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Hematological responses of Goldfish (*Carassiusauratus*) to different acute concentrations of Silver Sulfate as a toxicant

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ABSTRACT: This study aimed to evaluate the efficacy of silver sulfate ($AgSO_4$) as a toxicant in goldfish (*Carassiusauratus*). One hundred and forty-seven live specimens of *C. auratus* were obtained and exposed to 1, 10, 100, 500, 1000 and 2000 ppm of $AgSO_4$for 96 hours. There was one control group (no $AgSO_4$) and three replicates. The physicochemical properties of water and the following parameters were constant: pH: 7.56±0.45 (TS1); temperature: 19±1°C; hardness: 293±2.35 ppm and dissolved oxygen: 8.80 ±0.06 mg L^{-1} (DO-5510). LC_1, LC_{10}, LC_{30}, LC_{50}, LC_{70}, LC_{90} and LC_{99} were calculated in 24, 48, 72 and 96 hours. For assessing the impact of$AgSO_4$ on physiological responses of goldfish hematological indices, blood glucose and cortisol levels were measured. Results showed that LC_{50} 96-h of $AgSO_4$ for goldfish was 687.81 ppm. In addition, the use of $AgSO_4$induces a significant decrease in MCHE after 48 hours, MCV and MCH after 96 hours and lymphocyte after 96 hours in contrast to the control group ($P<0.05$). Furthermore, increased lymphocyte was significant after 24 hours exposure ($P<0.05$). In addition, glucose increased significantly at $P<0.05$ with time increase24 hours after experiment but this (). In conclusion, the study showed that acute toxicity of $AgSO_4$induced hematological alterations in goldfish and offers a tool for the evaluation oftoxicity-derived alterations.

Keywords: *Carassiusauratus*, hematological parameters, silver sulfate, stress response, toxicity.

INTRODUCTION

Aquatic ecosystems are the largest natural environments constantly faced with threats of deterioration in genetic and biological diversity (Vinodhini and Narayanan, 2009; Shahbazi Naserabad et al., 2015). Silver (Ag^+) is one of the most toxic metals known to aquatic organisms and of concern in various aquatic ecosystems because of the severity of its contamination in the water column, sediments and biota (Eisler, 1996).Silver is used as halide in the manufacture of photographic imaging materials, jewelry, coins, indelible inks, eating utensils; and used as silver salt in caustics, germicides, antiseptics, and astringents production (Klaassen et al., 1986). It is also a waste product from heavy metal mining and milling processes (Lima et al., 1982). Most of Ag in the environment is bound to particles, thiosulfate, organic colloids, dissolved

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organic matter (DOM), sulfide, and chloride, with the latter two representing the major forms of Ag in oxic natural waters where fish live (Wood et al., 1999).In contrast, the proportion of uncomplexed ionic Ag (Ag^{+}) is normally a very small percentage of the total Ag amount in waters resources (Purcell and Peters, 1998; Shafer et al., 1998; Lytle, 1984).

Ecotoxicology is the study of the impact of environmental contaminants on ecosystems. Understanding the effect of toxicants on fish supports the larger ecotoxicological goal of comprehending the action of ecotoxicans on fish populations (Bols et al., 2001). It is important to examine the toxic effects of heavy metals on fish since they constitute an important link in the food chain and their contamination by heavy metals causes imbalances in the aquatic system (Ahmad, 2011, Khabbazi et al., 2014). *Carassiusauratus* (Goldfish) is a freshwater, benthopelagic fish of the Family Cyprinidae, Order Cypriniformes. It is an exotic and invasive fish inhabiting the Inland waters of Iran, with a wide distribution (Esmaeili et al., 2014). *C. auratus* is considered as the most popular and favorable aquarium fish in Iran and approximately 5 million goldfish are reproduced during the new year's holidays (Nowruz) (Coad and Abdoli, 1993).The toxicity of Ag+ to fishes is relatively well documented. Many researchers have reported silver toxicity in various aquatic organisms (Birge and Zuidervee, 1995, Davies et al., 1978; Ratte, 1999; Shaw et al., 1998; Lee et al., 2005; Asharani et al., 2008; Fabrega, 2011), but data on acute toxicity and the effects of $AgSO_4$ on hematological parameters of *C. auratus* are scarce.

