# ACUTE IN VITRO EXPOSURE OF POTASSIUM DICHROMATE ON OXIDATIVE STRESS, CELL DENSITY, AND CELL VIABILITY OF *Osteochilus vittatus*

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**Abstract.** The harmful effect of $K_2Cr_2O_7$ has been reported in animals and humans but the mechanism in exerting such an effect is unclear. An experimental study applying a completely randomized design was conducted to evaluate the impact of *in vitro* exposure to $K_2Cr_2O_7$ on MDA level, cell density, and cell viability in *Osteochilus. vittatus* testis. The testis fragments were cultured in DMEM supplemented with 10% FBS and 5% pen-strep for 24 hours and 72 hours at 29°C; 5 concentrations of $K_2Cr_2O_7$: 0µg.mL$^{-1}$, 0.125 µg.mL$^{-1}$, 0,250 µg.mL$^{-1}$, and 0.50µg.mL$^{-1}$ were tested. The result showed that the exposure of 0.500 µg.mL$^{-1}$ $K_2Cr_2O_7$ for 24 hours did not significantly affect the MDA level ($p>0.05$), but it significantly reduced cell density ($p<0.05$) and cell viability ($p<0.01$). Exposure to 0.500 µg.mL$^{-1}$ $K_2Cr_2O_7$ for 72 hours significantly increased the DMA level ($p<0.05$), cell density ($p<0.05$), and cell viability (0.01). These results indicated that $K_2Cr_2O_7$ induces oxidative stress, cell proliferation, and cell survival of *Osteochilus vittatus* testis.

**Keywords:** cell proliferation, cell viability, MDA, *Osteochilus vittatus*, oxidative stress

## A. Introduction

Chromium has been widely used in various industries, one of the most prominent being stainless steel manufacture due to its corrosion resistance [1]. Other industrial activities such as electroplating, leather tanning, wood preservation, the paint industry, and mine tailings also use chromium. Untreated or partially treated industrial effluent is discharged into surface water sources [2, 3]. The polluted water will eventually end up in the sea. Such conditions will put fresh water and marine aquatic organisms at risk. United Nations 2024 SGDs Goal 14 progress report informed an alarming trend of fish stock decrease. This calls for urgent actions to reduce pollution for the protection of life and the ecosystem for future generations. In supporting the SGDs 14 (life below water) the release of waste containing chromium has to be well regulated.

In the environment, Chromium exists in 6 oxidation states, among them Cr (III) and Cr(VI) are the most stable forms of chromium [4]. Cr(VI) exists in the form of three major ionic species namely $CrO4^{2-}$, $HCrO4^{-}$, and $Cr2O7^{2-}$ [5]. Cr (VI) is considered more toxic than Cr(III) and highly soluble in water [2]. Cr(VI) toxicity is due to its higher oxidation state and its ability to diffuse across the plasma membrane [6]. The exposure to Cr(VI) in humans and animals is mainly through drinking of Cr(VI)-contaminated water, ingestion of contaminated food, and direct skin contact [4 Chen]. Bioaccumulation of Cr has been reported in several organisms such as mussels Periwinkle (*Littorina littorea* Linnaeus) [7], soft tissue of Mussels (*Perna viridis*, Linnaeus 1758) [8], and catfish [9]. Continuous Cr(VI) accumulation along food chains leads to its biomagnification. This puts human health at risk due to dermatitis, kidney and gastrointestinal impairment, irritation to the respiratory tract and eyes, and lung cancer [10].

Cr(VI) also affects reproductive aspects in humans and animals. Exposure of Cr(VI) decreased sperm count per mL and sperm viability in human workers [11], reduced sperm

motility and subsequent embryonic development in mica [12], degenerated oocytes, delayed first polar body extrusion, and caused infertility in the rat [13]. Our previous studies showed that exposure to Cr(VI) increased embryonic abnormality, reduced embryonic hatching and larval survival rates, and decreased the juvenile growth and survival rates of *Osteochilus vittatus* [14]. Exposure of Cr(VI) to larvae and juveniles of *Osteochilus vittatus* reduced the germ cell number and delayed their gonadal development [15].

