00 and 16.00 – 17.00.

2.2. LC₅₀ Determination

The tested chemical was K₂Cr₂O₇ (MW=294.21, Merk Cat.no. 024-002-00-6). The procedure for LC₅₀ testing was conducted according to OECD [11, 12]. The 2-cells stage embryos were selected for the LC₅₀ test to make sure that only normal embryos were used in this study. A series of K₂Cr₂O₇ concentrations consisted of 0, 1, 5, 10, 20, 40, 80, 160, 340, 680, and 1260 ppm were prepared in triplicate. Two hundred

embryos were placed into each replicate, the embryos were aerated. The number of hatched embryos for mortality determination. The corrected embryo mortality (CM) was determined according to Equation (1).

$$CM = \frac{\%M \text{ of treated} - \%M \text{ of Control}}{100 - \%M \text{ of control}} \times 100\% \quad (1)$$

The LC₅₀ of larvae was determined using 7 days post-hatching (dph) larvae. The tested concentration of K₂Cr₂O₇ were 0, 5, 10, 15, 20, 40, 60, and 80ppm. Three replicates each containing 10 larvae were provided for each test unit. The fries were not given food during the LC₅₀ 96h determination. The number of dead fries were recorded to determine the larvae mortality using the formulae as in Equation (1)

The juvenile LC₅₀ was determined using fries with an average total body length of 9.91±0.14cm. The tested concentration of K₂Cr₂O₇ were 0, 5, 10, 15, 20, 40, 60, and 80ppm. The procedure was conducted for 4days, the number of dead juveniles were recorded every day for mortality determination according to Equation (1)

Data were subject to probit analysis according to Finney's methods [13]. The probit of mortality was denoted as y and the log of K₂Cr₂O₇ concentration was denoted as x. The y and x were subjected to regression analysis to determine the LC₅₀. The probit analysis was performed using Microsoft Excel version 2016.

2.3. Effect of sub lethal exposure of K₂Cr₂O₇ on embryonic development

Based on the embryonic LC₅₀, a completely randomized design experiment was conducted to examine the sublethal effect of K₂Cr₂O₇ on embryonic development of *O. vittatus*. Five groups of 2-cells stage embryos were exposed to, 0, 20, 40, 60, and 80ppm of K₂Cr₂O₇, 8 units replicates each containing 200 embryos were provided, the measured parameters were hatching rate (HR) and survival rate (SR) of 4dph larvae. The HR and SR were determined using Equation (2) and Equation (3) respectively. The larvae were not fed since they still have a yolk.

$$HR = \frac{\text{Number of hatched embryos}}{\text{Number of fertilized eggs}} \times 100\% \quad (2)$$

$$SR = \frac{\text{Number of 4dph larvae}}{\text{Number of hatched embryos}} \times 100\% \quad (3)$$

2.4. Effect of sub lethal exposure of K₂Cr₂O₇ on Juvenile development

Three developmental stages of juvenile i.e. 30dph, 60 dph, and 90 dph fry were exposed to 0, 2.5ppm, and 5.00ppm of K₂Cr₂O₇, for 30 days. The fries were reared in aerated aquaria and fed with commercial fish pellets as much as 3% of total biomass daily. The aquaria were syphoned every 3 days to maintain the water quality. Water temperature and pH were monitored every 7

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Table 1. The Survival Rate of *O. vittatus* juveniles exposed to 0ppm (control), 2.5 ppm, and 5.00ppm K₂Cr₂O₇ for 30 days starting at 30dph, 60 dph, and 90dph

Parameter	Concentration of K ₂ Cr ₂ O ₇	60 dph	90dph	120dph
Survival Rate (%)	0 (Control)	88.83±11.39	83.33±5.77	40.83±6.31
	2.5 ppm	68.33±3.33	79.17±6.87	7.50±8.77**
	5.0 ppm	18.33±14.78*	39.17±9.95**	5.00±4.30**
Total body length (mm)	0 (Control)	14.67±1.53	26.75±3.88	37.00±1.7
	2.5 ppm	12.33±2.52	20.25±3.84	35.00±2.16
	5.0 ppm	10.00±0.82	20.3±3.85	33.33±1.53
Body weight (g)	0 (Control)	71.00±19.00	361.50±32.48	842.60±23.75
	2.5 ppm	51.33±20.25*	207.88±49.45**	694.00±136.15
	5.0 ppm	25.75±9.88*	130.75±52.89**	370.67±80.75**

* $p<0.05$; ** $p<0.01$

days. The data in form of total body length, body weight, and SR were collected after 30 days of K₂Cr₂O₇ exposure.

