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Germ Cells and Gonadal Development in a Teleost, *Osteochilus vittatus* (Valenciennes, 1842) Exposed to Potassium Dichromate

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ABSTRACT

Potassium dichromate, $K_2Cr_2O_7$, is a well-known heavy metal, commonly used as an oxidizing and tanning agent in industrial applications. Prolonged or repeated exposure of hexavalent chromium is deemed very toxic for aquatic biota, with long lasting effect. This substance induce damage to DNA and tissue structures, as well as disruption of survival and growth rate. The present research exposed *Osteochilus vittatus* in larval and juvenile stages, to varying concentration of $K_2Cr_2O_7$. This experiment was aimed to evaluate the effect of chromium on primordial germ cells (PGCs) and subsequently, to the subject gonadal development. The evaluation was based on paraffin-embedded section, stained with Haematoxylin-Eosin. $K_2Cr_2O_7$ of 2.5 and 5 ppm were applied to four crucial developmental stages; post-hatching larvae, 1-month, 2-months, and 3-months juvenile, for 30 days. There was a consistent pattern in all test subject, in which higher concentration of $K_2Cr_2O_7$ resulted in lower PGCs number and delayed gonadal appearance. Our results suggested that sublethal Cr exposure to larval stage potentially decrease PGCs and thus, hinder the formation of gonad. Regulation of Cr-containing waste disposal should be issued in near future, to prevent further damage on local freshwater fish.

Keywords: *Chromium, Cyprinids, Juvenile, Larvae, PGC*

ABSTRAK

Potasium dikromat, $K_2Cr_2O_7$, adalah logam berat yang umumnya digunakan sebagai zat pengoksidasi dan penyamak dalam industri. Paparan kromium heksavalen dalam waktu lama atau berulang bersifat toksik bagi biota air, dengan efek jangka panjang. Zat ini menginduksi kerusakan DNA dan struktur jaringan, mengganggu kelangsungan hidup serta laju pertumbuhan dari organisme yang terpapar. Penelitian ini memaparkan *Osteochilus vittatus* fase larva dan juvenil pada berbagai konsentrasi $K_2Cr_2O_7$. Eksperimen ini bertujuan untuk mengevaluasi efek kromium pada sel germinal primordia (SGP) dan perkembangan gonad subjek. Evaluasi didasarkan pada hasil sediaan histologis yang diwarnai dengan Hematoksilin-Eosin. $K_2Cr_2O_7$ dengan konsentrasi 2,5 dan 5,0 ppm dipaparkan selama 30 hari pada empat tahap perkembangan penting, yakni; larva 1 hari pasca penetasan, 1 bulan, 2 bulan, dan 3 bulan juvenile. Terdapat pola yang konsisten pada semua subjek uji, yaitu konsentrasi $K_2Cr_2O_7$ yang lebih tinggi berakibat pada jumlah SGP yang lebih rendah dan kemunculan gonad yang tertunda. Hasil penelitian menunjukkan bahwa paparan $K_2Cr_2O_7$ sublethal pada tahap larva berpotensi menurunkan SGP dan mengakibatkan terhambatnya pembentukan gonad. Batasan konsentrasi Cr dalam limbah yang dibuang ke perairan perlu dikendalikan untuk mencegah kerusakan lebih lanjut pada ikan air tawar.

Kata kunci: Cyprinidae; juvenil; larva; kromium, PGC

1. Introduction

Waste disposal has long become a concerning issue, especially in developing countries with ever growing industry. The most anticipated wastes are those containing heavy metal. Unlike organic contaminants, heavy metals are not biodegradable and tend to accumulate in living organisms (Bakshi & Panigrahi, 2018). Among those heavy metals, chromium is notorious to contaminate water bodies. In Indonesia, chromium is introduced into rivers through the effluent of batik industry

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(Jannah & Muhimmatin, 2019; Lestari et al., 2020).

In its hexavalent form, chromium is a harmful compound which both mutagenic and carcinogenic (Bakshi & Panigrahi, 2018). Precedent researches report, exposure of chromium to freshwater cyprinids cause acetylcholine inhibition (Nisha et al., 2016), gill disintegration (Solangi et al., 2012), hepatocytes pyknosis (Nazir et al., 2012), ROS (reactive oxygen species) production (Sfakianakis et al., 2015; Hao et al., 2022), and reproductive system disruption (Ebrahimi & Taherianfard, 2009; Khilare, 2017; Guo et al., 2020; Taslima et al., 2022).