The mechanism of Cr(VI) inducing harmful effects on animals and humans is still open for studies. Research in animals suggested that Cr(VI) exerts its effect by inducing lipid peroxidation leading to oxidative stress [16, 17] as indicated by an increase in malondialdehyde (MDA) level [16]. This research evaluated the effect of *in vitro* exposure of Cr(VI), in the potassium dichromate, on MDA level, cell proliferation, and cell viability of *Osteochilus vittatus* testis.

## B. Methods

### 1. Experimental design

This research was conducted experimentally by applying a completely randomized design with 4 treatments i.e. 0 $\mu g.L^{-1}$, 0.125 $\mu g.L^{-1}$, 0.250 $\mu g.L^{-1}$, and 0.500 $\mu g.L^{-1}$ of $K_2Cr_2O_7$ as independent variables; the dependent variables were oxidative stress, cell proliferation, and cell density. The measured parameters were the MDA level of the testis explants, cell density, and cell viability of the outgrowth after 24 hours and 72 hours of culture. Six replicates were provided for each treatment.

### 2. Explant and $K_2Cr_2O_7$ Preparation

The testis explants were prepared from adult *Osteochilus vittatus* length of 17±2.77cm; and weight of 63.6±35.4 g. The fish was purchased from a local fish farmer and acclimated to the laboratory condition for 7 days. The fish were sacrificed humanely by decapitation using a sharp knife, and the abdominal cavity was aseptically dissected to remove the testes. The testes were placed on a sterile petri dish filled with a handling medium containing DMEM low glucose with pyruvate (Gibco Cat No. 11-885-084) and 5% penicillin/streptomycin (Sigma, #P4333). The testes were moved to another sterile petri dish containing handling medium, they were cut into small fragments (0,01g per fragment).

The 1 ppm of chromium was prepared by dissolving 5.657 mg of $K_2Cr_2O_7$ powder in 1 L of sterile distilled water. The potassium dichromate (Molar mass: 294.19 g/mol) was obtained from Merck© with catalogue number 104864, CAS number 7778- 50-9. The $K_2Cr_2O_7$ solution was sterilized using the sterile Cellulose Acetate syringe filter with pore size of 0,22 µm (HIMEDIA SF172-50NO).

### 3. Testis Explant Culture

Four sterile 24-well plates were prepared for culture, 2 well plates were designated for 24 hours culture and the other 2 well plates were designated for 72 hours culture. Each well was filled with 1 mL culture medium consisting of low glucose DMEM low glucose with pyruvate (Gibco Cat No. 11-885-084) supplemented with 10% Fetal Bovine Serum (Sigma F2442), 5% penicillin/streptomycin (Sigma, #P4333), and 5% L-Glutamine (Merk G7513). The sterile $K_2Cr_2O_7$ solution was added into the culture medium to the final concentration of 0 $\mu g.L^{-1}$, 0.125 $\mu g.L^{-1}$, 0.250 $\mu g.L^{-1}$, and 0.500 $\mu g.L^{-1}$ $K_2Cr_2O_7$. The culture medium was equilibrated and a testis fragment was put into each well. The testis explants were cultured in a $CO_2$ incubator at 29°C, with 5% $CO_2$, and saturated humidity.

4. Malondialdehyde Measurement

The testis explants were removed from the culture media, washed 3 times in phosphate-buffered Saline (PBS) then homogenized in 1 mL PBS solution. The homogenates were centrifuged at 3000 rpm for 10 minutes, the supernatants were removed and placed into sterile 1.5 mL centrifuge tubes for DMA measurement.

The MDA measurement was performed according to Randox MDA Manual [18], in brief, a series of tetraethoxypropane (TEP) concentrations consisting of 0.0 $mg.L^{-1}$, 0.5 $mg.L^{-1}$, 1.0 $mg.L^{-1}$,1.5 $mg.L^{-1}$, 2.0 $mg.L^{-1}$, 2.5 $mg.L^{-1}$, and 3.0 $mg.L^{-1}$ were prepared. For MDA measurement, 400 µL each concentration of TEP standard and 400 µL of each sample was added with 400 µL of 20% trichloroacetic acid, the mixtures were vortexed and were centrifuged at 6000 rpm for 10 minutes. 400 µL of the supernatants were added to 1 mL of 0.67 % thiobarbituric acid. The mixtures were heated at 100°C for 10 minutes. The standard solutions and the samples were measured using a spectrophotometer at 532 nm wavelength. The standard absorbances were subjected to regression analysis to obtain the standard curve equation. The MDA level of the samples was determined using the standard curve equation.