2.4. Data Analysis

The data in form of HR, SR, and total body length were tested for normality using the Kolmogorov-Smirnov test and homogeneity using Levene's test. The data were confirmed to be homogenous and normally distributed then they were analyzed using Anova followed post hoc test (Tukey).

3. RESULTS

3.1. LC50 of Embryo, Larvae, and Juvenile

The LC₅₀ for K₂Cr₂O₇ in *Osteochilus vittatus* was varied according to the developmental stages. The LC₅₀ for embryos was 100ppm, LC₅₀ for larvae was 14.37ppm ($y=4.76x-0.507$, $R^2=0.930$) and LC₅₀ for juveniles was 8.1 ppm ($y=8.853x-3.043$, $R^2=0.847$).

3.2. Effect of sub lethal exposure of K₂Cr₂O₇ on embryonic development

The effect of K₂Cr₂O₇ exposure on embryonic development of *O. vittatus* was evaluated based on their HR and SR of 4dph larvae (yolk sac larvae). The HR of control embryos and those treated with 0, 20, 40, 60, and 80ppm of K₂Cr₂O₇ were 90.61±2.52%, 81.13±0.70%, 87.21±4.62%, 76.30±3.20%, and 77.30±4.12% respectively. The HR of embryos exposed to 60ppm and 80ppm of K₂Cr₂O₇ were significantly lower than control embryos ($p<0.01$) and those exposed to 20ppm and 40ppm of K₂Cr₂O₇ ($p<0.05$). The SR of 4 dph larvae of the control and the treated embryos were 100%, 99.66±0.29%, 98.71±0.84%, 98.31±1.79, and 94.61±1.75 respectively. The SR of 4 dph larvae was significantly lower than control and those exposed to 20 – 60ppm ($p<0.05$).

These data showed that K₂Cr₂O₇ exposure affected embryonic development as indicated by the decreased of HR and SR. Morphological evaluation on prehatching embryos and newly hatched larvae showed that K₂Cr₂O₇ exposure induced embryos and larvae abnormality. Their body length was shorter with oedema at the pericardial cavity and abnormal vertebrae curvature showed by the bent tail and hook-like tail.

3.2. Effect of sub lethal exposure of K₂Cr₂O₇ on Juvenile development

The exposure of 2.5ppm, and 5.0ppm K₂Cr₂O₇ for 30 days to the 30dph, 60 dph, and 90dph juvenile significantly decreased their SR and growth in concentration-dependent manner (Table 1). The lowest SR was observed in the juvenile exposed to 5.0ppm K₂Cr₂O₇ regardless of their developmental stages (age). The growth of the survivor as measured by their total body length and body weight was also impaired.

4. DISCUSSION

The LC₅₀ value in the different developmental stages of *O. vittatus* is interesting. Initially, it was predicted that the LC₅₀ of the embryo will be lower than the larvae and juvenile because several studies suggested that the early stage of fish is more prone to toxicants [14]. The data in this study, however, did not match the prediction. A favorable explanation for such a condition is that the embryos were exposed to K₂Cr₂O₇ at the 2-cells stage in which the developing embryos are protected by the chorion and the substance present in the perivitelline cavity. A study in *Oryzias latipes* showed that embryonated chorion (water hardened chorion) capable of accumulating cadmium exposed to the embryos and reduced the amount of this heavy metal reaching the embryo [15]. To test if the same condition also occurs in *O. vittatus*, in a separate experiment, we fertilized *O. vittatus* oocytes in media

containing $K_2Cr_2O_7$ at a concentration up to 80ppm. The results showed that the fertilization rate and the HR of the embryos were comparable to the HR obtained from the sublethal exposure (data not shown).