Early developmental stage is deemed the most sensitive to chemical pollutant. During this time, cells differentiated and vital organs formed. If during the embryonic and larval phases, the subject is exposed to chemical pollutant which also endocrine disruptor agent, irreversible changes and delay in expression of crucial gene will happen (Rolland, 2000). Nguyen and Janssen (2002) experiment on *Clarias gariepinus* showed, exposure of chromium to newly hatched larvae for only 5 days led to deformed body axis of all the treated individuals. Lethal exposure may cause massive larval death (Jin et al., 2015), but sublethal exposure cause a worse consequence. Instead of obvious calamity, the damages in early stages led to major deformity in later development stage which cost the individual an important organ system. Mishra and Mohanty (2012) found that acute exposure of hexavalent chromium resulted in atresia of oocyte, hypertrophy of gonadotrophs, and decrease of 17β-estradiol level. These events in unanimous led to follicular apoptosis and ovary deformation in a much later stage. Research conducted by Khillare (2017), also approved that exposure of chromium to Cyprinid cause gonadal anomalies, marked by severe oocytes atresia. The presence of atretic oocytes reduce the number of healthy oocytes, which directly affect the reproduction capacity of the fish. Our previous study showed that the gonad primordia of *Osteochilus vittatus* (previously known as *Osteochilus hasselti*) is identifiable in histological section at week 8^{th} post hatching. The first sign of gonadal differentiation as indicated by the appearance of testicular structure was observed at week 16^{th} post hatching. The gonadal maturity of male was initiated at 7 months while in the female 9-10 months (Wijayanti et al., 2015).

The present study was set out to discover the potential of $K_2Cr_2O_7$ to induce gonadal deformity in the early developmental stage of Bony-lipped barb fish (*Osteochilus vittatus)*. Locally known as *Nilem* fish, it is commercially breed for consumption. As indigenous fish of Indonesia, this species of Cyprinids have been well distributed throughout Java, Sumatra, and Borneo (Hasan et al., 2019). Various sublethal exposure (2.5 ppm; 5 ppm) was given as treatment to the fish, ranging from newly hatched larvae, to 3-months old juvenile. The evaluation focused on primordial germ cells (PGCs) number and appearance of gonad. The results bring insights upon how severe chromium exposure affect reproductive development of local Cyprinid.

2. Material and Methods

2.1. Period and Location of Research

This research was conducted in March until August 2020. Procedure for LC50 determination, fish rearing, tissue processing, and histological evaluation were conducted at the Laboratory of Animal Structure and Development, Faculty of Biology. Specimen sectioning was conducted at the Jenderal Soedirman Research Laboratory.

2.2. Animal Subject Spawning, Handling, Rearing and during Treatment

The subjects used in this research were newly hatched yolk larvae or 24 hours post fertilization (4.8 mm±0.02), 1-month old larva (11 mm± 0.87), 2 months (15 mm±1.53) and 3 months old (27 mm±3.88) juvenile. The newly hatched larvae were obtained from induced spawning using GnRH analog (Ovaprim©, Syndel Lab), 0.5 mL/kg body weight for female, and 0.3 mL/kg body weight for male (Simanjuntak & Wijayanti, 2005). The larvae and juvenile were obtained from freshwater fish breeding center in Tambaksogra and Pandak, Banyumas, Central Java, Indonesia. The subjects were acclimatized for 7 days in 150 L aquaria, equipped with aeration system (Armada©; Resun©) to maintain oxygen supply in the water. During acclimatization, the subjects were fed with commercial pellet (Fengli©) containing 41% protein, 7% fat, 3% fiber, 13% ash, and 10% water content. The feed was given twice per day as much as 5% of total biomass (Lawrence, et al., 2012).

2.3. Determining the Concentration of Potassium Dichromate as Treatment

The potassium dichromate (Molar mass: 294.19 g/mol) was obtained from Merck© with catalogue number 104864, CAS number 7778-

50-9; 1 ppm of $K_2Cr_2O_7$ was prepared by dissolving 5.657 mg of $K_2Cr_2O_7$ powder in 1L distilled water.

The lethal concentration 50% (LC_{50}) of potassium dichromate was determined by evaluating the survival rate of larvae reared in medium with different concentrations of $K_2Cr_2O_7$ for 96 hours according to OECD (2019). Potassium dichromate concentrations used for LC50 determination were 0 ppm (control), 5 ppm, 10 ppm, 15 ppm, 20 ppm, 40 ppm, 60 ppm, and 80 ppm (Wijayanti et al., 2022). Each concentration was prepared in triplicates. Each replicate was filled with 10 larvae, reared with aerator, without feeding (OECD, 2019). The percentage of survived larvae was count corrected with control treatment (0 ppm). If the 0% < mortality of control treatment < 20%, then Abbot formula was used to look for corrected mortality, as follows (WHO, 2016):

$$\boldsymbol{P} = \frac{\boldsymbol{P' - C} \times \mathbf{100}\%}{\mathbf{100 - C}}$$

Notes :
P = corrected mortality (%)
P' = larvae mortality from each treatment (%)
C = larvae mortality from control treatment (%)

The obtained result was used to determine LC_{50} using Probit method with formula as follows:

$$\boldsymbol{LogLCn} = \boldsymbol{X_1} + \boldsymbol{X_2}\frac{\boldsymbol{n - P_1}}{\boldsymbol{P_2 - P_1}}$$

Notes :
X_1 = value from Log of concentration < than mortality "n"%
X_2 = difference from Log of mortality concentration > "n"% and < "n"%
n = percentage of cumulative value "n"%
P1 = percentage of cumulative value < "n"%
P2 = percentage of cumulative value > "n"%
LCn = the value of LC