Silver is very reactive or catalytic and is able to pass through cell membranes in organisms. Furthermore, its interactions in biological systems are relatively unknown. Therefore, the aim of this study was to determine the potential toxicity of $AgSO_4$ in *C. auratus* and its impact on hematological parameters. These data can be useful in aquatic toxicity management and environmental safety.

MATERIALS AND METHODS

Ethics Statement

All experiments performed on fishes in this study complied with the standards of the Organization for Economic Cooperation and Development (OECD). All analyses were accomplished to minimize suffering. Fish were anaesthetized before blood sampling was carried out.

One hundred and forty-seven live specimens of *C. auratus*weighing56.33±12.05 g were used for this study. They were acclimatized randomly in a 400 L fiberglass aquarium for one week. Six aquariums were treated with 1, 10, 100, 500, 1000 and 2000 ppm of $AgSO_4$ with one control group (no $AgSO_4$). $AgSO_4$was purchased from Merck Company (Frankfurter, Germany).No feeding occurred during the period of the test (96 hours). There were no significant differences in water quality among the aquariums and the following were constant: pH: 7.56±0.45 (TS1); temperature: 19±1°C; hardness: 293±2.35 ppm and dissolved oxygen: 8.80 ±0.06 mg L^{-1} (DO-5510). 80% of water in the aquariums were changed every 12 h with re-dosing after each change and the photoperiod was adjusted to 12 h light and 12 h dark. Static acute toxicity test was performed following the guidelines of OECD standard method (OECD, 1989). Mortality rates were recorded after 24, 48, 72 and 92 hours and dead specimens were quickly removed from the aquarium. The nominal concentration of toxin causing mortality (LC1،LC10 ، LC30 ، LC50 ،LC70 ،LC90 and LC99) within 24, 48, 72 and 92 hours was recorded. LC50 values for 24, 48, 72 and 96 h exposures were computed and analyzed with probit analysis version 16.0 (Finney, 1971).

Fish were anaesthetized with 200 ppm eugenolin 5 L tanks and blood samples were collected 24, 48 and 96 hours after

exposure. Hematological parameters were estimated according to routine clinical methods (Wintrobe, 1974). The acid-hematin method of Sahli in hemometer was used to analyze hemoglobin percentage and Naeubaur's double hemocytometer to enumerate the erythrocytes (Mukherjee, 1988). Mean cell hemoglobin (MCH), mean corpuscular volume (MCV) and mean cell hemoglobin concentration (MCHC) were calculated according to Decie and Lewis (1991). One-way analysisof variance (ANOVA) wasused to analyze hematological parameters.

Cortisol and Glucosetests were carried out as described by in Shaluei et al. (2012). The blood samples were measured by placing in tubes and allowed to clot at 22-24°C for 30 min. Serum was removed from the clotted sample after centrifugation at 5,000 rpm for5 min and frozen at -80°C until analysis. Glucose was measured using a spectrophotometric method (WPAS2000-UV/VIS, Cambridge, UK) with reagents provided in standard analyses kits (Pars Azmon, Iran). Cortisol was determined with a commercial kit (ELISA, DRG Diagnostics, Mountainside, NJ, USA) (King et al., 2005; Teles et al., 2007; Caruso et al., 2010). ELISA kit was validated for use by linear response tests of the sample and cortisol overload (Weber et al., 2011).

All results are expressed as mean ± SD. Statistical analyses were carried out using SPSS 18.0. Normality of data was first estimated using a Kolmogorov–Smirnov's test and homogeneity of variance was assessed with Levene's test. Differences between means were determined using one-way ANOVA followed by Tukey's multiple range test at 5% probability level.

RESULTS

No mortality was observed during the acclimatization. Results showed that within 96 h, LC_{50} value in goldfish declined (687.81 ppm) with increasing toxin concentration and duration of exposure. The nominal concentration of toxin-causing mortality (LC1, LC10, LC30, LC50, LC70, LC90 and LC99) within 24, 48, 72 and 92 hours are presented in Tables 1 and 2. Hundred percent mortality of fish occurred 72 hours after exposure to 2000 ppm concentration of the contaminant. Effects of different concentrations of silver sulfate on hematological indices of goldfish are presented in Table 3.Values of MCHE after 48 hours, MCV and MCH after 96 hours and lymphocyte after 96 hours showed significant reduction in contrast with the control group ($P<0.05$).Furthermore, increase in lymphocyte was significant after 24 hours exposure ($P<0.05$). In addition, glucose increased with increasing time but this increase was significant at 24 hours after exposure ($P<0.05$) (Fig. 1). Figure 2 shows the minimum and maximum levels of lethal concentration of $AgSO_4$ for goldfish.