5. Cell Density and Viability

After 24 hours and 72 hours of culture, the culture medium was removed from the well plate. The explants were taken out, and the cell outgrowth was washed 3 times in PBS solution; 500 µL of 0.25% trypsin/EDTA solution was added to each well, and then incubated for 5 minutes to detach the cells from the substrate. The detached cells were removed from the well, put into the centrifuge tube, and centrifugated at 3000 rpm for 5 minutes. The supernatant was removed, and the cells were resuspended in a washing medium containing DMEM, 10% FBS, and 5% penicillin/streptomycin. This step was repeated 3 times to make sure that there was no trace of Trypsin. The cells were resuspended in the culture medium, 20 µL of cell suspension was put into a 0.5 mL tube then 20 µL of trypan blue was added. The cell mixture was homogenized then loaded to the hemocytometer to calculate cell density and cell viability according to the following formula:

Cell density per mL= $X.(1/4.10^{-3}).10^{3}.DF$

X= average of cell amount of 5 counting chambers (volume of the counting chamber is $10^{-3}mm^{3}$); DF= dilution factor is 2

$$\text{Cell viability} = \frac{\text{Number of life cell}}{\text{Number of life+dead cell}} \times 100\%$$

6. Data Analysis

The MDA concentration, cell density, and cell viability data were subjected to normality tests using Kolmogorov-Smirnov and homogeneity tests using Lavene's. As the data were normally distributed ($p>0.05$) and homogenous ($p>0.05$) the analysis was preceded to oneway Anova.

## C. Results And Discussion

1. Malondialdehyde Level in the Testis Explants

The MDA level in the testis explant cultured for 24 hours ranged from 7.97±0.15 $\mu g.mL^{-1}$ to 8.18±0.29 $\mu g.mL^{-1}$. The MDA level in the testis explant cultured for 72 hours ranged from 7.13±0.65 $\mu g.mL^{-1}$ to 9.67±1.46 $\mu g.mL^{-1}$ (Figure 1).

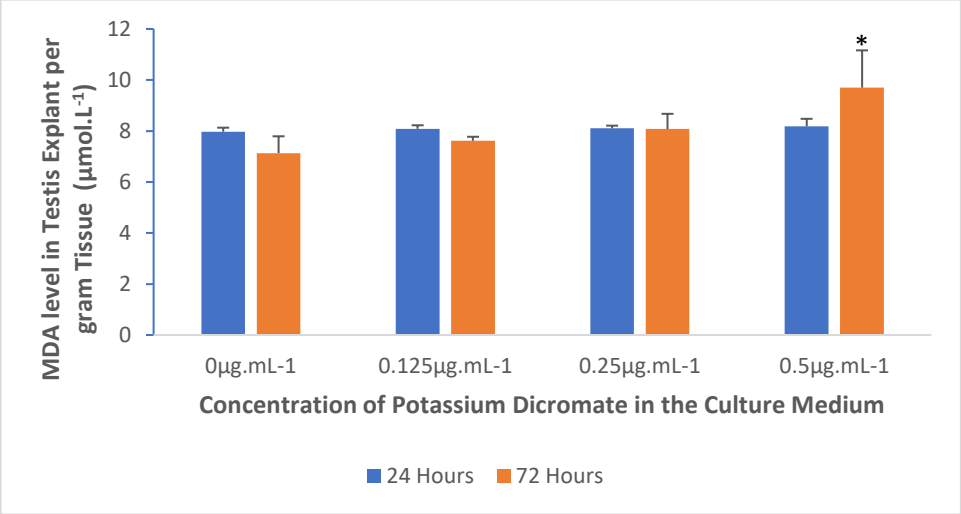


Figure 1. The MDA level in the testis explant after in vitro exposure of $K_2Cr_2O_7$ for 24 hours and 72 hours in DMEM supplemented with 10% FBS, 5% penicillin/streptomycin, and 5% L-Glutamine

The MDA level in the testis explant exposed to 0.125 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ for 24 hours was not significantly different to the MDA level of the control ($p>0.05$). Extended exposure of 0.125 µg.mL$^{-1}$ to 0.250 µg.mL$^{-1}$ $K_2Cr_2O_7$ to 72 hours did not significantly affect the MDA level of the testis explants but exposure of 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ significantly increased the MDA level in the testis explant ($p<0.05$).