The value of Log LCn was converted into Antilog to obtain LCn. The value of LC_{50} was 14.37 ppm. On the first attempt, the subjects under 10 and 15 ppm treatment could not survive even in the first week of exposure. The mortality happened despite the fact that all subjects had been acclimatized for 7 days, following OECD (2019) procedure. Therefore, to ensure that the fish survive for at least 1 month for evaluation of germ cells and gonadal development, the tested concentration of $K_2Cr_2O_7$ were adjusted to 0 ppm, 2.5ppm, and 5 ppm

2.4. Exposure of $K_2Cr_2O_7$

There were 4 developmental stages used in this research, day-1 yolksac larvae, 1-month old larvae, 2-months old post larvae, and 3-months old juvenile. These stages correspond to the period of primordial germ cell migration and gonadal development in this species. The subjects of each developmental stage were exposed to $K_2Cr_2O_7$ at the concentration of 0ppm (control), 2,5 ppm, and 5 ppm for 1 month. Therefore, at the time of data collection the fish have reached 1-month old, 2-months old, 3-months old, and 4-months old respectively. The initial density was 15 larvae/L for the day-1 yolk-sac larvae (reared in 2L container) and 10 fish/L for the juvenile (rear in 4L container). Three replicates were provided for each treatment. During the $K_2Cr_2O_7$ exposure, the subjects were fed with commercial pellet (Fengli©). The feed was given twice per day as much as 5% of total biomass (Lawrence, et al., 2012). The water quality of rearing media was monitor by measuring the water pH and temperature every week, the rearing media were syphoned and refreshed every 3 days. These treatments have been ethically approved by The Ethical Committee of Medical Research, Medical Faculty, Universitas Jenderal Soedirman (Number 192/KEPK/IX/2020).

2.5. Histological Specimens Preparation for Germ cells and Gonad evaluation

In accordance to the research design, there were 12 treatment combinations originated from three concentrations of $K_2Cr_2O_7$ (0, 2.5; 5 ppm) and four different developmental stage (1; 2; 3; 4 months-old). Therefore, three samples of each treatment combination were randomly taken for histological examination. The fish were chilled for 5 minutes to death and fixated in neutral buffered formalin (NBF) for 48 hours. Post-larvae and larvae samples (1 and 2-months old) were directly proceeded for embedding protocol, while juvenile samples (3 and 4-months old) were firstly decalcified. These specimens were submerged in a mix of decalcificant (8% of Hydrochloric acid + 8% Formic acid) for 24 hours (for 3 months old juvenile) and 40 hours (for 4 months old juvenile). Considering the presumed position of migrating PGCs (ventral to vertebrate and kidney, posterior to intestinal tract), the head (from mouth to operculum) and fins (ventral, abdominal, anal, and caudal) were removed for practical reason. The whole specimen body were further processed for embedding.

Embedding protocol began with dehydration of the samples in a series of alcohol concentration (70% to absolute). This step was followed by submerging the specimen in a series of alcohol:xylol (3:1; 1:1; 1:3 v/v; and pure xylol) to remove the alcohol from the tissue and infiltration which was submersion of specimens in series of xylol:paraffin (3:1; 1:1; 1:3 v/v; and pure paraffin). The specimens were then embedded in paraffin (Merck© CAS-no: 64742-51-4) using embedding mold and and cassette (SPL Life Science©). The specimen was sectioned in sagittal orientation using rotary microtome (Slee-Mainz CUT 4062©) at 5 µm thickness. The specimen sections were affixed on an object glass (GEA Medical) which already been coated with 1% gelatin. The tissue sections were left to dry at room temperature for 24 hours before staining. Staining procedure began with deparaffination in xylol, and rehydration in a series of alcohol concentration (absolute to 70%). The specimen was stained with Carazzi's Haematoxylin – Eosin 0.5% (Merck©). The specimen was dehydrated once again in a series of xylol concentration, and finally mounted with entellan (Merck©), and covered with cover glass (Menzel©). The detail of paraffin-embedding to staining procedure can be referred to Wijayanti et al. (2017)**.**

The specimen was observed under a light microscope (Olympus CH20©, ocular lens magnification: 10X) with 4, 10, and 40 objective magnifications. The histological features being observed include the presence of PGCs at the migratory pathway, the presence of gonad, and the number of PGCs entering the gonad. The characteristic of PGCs were the cells size is bigger than other cell type with metachromatic nucleus and higher ratio of nucleus:cytoplasm (Braat et al., 1999, Wijayanti et al. 2015). The amount of PGCs were counted manually, by observing all slides in which PGCs are visible. From each slide, 5 individual sections were observed, and the PGCs of these sections were averaged as cumulative data. The presence of gonad is expected to be located under the posterior kidney ventral to the swim bladder. The gonad developed in pair and connected to the dorsal wall of the body by a mesentery (Braat, et al., 1999, Saito & Psenicka, 2015, Wijayanti et al. 2015).

2.6. Statistics

The qualitative data, including histological structure of fish gonad, the presence of germ cells, and abnormalities in formation of gonad were analyzed descriptively and represented in figures. The quantitative data, the amount of germ cells, was analysed statistically by using Excel (Microsoft). The normality of amount of germ cells was evaluated using *Kolmogorov-Smirnov* method and homogeneity was evaluated using *Levene* statistics. As the data was proven to be normal and homogenous ($p > 0.05$), data analysis was proceeded to one-way analysis of variance (ANOVA). ANOVA result with p value <0.05 was further tested with t-test of 95% level of confidence.