The LC_{50} of Larvae was lower than the embryo but higher than the juvenile. After hatching, the larvae are no longer protected by the chorion thus more exposed to $K_2Cr_2O_7$. On the first two days post-hatching the mouth of *O. vittatus* larvae is still closed and the movement is still limited. As the development proceeds, the postlarvae and juveniles become more active. An increase in swimming, breathing, and feeding activities increase the area of exposure to $K_2Cr_2O_7$ through the skin, gill lamella, and mouth epithelium. The wider surface area in contact with water containing $K_2Cr_2O_7$ will increase chromium intake and its accumulation in various tissue. This condition might in part contribute to lower LD_{50} in the *O. vittatus* juvenile indicating that the juvenile of *O. vittatus* is less tolerant to $K_2Cr_2O_7$ compared to embryo and larvae. A similar condition was also reported in *Pagrus major* in which 1-day old fish were more tolerant to cadmium than older larvae [16].

The exposure of $K_2Cr_2O_7$ at a sublethal concentration significantly decreased the HR and the SR of 4dph larvae. $K_2Cr_2O_7$ dissolved in water will produce $Cr(VI)$ which is capable to pass through plasma membrane using a concentration gradient of divalent anion in chloride phosphate and channel as a tetrahedral divalent CrO_4^{2-} anion. This anion is structurally analogous to PO_4^{3-} , anions transported across cytoplasmic membranes through the chloride phosphate channel carrier, which integrates itself onto the organelle membrane structure and passes its content into various subcellular compartments [17]. Inside the cells, $Cr(VI)$ can be reduced to intermediate forms such as $Cr(V)$, $Cr(IV)$, and $Cr(III)$. In this reduction process, ROS are produced and lead to lipid peroxidation, DNA damage and apoptosis [18, 19a, 20], disfunction of Ca^{2+} mobility, cell cycle regulation, and metabolism [21]. These reactions might result in failure of cell function related to developmental disturbance responsible for the decrease of HR. There is also evidence that $K_2Cr_2O_7$ exposure to embryos slows down the embryonic development. In a normal laboratory condition (water temperature $26 - 28^\circ C$, pH 7 – 9, and saturated Oxygen), *O. vittatus* embryo hatch 23-25 hours post-fertilization [22]. In the current experiment, embryos exposed to 60ppm of $K_2Cr_2O_7$ hatched 35.2 ± 0.0 hours post-fertilization, and those exposed to 80ppm of $K_2Cr_2O_7$ hatched 46.28 ± 0.03 hours post-fertilization. The developmental disturbance may result in low quality of larvae and disruption of yolk utilization leads to reduced SR of the larvae.

The exposure of 5ppm $K_2Cr_2O_7$ to 30dph, 60dph, and 90dph *O. vittatus* juvenile for 30 days significantly

decreased their survival. The survivors of this exposure have significantly decreased ($p < 0.01$) in body weight (Table 1). The effect of chromium on fish growth have also been reported in *Platichthys stellatus*. In this species, exposure of 400ppb $K_2Cr_2O_7$ resulted in decreased of in daily length gain and daily weight gain in 2 weeks [23]. A study in *Oreochromis niloticus* showed that exposure of $15\mu/L - 3015\mu/L$ for 28 days significantly reduced the survival rate [4]. $K_2Cr_2O_7$ exposed to fish are accumulated in the gill, skin, and muscle [4]. Accumulation of Chromium in the gill affect the basement membrane and submucosa, in the more severe condition the gill filament undergoes hypertrophy [20]. This condition caused the fish to avoid taking food and reduce oxygen intake. The low food and oxygen intake leads to growth inhibition and increase mortality.

5. CONCLUSION

The LC_{50} of $K_2Cr_2O_7$ for embryo, larvae, and juvenile of *O. vittatus* indicate that there is a differential tolerant tolerance to $K_2Cr_2O_7$ in this species. Exposure of sublethal $K_2Cr_2O_7$ disturbs embryonic development, slow down fish growth, and increased mortality.

AUTHORS' CONTRIBUTIONS

GEW contributed on LC_{50} determination, data analysis and manuscript preparation, SH contributed on juvenile rearing and body length measurement, AS, AA and AES equally contributed on embryo incubation, HR and SR calculation.

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