2. Cell Density and Cell Viability

The cell proliferation in the testis explants exposed to $K_2Cr_2O_7$ was evaluated based on the cell density of the outgrowth. The cell density of explant outgrowth in the control group after 24 hours of culture was 8.23±1.86x10$^8$ cells.mL$^{-1}$ while in the explants exposed to 0.125 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ was ranged from 6.93±0.86x10$^8$ cells.mL$^{-1}$ to 4.79±1.10x10$^8$ cells.mL$^{-1}$. The cell density of explant outgrowth in the control group after 72 hours of culture was 6.44±0.55x10$^8$ cells.mL$^{-1}$ while in the explants exposed to 0.125 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ was ranged from 5.79±1.32x10$^8$ cells.mL$^{-1}$ to 3.21±0.80x10$^8$ cells.mL$^{-1}$ (Figure 2.).

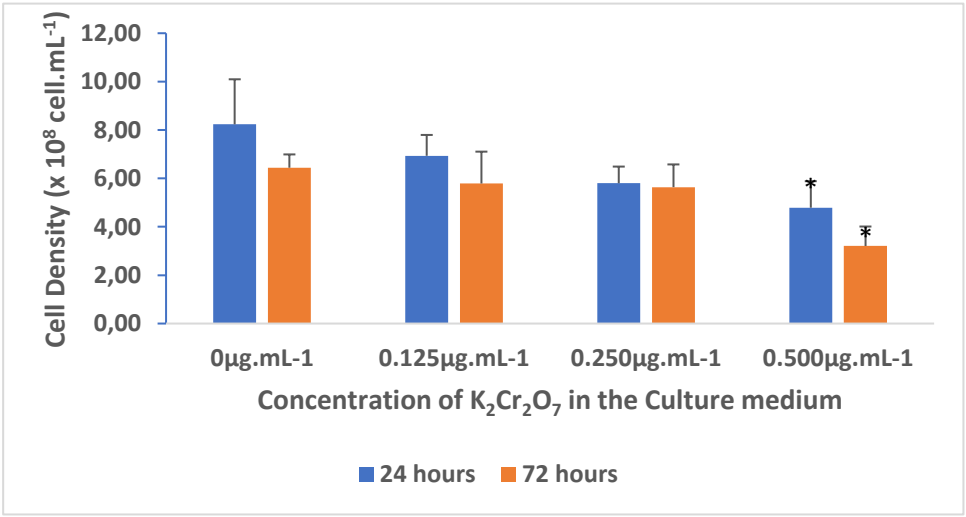


Figure 2. The cell density of the testis explant outgrowth cultured for 24 hours and 72 hours in DMEM supplemented with 10% FBS, 5% penicillin/streptomycin, and 5% L-Glutamine containing 0.00 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$

The cell viability of the testis explant outgrowth after 24 hours of culture in the presence of 0.00 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ was ranged from 98.88±0.58% to 75.33µ±5.03%. After 72 hours of culture in the presence of 0.00 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ the cell viability was ranged from 93.00±0.58% to 51.67±1.53% (Figure 3).

Culturing the testis explants in the presence of 0.125 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$ for 24 hours significantly decreased the cell viability ($p<0.01$). The cell viability decreased as the concentration of $K_2Cr_2O_7$ in the medium increased ($y=-45,6x + 98,6$; $p<0.01$). The same pattern was observed after 72 hours of exposure of the testis explants to 0.125 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$, the cell viability decreased in concentration dependent manner ($y = -85,181x + 94,8$;$p<0.01$).