3. Results and Discussion

3.1. The Effect of $K_2Cr_2O_7$ in decreasing the amount of PGCs

Histological examination on *O. vittatus* fish age of 1 month, 2 months, 3, months and 4 months showed the present of PGCs characterized by larger size of cell compared to the somatic cells and prominent metachromatic nucleus. The germ cells were located extra gonadal. PGCs are commonly observed at the dorsal side of coelom, where gonad will be established in later development. The PGCs of *Osteochilus vittatus* during and its migration route are displayed in histological specimen (Figure **1**1A, Figure **1**1B). The number of PGCs increased with age in both control fish and those exposed to 2.5ppm and 5.0ppm $K_2Cr_2O_7$ (Figure 2, Figure 3).

The exposure of $K_2Cr_2O_7$ at 4 different ages (1, 2, and 3 months) resulted in a lower PGCs number. There was no PGCs detected in 1 month and 2 months *O. vittatus* exposed to 5.0ppm $K_2Cr_2O_7$. In 1 month fish, exposed to 2.5 ppm $K_2Cr_2O_7$ only 1 out of 4 individuals showed the presence of 3 PGCs (Table 1).

The data presented in Table 1 showed that $K_2Cr_2O_7$ inhibit PGCs development in concentration dependent manner. The increased of $K_2Cr_2O_7$ concentration in the rearing media lead to a significant decreased of PGCs number ($y = -52.058x + 321.82$; $p < 0.001$) (Figure 4). The degree of inhibition on PGCs development was related to the age of initial exposure of $K_2Cr_2O_7$ and occurred in biphasic way. Exposure of $K_2Cr_2O_7$ to newly hatched larvae resulted in severe inhibition of PGCs development leads to no PGCs was observed in fish exposed to 5.0ppm $K_2Cr_2O_7$ and only 1 out of 4 fish showed a very limited number of PGCs in fish exposed to 2.5ppm $K_2Cr_2O_7$. Exposure of $K_2Cr_2O_7$ to 1 month, 2 months, and 3 months fish showed that the inhibition effect of 2.5ppm $K_2Cr_2O_7$ on PGCs development increased with age of initial exposure (Table 2).

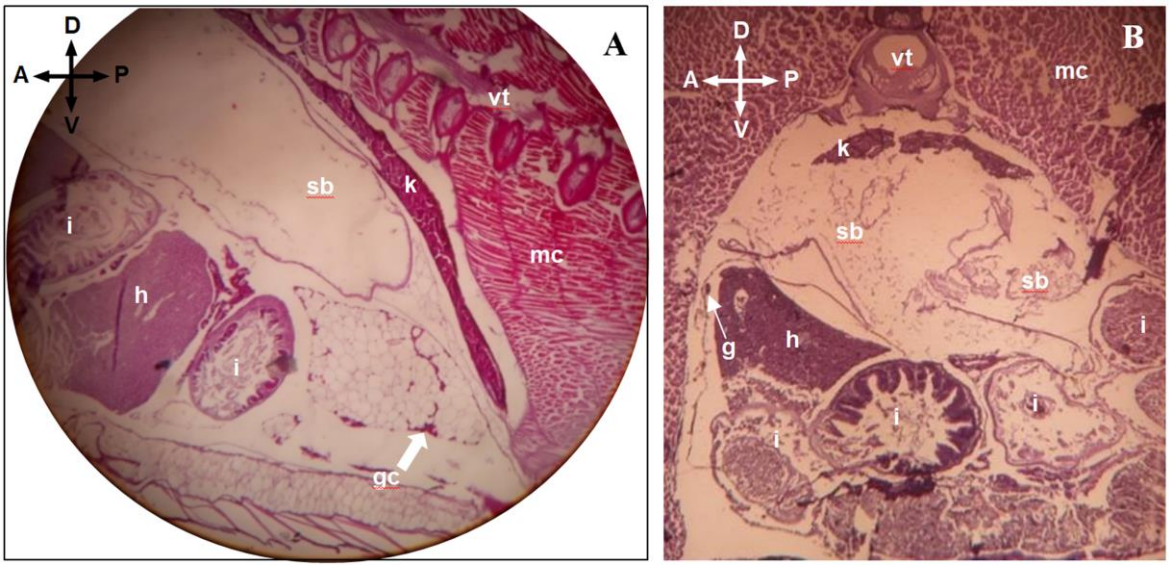

Figure 1. Histological Features of Untreated *Osteochilus vittatus* (Magnification: 72x) (I) Sagittal-section of 3-months old sample. (II) Cross section of 4-months old sample. A, anterior; D, dorsal; P, posterior; V, ventral; g, gonad; gc, primordial germ cell; h, hepatopancreas; i, intestine; k, kidney; mc, muscle tissue; sb, swim bladder; vt, vertebrae.