Table 1. Cumulative mortality of Goldfish (n=21, each concentration) exposed to acute $AgSO_4$

$AgSO_4$ lethal concentrations (ppm)	**Number of samples**	**Mortality (No.)**			
		24 hours	**48 hours**	**72 hours**	**96 hours**
0	21	0	0	0	0
1	21	0	0	0	0
10	21	0	0	0	0
100	21	0	0	0	0
500	21	0	3	8	8
1000	21	0	5	11	17
2000	21	19	19	21	21

Table 2. Lethal Concentrations (LC1-99) of $AgSO_4$ depending on time (24-96h) for Goldfish (mean ± SE)

	Concentrating (ppm) (0.05 Significant level)			
LC	**24 hours**	**48 hours**	**72 hours**	**96 hours**
LC_1	1360	75.06 (0-340.31)	0	28.77 (0-189.04)
LC_{10}	1670	627.05 (376.15-822.63)	355.11 (0-585.50)	324.75 (157.08-440.13)
LC_{30}	1890	102000 (80..10-1231.12)	645.16 (396.06-958.90)	539.25 (421.71-649.19)
LC_{50}	**2050**	**130000(1109.94-1558.21)**	**861.27 (630.62-1330.17)**	**687.81 (580.20-818.78)**
LC_{70}	2200	158000(1360.78-1912.30)	106.17 (810.52-1756.09)	836.37 (720.0-1007.5)
LC_{90}	2430	1850 (1698.52-2447.99)	1360 (1033.64-2407.69)	1050 (903.5-1297.23)
LC_{99}	2740	2530 (2144.94-3206.82)	1780 (1317.43-3330.92)	1340 (1142.25-1712.13)

Table 3. Hematological parameters of goldfish under LC_{50} $AgSO_4$ concentration

Hematological parameters	**0**	**24 hours**	**48 hours**	**96 hours**
RBC (10^6 mm^3)	0.58±0.11	1.53±0.29	0.81±0.06	0.94±0.16
WBC (10^3 mm^3)	13100±2133.88	10100±1016.02	11400±960.06	12300±1284.30
MCH (10^{-5}pg)	92.55±1.52^{a}	52.56±1.51^{a}	63.72±0.51^{ab}	61.61±10.48^{b}
MCHC (g/dl)	33.71±1.25^{a}	32.39±0.74^{ab}	30.71±1.06^{b}	32.55±0.62^{ab}
MCV ($10^{-4}$$mm^3$)	269±22.61^{a}	271±10.21^{a}	242±4.29^{ab}	225±4.21^{b}
HB (g/dl)	5.34±1.06	7.74±0.5	6.39±0.92	7.49±1.85
HCT (%)	15.55±1.04	18.02±2.78	16.10±2.28	17.32±1.27
Lymphocyte (%)	90.33±0.57^{bc}	95.33±1.15^{a}	92.66±1.15^{ab}	88.66±2.08^{c}
Monocyte (%)	33±0.57^{a}	0^{b}	0.66±57^{c}	0.66±0.57^{c}
Neutrophil (%)	9.33±0.57^{ab}	4.66±1.15^{c}	6.66±0.57^{bc}	10.66±1.52^{a}

Each value is a means ± standard error. Different superscript letters indicate significant ($P < 0.05$) difference between the groups.