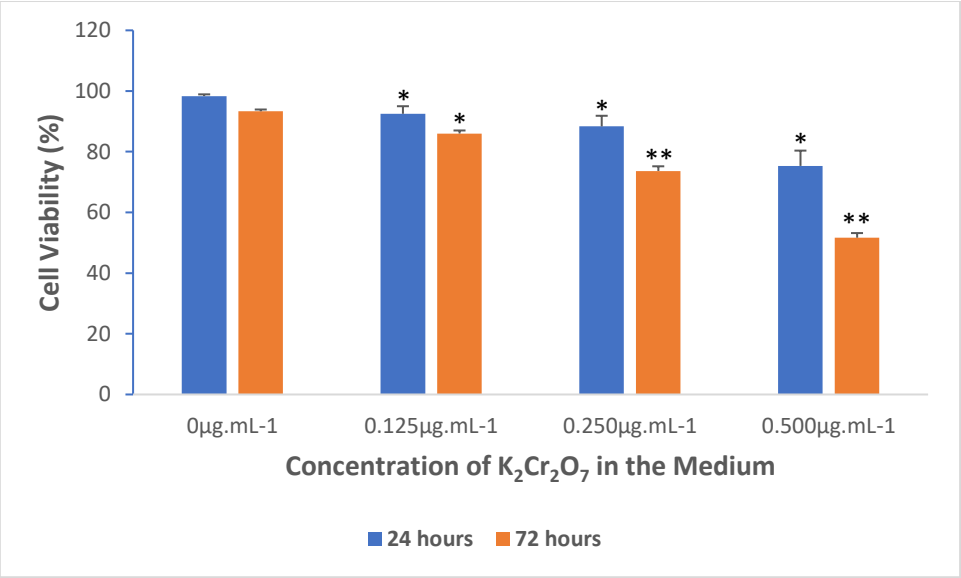


Figure 3. The cell viability of the testis explant outgrowth cultured for 24 hours and 72 hours in DMEM supplemented with 10% FBS, 5% penicillin/streptomycin, and 5% L-Glutamine containing 0.00 µg.mL$^{-1}$ to 0.50 µg.mL$^{-1}$ $K_2Cr_2O_7$

MDA is produced by the cells suffering from oxidative stress. When the $K_2Cr_2O_7$ solution was added to the medium the molecule would form Cr(VI). There is a possibility that this anion enters the cell via an anion transporter or other surface receptor phosphate transporter [19]. When Cr(VI) enters the intracellular space, it is subsequently reduced to Cr (III) [20]. During the reduction process, ROS are generated, which cause cell toxicity. Studies suggest that Cr(VI) toxicity is mainly due to an increase in ROS production. ROS are the primary effector molecules of oxidative stress. The presence of ROS might induce lipid peroxidation. A study in human showed that MDA can be formed during lipid peroxidation of polyunsaturated fatty acids (PUFAs) by the action of human platelet thromboxane synthetase on prostaglandins PGH2, PGH3, and PGG2, and by the action of polyamine oxidase and amine oxidase on spermine [21].

Even though the exposure of $K_2Cr_2O_7$ did not drastically increase the MDA it might induced an effect on the cells outgrowth. MDA can react with cellular components such as proteins, DNA, and lipids, leading to cellular damage and dysfunction [22]. The cellular damage might affect cell fitness and survival. In the present study the effect of $K_2Cr_2O_7$ was more severe on cell survival. The decrease of cell fitness will slow down cell proliferation as indicated by the decrease of cell density. $K_2Cr_2O_7$ may affect cell proliferation by regulating the cell cycle. Exposure of the human cell line NHIK 3025 to 8 µmol.l$^{-1}$ $K_2Cr_2O_7$ inhibits cell proliferation, the effect was observed within 6-9 hours of exposure [23]. The inhibition of cell proliferation and cell survival in the testis has led to decreased fertility due to the reduction of sperm production in this species.

## D. Conclusion

This finding showed that the exposure of $K_2Cr_2O_7$ induced a harmful effect on *Osteochilus vittatus* testis by stimulating the production of MDA, and decreasing cell survival leading to decreased in cell proliferation.

## E. Acknowledgement

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