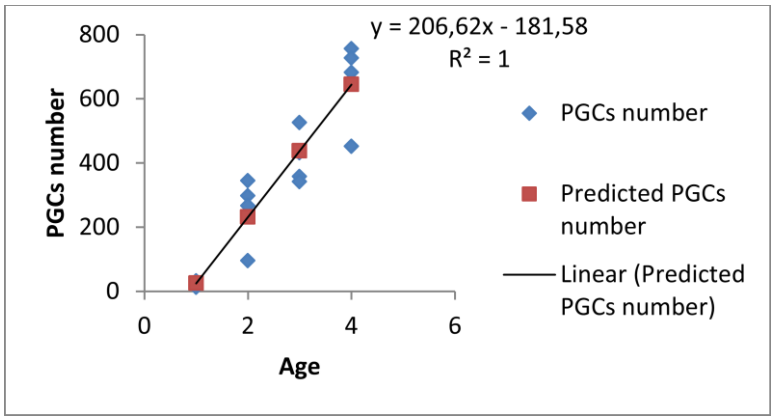

Figure 2. The Line Plot of regression showing the increase of PGCs number from 1 to 4 months old control samples

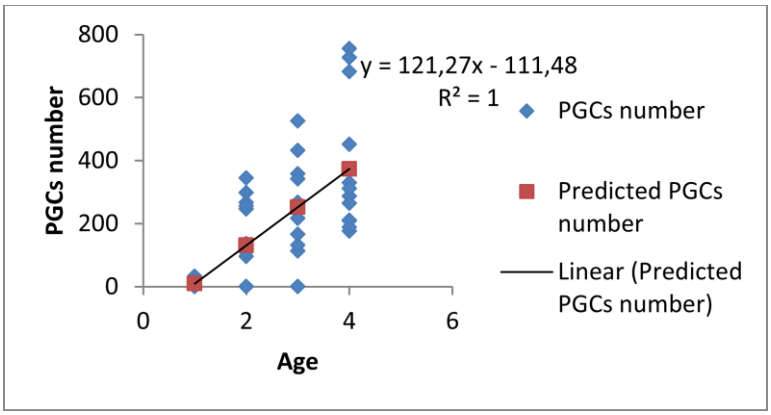

Figure 3. The Line Plot of regression showing the increase of PGCs number from 1 to 4 months old treatment samples

Table 1. Number of Primordial Germ cells (PGCs) of *O. vittatus* age of 1, 2, 3, and 4 months after 1 month exposure of $K_2Cr_2O_7$

Concentration of $K_2Cr_2O_7$	PGCs number and age of *O. vittatus* at the time of data collection			
	1 month (n=12)	2 months (n=12)	3 months (n=12)	4 months (n=12)
0 ppm (control)	19.75±9.21	251.5±108.51[a]	414.5±84.04[a]	654.15±138.13[a]
2.5 ppm	3*	187.0±74.24[b]	234.0±29.44[b]	298.25±28.30[b]
5.0 ppm	0	0	137.0±26.85[c]	196.0 ±16.91[c]

Note: *PGC was observed only in 1 out of 4 individuals. Different notation in the same column indicates significant different ($p<0.01$)

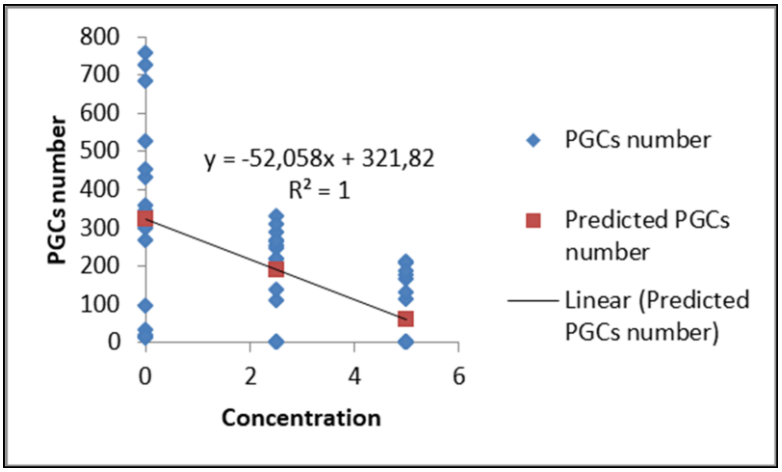

Figure 4. The Line Plot of regression showing the increase of K2Cr2O7 Concentration Leading to the Decrease of PGCs number from 1 to 4 months old samples

Table 2. Reduction of primordial germ cell number in *O.vittatus* age of 1, 2, 3, and 4 months after 1 month exposure of 2,5ppm and 5.0ppm $K_2Cr_2O_7$

Concentration of $K_2Cr_2O_7$	Percentage of PGCs reduction relative to control at the time of data collection			
	1 month	2 months	3 months	4 months
2.5 ppm	80.25%	25.65%	43.55%	54.41%
5.0 ppm	100%	100%	66.91%	70.03%