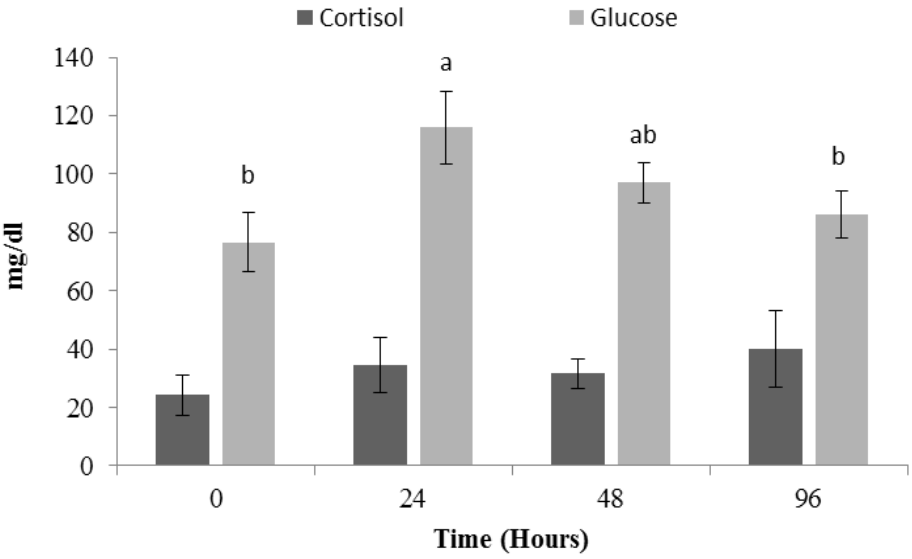

Fig. 1. Levels of blood glucose and cortisol of Goldfish in different times exposed to LC_{50} $AgSO_4$ concentration (Different superscript letters indicate significant ($P < 0.05$) difference between the groups)

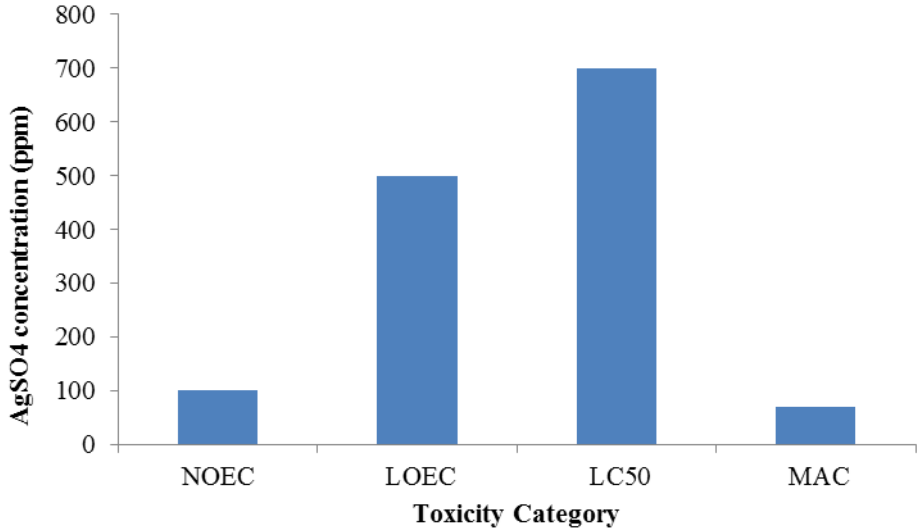

Fig. 2. $AgSO_4$ toxicity category for Goldfish

Exposure time is one of the effective factors in toxicity studies (Larkin and Tjeerdema, 2000). When fish are exposed to a constant concentration of toxin, fish tolerance diminishes over time and the toxin is more effective. However, while the toxin accumulated in fish tissue also increases its adverse effects on the body and thereby causes a decrease in LC_{50}values in 96h. Overall, LC_{50} for silver sulfate in goldfish showed a decreasing trend over 96 hours and in physicochemical conditions. Contrasting results are limited on the toxicity of silver sulfate in fishes. Davies et al. (1978) studied the acute toxicity of silver on *Salmogairdneri*. They stated that the mean 96-h LC_{50} of silver in rainbow trout were 6.5 and 13.0 µg 1^{-1} in soft water (approximately 26 mg 1^{-1} hardness as $CaCO_3$) and hard water (350 mg l^{-1} hardness as $CaCO_3$), respectively. In addition, Birge and Zuiderveen (1995) reported LC_{50} value for *Oncorhynchusmykiss*, *Ictaluruspunctatus* and *Micropterussalmoides*as 0.01, 0.01 and 0.11 mg/L, respectively.

Zhao et al. (2011) reported that the calculated $AgNO_3$48-h $LC_{50}$was 2.51 µg/L. Erickson et al. (1998)studied the acute toxicity of silver nitrate on *Pimephalespromelas* and *Daphnia magna* in laboratory water (pH: 7.94; hardness: 48mg l^{-1}) and St. Louis River (pH: 8.02; hardness: 81mg l^{-1}). They stated that LC_{50} of silver nitrate for *P. promelas* was 10.4 and 106µg Ag/L in laboratory and river conditions, respectively. In addition, these values were 0.58 and 35µg Ag/L for *D. magna*, respectively. However, various factors may influence bioassay techniques like differences in fish (e.g., species, weight, size) and other environmental factors viz. temperature, variations in pH of the water, total hardness of water and dissolved oxygen (Bat et al., 2000; Pandey et al., 2005).

In recent years with developing nanotechnology, researchers have reported the acute toxicity of nano silver to many fishes. The 96h-h LC_{50} values of nano silver was 5 mg/L for *Oncorhynchusmykiss* (Soltani et al., 2010) and at 72-h for *Daniorerio*, it was 10–20 µg/L (Yeo and Yoom, 2009). Results showed that silver sulfide is much more toxic than the other two forms of silver (nano silver and silver nitrate). However, the LC_{50} value is not constant due to various factors such as age, length, weight and environmental factors and measurement of blood factors is required to assess toxicity of the substrate.

Hematological indices

Increase in erythrocyte indices (red blood cells, hemoglobin and hematocrit) often occur due to dehydration and hypoxia and subsequently increase the movement of red blood cells in blood flow. Another simple mechanism also occurs with alkalinity and increased oxygen demand as the mechanism forces kidney sensors to detect hypoxia and increase the movement of red blood cells (Di Giulio and Hinton, 2008).In addition, under stress conditions, immature red blood cells are released from the spleen and consequently, RBC, HB and HCT will increase (Molinero and Gonzalez, 1995; Shaluei et al., 2012).The results are in agreement with Khabbazi et al. (2015). They reported increase in RBC, HB and HCT of *O.mykiss* exposed to CuO nanoparticles. Also, Casillas et al. (1995) declared that RBC, HB and HCT of *O. mykiss* increased under stress condition. However, increasing red blood cells, hemoglobin and hematocrit were not significant in this study ($P>0.05$). Furthermore, MCH and MCHC decreased significantly ($P<0.05$) with increasing exposure time. Reduction in erythrocyte indices often occur due to anemia. In anemia, reduction in the number of red blood cells, hemoglobin and hematocrit are observed and may be due to bleeding, hemolysis or decreased generation of red blood cells (Di Giulio and Hinton, 2008). Heavy metals might alter the properties of hemoglobin by decreasing their affinity towards oxygen binding capacity thus rendering the erythrocytes more fragile and permeable probably resulting in cell welling deformation and damage (Witeska and Kosciuk, 2003). Many researchers have reported a significant decrease in MCH and MCHC infresh water fish exposed to heavy metals (Vutkuru, 2005; Shalaby, 2001). Overall, the perturbation in these blood indices may be attributed to a defense reaction against toxicity through the stimulation of erythropoiesis (Vinodhini and Narayanan, 2009). Fluctuation in hematological indices and decrease in MCH and MCHC proved that the toxic effect of $AgSO_4$ affects both metabolic and hemopoietic activities of goldfish.

The blood of goldfish showed significant increase in glucose during 96-h of silver sulfate in toxication. This might be due to the vulnerable stress induced by the silver sulfate which resulted in hyperglycemia (Fig. 1). Hyperglycemia is a common response to stress that occurs as a result of the effects of catecholamines and cortisol (Barton, 2002).The increased plasma glucose levels are consistent with those reported in Senegalese sole (*Soleasenegalensis*) after exposure to 2-phenoxyethanol (an anesthetic) (Weber et al., 2011) and in great sturgeon (*Husohuso*) (Shaluei et al., 2012). In addition, Almeida et al. (2001) declared that heavy metals increase blood glucose content because of intensive glycogen lysis and the synthesis of glucose from extra hepatic tissue proteins and amino acids. In this study, blood glucose increased after exposure to silver sulphate. However, this increase was significant at 24-h exposure ($P<0.05$). Insulin, the main factor in the balance of glucose is very low in fish (Velíšek et al., 2005). Many factors except toxicological factors (such as nutrition, time of blood sampling, bloodletting from dead fish, manipulation stress, procrastination in serum removal and blood serum integration) affect blood glucose (Rabitto et al., 2005). Significantly, the authors tried to consider all these factors in this study. Accordingly, feeding was stopped 24 h before blood sampling and bloodletting after which anesthesia and serum were removed immediately.