Our previous studies on PGCs migration in *O.vittatus* showed that during the first month post hatching most of the PGCs were still in migration toward the developing gonad (Wijayanti et al., 2015). While migrate, the PGCs also undergo cell division. There is a possibility that lower PGCs amount in $K_2Cr_2O_7$ treated fish is due to inhibition PGCs proliferation or induction of PGCs death through apoptosis. There are several factors affecting PGCs survival during their migration such as signaling molecules along the migratory pathway and signaling molecules for cell proliferation. The mechanism by which $K_2Cr_2O_7$ inhibit PGCs development in this species is unclear. Some studies in mammalian, however, shed some light upon how chromium exposure might cause the damage in germ cell development. An in vitro study using rat granulosa cells showed that exposure of 10µM potassium dichromate for 48 hours significantly decreased proliferation of granulosa cells (Stanley et al., 2011). This effect was resulted from cell cycle arrest in G1-progression through down regulation of cyclin-dependent kinases (CDK), cyclins, and PCNA while up-regulating CDK-inhibitors (Stanley et al., 2011). The inhibition of cyclin B3, one of protein involves in cell proliferation, was also reported in male clam *Geloina coaxans* (Guo et al., 2020). These finding support the hypothesis that CrVI interfere with germ cell proliferation leading to the decrease of PGCs number in $K_2Cr_2O_7$ treated fish.

The hypothesis that $K_2Cr_2O_7$ exposure induce apoptosis in *O.vittatus* PGCs initially inspired by an in vivo study showed that CrVI given to gravid rats capable of crossing the

placenta and induced defect to the mother and the produced offspring (Shivakumar et al., 2014). They proposed that these events happen due to hexavalent upregulate and downregulate the crucial gene expression. Cr(VI) downregulate p-AKT/p-ERK, SOD2, and XIAP, while at the same time it upregulate Casp-3, BAX, P27, and P53. P-AKT/p-ERK. The P53/KAT is a signaling transduction pathway which promotes survival and growth in response to extracellular signals. Its role to gonadal development is governing growth, differentiation and survival of the follicles during oogenesis. When p-AKT failed during this process, the subjects will have a significantly shorter reproductive lifespan, even some can be infertile (Shivakumar et al., 2014). Apoptosis due to this downregulation might still be redeemed with the expression of inhibitor apoptosis protein (IAPs) and the work of SOD2 (mitochondrial SOD). However, these inhibitors are also disrupted by chromium. By hindering IAPs, hexavalent chromium leads the release of Caspase 3 and 7. SOD2 failure to neutralize mROS will cause damage of mDNA, which therefore lead to cell apoptosis (Lakhani et al., 2006; Li et al., 2019; Hao et al., 2022). These caspases are responsible in enhancing germ cell death and early germ cell cyst breakdown. Even worse, chromium also upregulates BAX. Reportedly, BAX regulates the number of primordial follicles by causing germ cell death. In normal development, BAX played role in the death of ectopic germ cells that lost during its migration to gonadal ridge, and thus preventing such cell to turns malignant. In spite of its noble function, BAX should not be upregulated, due to the risk of killing non-ectopic germ cells. This is exactly what happens in subjects enduring sublethal chromium. Even though Sivakumar et al. (2014) research is based on mammals, Eimon and Ashkenazi (2010) approved of such proteins to be exist and have a same exact role in zebrafish. Wang et al. (2016) also reported the presence of BAX in grass carp (*Ctenopharyngodon idellus*).

3.2. The Effect of $K_2Cr_2O_7$ in disrupting PGCs migration into gonadal ridge and formation of functional gonad

Gonadal development was evaluated in serially sectioned paraffin embedded specimens. The histological feature of the developing gonad in *O.vittatus* is varied according to age and orientation of sectioning, its sometimes appear as irregular, triangular or ovoid structure attached to the dorsal mesentery. In early development the gonad hardly being recognized without topographical orientation. As the fish developed, the gonad apparent as thickening area in the mesentery at the ventro-lateral aspect of the swim bladder.

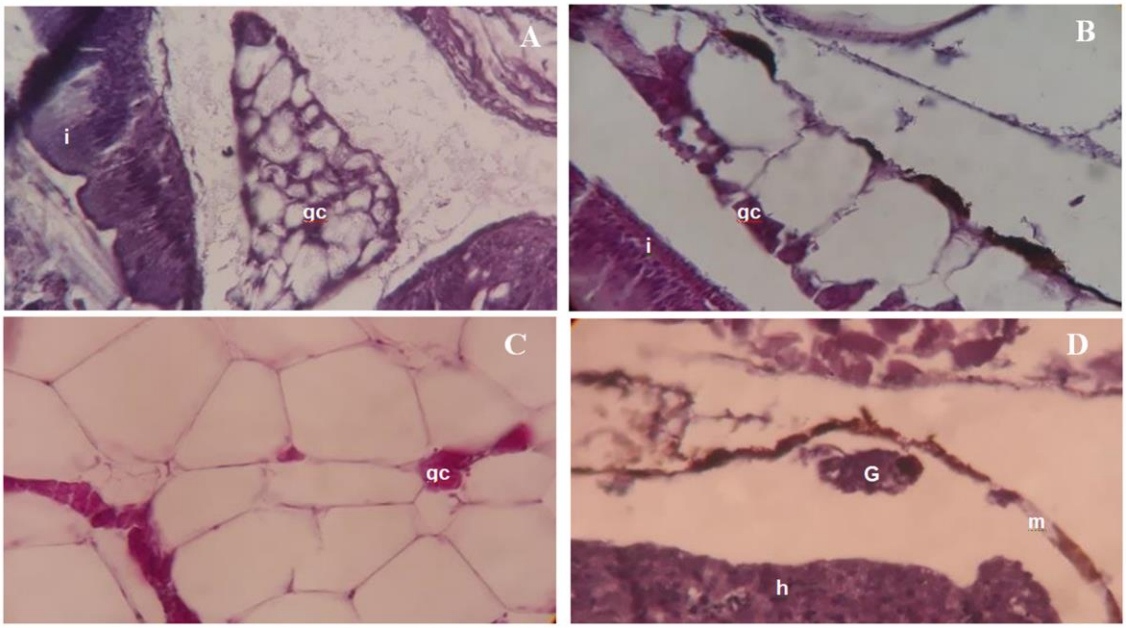

Figure 1. Sagittal Sections of 1, 2, 3, and 4 months old *Osteochilus vittatus* showing Primordial Germ cells and Gonad in Control Group (Magnification: 720x) (A) 1 month old; (B) 2 months old; (C) 3 months old; (D) 4 months old; G, gonad; gc, primordial germ cells; i, intestine; h, hepatopancreas; m, mesentery.

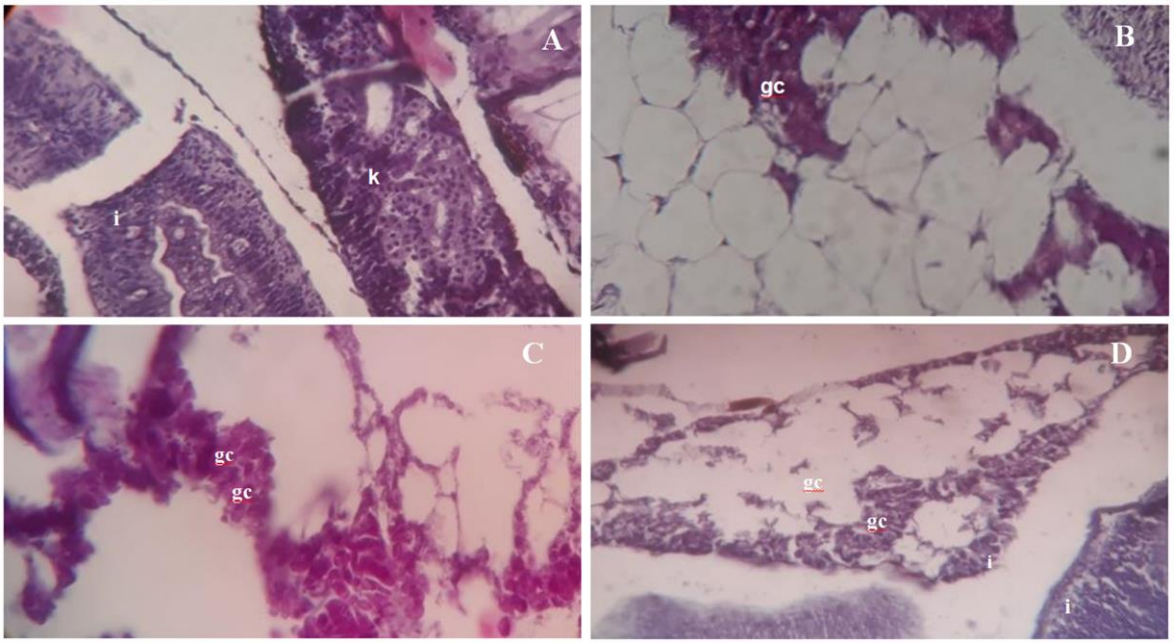

Figure 3. Sagittal Sections of 1, 2, 3, and 4 months old *Osteochilus vittatus* showing Primordial Germ Cells in 2.5 ppm K2Cr2O7Treatment Group (Magnification: 720x) (A) 1 month old; (B) 2 months old; (C) 3 months old; (D) 4 months old; gc, primordial germ cells; i, intestine; k, kidney.