Cortisol is the most common hormone indicator of stress intensity in fishes (Shaluei et al., 2012). It is the principal glucocorticoid secreted by the inter-renal tissues (steroid genic cells) located in the head-kidney of teleost fish (Iwama et al.,

1999).Cortisol has significant negative correlation with blood glucose (Lehninger, 1975). In response to blood glucose reduction, increased cortisol is secreted from the adrenal gland cortex. Cortisol activates glycogenolys is and gluconeogenesis processes in fish but also causes an increase in the release of catecholamines from chromaffin cells which further increases glycogenolys is and modulates cardiovascular and respiratory functions (Reid et al. 1992, 1998). This process increases the substrate levels (glucose) to produce enough energy according to demand. However in this study, this increase was not significant ($P>0.05$). Furthermore, Martinéz-Porchas et al. (2009) stated that some factors can affect the intensity of response. They declared that factors that affect/modulate cortisol response may be from intrinsic nature when some factors depend basically on the genotype or phenotype of the organism and from the extrinsic nature when response is affected by external factors. However none of these factors were considered in this study.

Fluctuations in leukocyte indices as a non-specific immune cell (WBC, lymphocytes, neutrophils and monocytes)is considered an appropriate indicator associated with response to stress in fish (Stoskopf, 1993). Normal values of WBC are an indicator of fish health and body preparedness. However, changes in the quantitative and qualitative characteristics of blood cells occur when anomalies in blood components interfere with normal functions such as clinical inflammation, invasion of parasites or bacteria (Khabbazi et al., 2015; Savari et al., 2011). In response to stress conditions, reduction in WBC counts may indicate immune suppression while increasing values indicate a response to stress or infection (Adams, 2002).The total WBC countdecreased which might be due to malfunctioning of the hematopoietic system caused by exposure to $AgSO_4$ ($P>0.05$). Al-Kahem (1995) reported a reduction in WBC count of fish exposed to chromium and noted it to be a consequence of significant decline in the number of lymphocytes and thrombocytes.

Lymphocyte is the most dominant differential leukocyte and responsible for many of the functions of the immune system in fish. Decrease in cell count, especially of lymphocytes usually occurs in fish subjected to stress (Elsaesser and Clem, 1986). Heavy metal intoxication always reduces white blood cells count, particularly lymphocytes (Witeska, 2003). According to Donaldson and Dye (1975), exposure to heavy metals in fish causes an increase in cortisol level which is responsible for a decrease in WBC, particularly in lymphocytes count and their activity. In fact, cortisol secreted during stress reaction shortens the life span of lymphocytes and promotes their apoptosis (Wyets et al., 1998, Verburg van Kemenade et al., 1999), and reduces their proliferation (Espelid et al., 1996). However in this study, lymphocytes increased significantly at 24 hours exposure to $AgSO_4$but decreased significantly after 96 hours.These results agree with those of Dick and Dixon (1985) and Vosyliene (1996). They reported a decrease in leukocyte count following acute metal exposure. In addition, many researchers have reported lymphocytes reductionin fish such as *heteropneustesfossilis* (Nath and Banerjee, 1996) and *Cyprinuscarpio* (Siwicki et al., 1990; Banaee et al., 2008) exposed to pesticides.

There was a significant decrease in neutrophil after 24 hours exposure ($P<0.05$) however, there was an increase after 48 and 96 hours. This shows that with increased exposure time, $AgSO_4$-induced infection and tissue damage increases. Banaee et al. (2008) stated that most infections result inneutrophilia. The degree of elevation often indicates the severity of the infection. Tissue damage from other causes also raises the neutrophil count.

Poisoning and severe disease, like kidney failure all cause neutrophilia (Holland et al., 1997). Ghosh and Banerjee (1993) reported that neutrophile and eosinophile increased in *heteropneustesfossilis* after they were affected by Dimethoate in 96 h LC50 concentration.

CONCLUSION

This study suggested that the presence of $AgSO_4$ in an aquatic environment is toxic and has significant influence on hematological parameters in goldfish. It is notable to state that fish are constantly exposed to environmental stress resulting to serious metabolic crises. The above results clearly indicate that the usage of this heavy metal has generated great concern in the scientific community on its possible toxic effects both to aquatic flora and fauna as well as to humans.

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