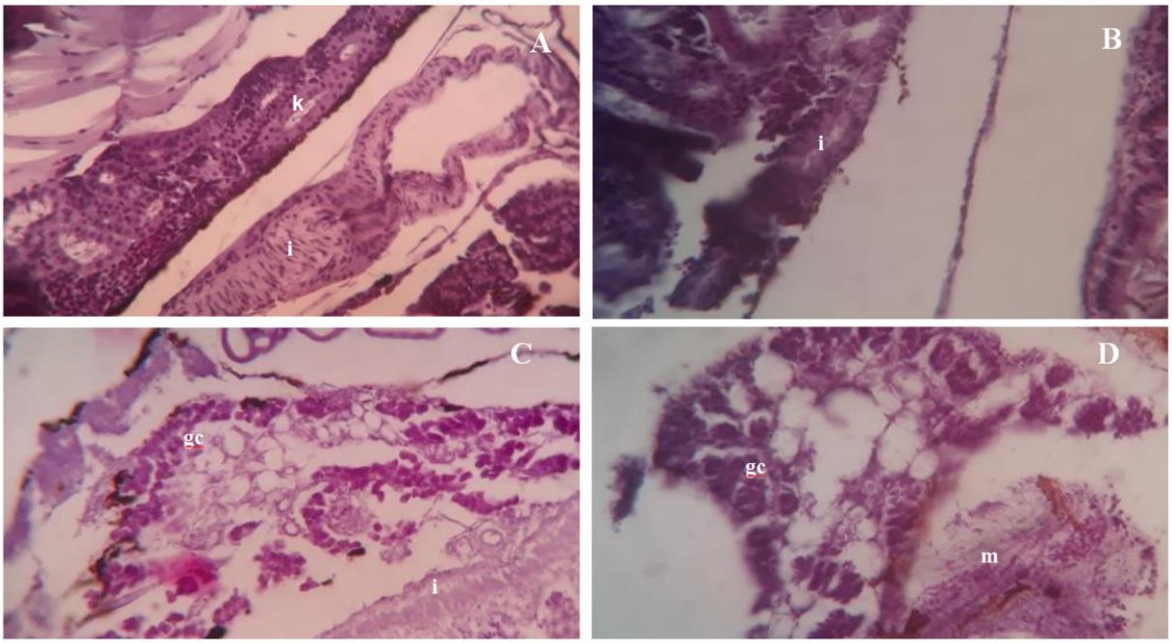

Figure 2. Sagittal Sections of 1, 2, 3, and 4 months old *Osteochilus vittatus* showing Primordial Germ Cells in 5 ppm K2Cr2O7 Treatment Group (Magnification: 720x) (A) 1 month old; (B) 2 months old; (C) 3 months old; (D) 4 months old; gc, primordial germ cells; i, intestine; k, kidney; m, mesentery.

Gonadal development of the control fish was comparable to our previous finding (Wijayanti et al., 2015). In the control fish, the gonad became more obvious. Most of the PGCs are entering the gonad, while several others are still located posterior to kidney and swim bladder, on track towards the gonad (Figure 5, white square; Figure 6D). In the treated fish most of the PGCs were still in their migratory pathway and no structure resembling gonad was detected. Figure 6D; Figure 7D). The development of *O.vittatus* gonad in this study closely correlated with the number of PGCs. The fish with lower number of PGCs as a result

of $K_2Cr_2O_7$ exposure tend to delay gonadal development. Role of PGCs in gonadal development have been studied in several species. In zebrafish, a complete absence of germ cells leads to sterile males (Tzung et al, 2015).

The development of fish gonad as in other vertebrates is regulated by several factors including genes. Studies in zebra fish (*Danio rerio*) showed that there are 21 genes involve in the development of primordial germ cells. Amongst them *dmrt* and *sox9a* are crucial for testis determination and development while *foxl2*, *brca2*, and *cyp17a1* are important for ovarian development (reviewed in Ye and Chen, 2020). In the testis, *sox9* has been shown to be an upstream positive regulator for *amh* and upstream negative regulator for *cyp19a1a* (Jorgensen et al., 2008). *brca2* is required for establishing or maintaining oocyte nuclear architecture and critical for ovarian development. In medaka (*Oryziaz latipes*), expression of *Foxl2* started in somatic cells surrounding germ cells in XX gonads, just after initiation of ovarian differentiation, and was maintained in granulosa cells throughout ovarian development. (Nakamoto et al., 2006). *Foxl2* also play a major role in ovarian development of Nile tilapia (Zhang et al., 2017).

The information on CrVI involvement in the regulation of genes expression needed for gonad development and differentiation is scare and call for research. It is worthy for a follow up research emphasizing in germ cell-related protein regulation and continued exposure of Cr(VI) starting from the fertilization, until the formation of gonad. Study on the effect of CrVI on early development of fish gonad is less compare to studies on the effect of CrVI in gonad of adult fish. Mishra and Mohanty (2008) had experimented with *Channa punctatus* being exposed to 4 mg/L CrVI for 1 month. Their subject showed retardation in both growth and ovary development. The gonadosomatic index (GSI) of fish under treatment is significantly lower, compared to control group. A higher dose of 20 mg/L cause severe oocyte atresia, despite of only exposed for 96 hours. These authors went further as to prove the gonadosomatic index (GSI) of exposed subject is very low, in contrast to control. Khillare (2017), treated mature females *Labeo rohita* with a high concentration of CrVI but in shorter duration of exposure leads to a severe damage in the ovary, including atretic oocytes, separation of ovarian follicles, appearance of vacant spaces in ovary, and reduction of fish ability to reproduce.

This study and other studies discuss in this paper clearly showed that the exposure of $K_2Cr_2O_7$ in critical stages of gonadal development might inflict turmoil in subject's reproductive capability in much later stages of life. Considering these consequences it is important to properly manage waste disposal containing CrVI before released it into the waters.

4. Conclusion

This study concludes that $K_2Cr_2O_7$ exposure cause detrimental effect to *Osteochilus vittatus* gonadal development. The concentration of 2.5 ppm of $K_2Cr_2O_7$ has induced intolerable effect on gonadal development, by means of significantly reducing the number of PGCs compared to control. Causatively, juvenile exposed to 2.5 and 5 ppm shows the absence of gonad in later development.

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