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Aqueous ozonation of herbicide, thiobencarb: Implications for oxidative stress on mosquito fish *Gambusia affinis*.

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Abstract: The role of aqueous ozonation among stimulation of the potential toxic effects of herbicide; thiobencarb on mosquito fish *Gambusia affinis* was examined after 96 hrs exposure. Two levels (1/5 and 1/10LC_{50}) of herbicide were used individuals or mixed with either 1 or 5 ppm of ozone (O_3). The aqueous ozonation of thiobencarb stimulated acetylcholinestrase (AChE) activity to reach the highest value (0.186 μmole.mg^{-1}.min^{-1}) in case of [1/5LC_{50} thiobencarb+O_3 (5 ppm)] treatment. All treatments induced increase of malondialdehyde (MDA) levels compared with untreated group which not exceeded than 1.52 mM.g^{-1} tissue. Catalase (CAT) and glutathione peroxidase (GP_x) showed variability in their response to ozonation process, especially GP_x which subjected to values lowest than those of untreated group. On the other hand, lactic dehydrogenase (LDH) activity increased in most treatments, where 1/10LC_{50} thiobencarb recorded the highest value (512.7 U. L^{-1}). The lowest one was recorded for [1/10LC_{50} thiobencarb+O_3 (1 ppm)] treatment to be 129.5 U. L^{-1}. This fact represents the cytotoxic effect of examined herbicide under ozonation condition. The all data revealed that, ozonation process for pesticide removal must be done under especial conditions and usage of catalytic agents to decline their potential toxic effects on organisms.
Key words: thiobencarb; aqueous ozonation; mosquito fish; oxidative stress; cholinesterase.

1. Introduction.

Aquatic environment is a sink for many environmental contaminants which may be absorbed by aquatic organisms such as fish, algae and others. The chemicals may induce cells disruption and oxidative stress in their components[1,2]. Moreover, herbicide run into aquatic media was found to affect cellular functions, growth and behavior of organisms[3]. Fish is considered a good bioindicator for ecosystem health[4], where it accumulates pesticides in the tissues causing different toxic responses. Thiobencarb (S-[(chlorophenyl) methyl] N, N-diethyl carbamothioate), CAS No: 28249-77-6 is traded as theactive ingredient of the contact herbicide Saturn®. It has highly effects against grasses, broadleaf weeds, fesses and sedges. This compound is always used in pre-emergence and early post-emergence weed control in rice fields[5]. After application, it has a half-life of 37 days in soil, but expected to be 37-days for aerobic or 306 days for anaerobic conditions. Furthermore, its release in water is expected to adsorb on suspended solid and sediments accounted for a half-life of 160 days.

Generally, aqueous ozonationprocessesof pesticides lead to a reduction in pesticide levels during water treatment. The literature indicated that, ozone application during treatment may achieve at least a partial destruction of many pesticides[6]. Ozone is apowerful oxidizing agent. The reactions of ozone with organic compounds in aqueous media had been under investigation by different authors[7,8]as a source of more radicals

stimulates pesticides degradation in aqueous media. On the other hand, Reactive Oxygen Species (ROS) are produced in organisms under series of physiological conditions or influence of environmental factors. Superoxide anion radical ($O_2^{\bullet-}$) and singlet oxygen ($^1O_2$) are the main forms of ROS. They are particularly transient species due to their high chemical reactivity and can react with cell components[9]. The ($^1O_2$) form is considered the main product of O_3hydrolysis in water resulting in pesticides destructive and stimulation of ROS generation in organisms.

Mosquito fish, *Gambusia affinis* is the smallest fish distributes in the freshwater bonds and canals near the crop fields. It is considered as the most widespread freshwater fish in the World. As documented previously, there are relationships between environmental stress with chemicals and ROS generation in organisms. Thus, this study aims to investigate the interaction of ozone treated water with herbicide; thiobencarb as implications for stimulation of oxidative stress in mosquito fish.

2. Materials and Methods.

2.1. Chemicals.

Active ingredient of herbicide; thiobencarb (95%) was supplied by Sumotomo Chem. Co., Osaka-Japan. Aqueous solution of O_3(Oxilite®99.7%) was purchased from El-Sharkia Co. Ltd, Egypt.SEP-PAK®, C_{18} cartridge for sample preparation was obtained from Waters Associates (Maple St. Milford, MA 01757, USA). Acetone and methanol for HPLC- plus gradient were supplied by Carlo Erba Reagent-SpA, 1-20090 Rodano. Acetylthiocholine iodide (ASChI), S-butyrylthiocholine iodide (BuSChI), 5,5-dithiobis (2-nitrobenzoic acid) (DTNB, Ellman's reagent) and foline reagent were obtained from Sigma-Aldrich Chemical (St. Louis, MO, USA). Sodium phosphate either mono or dibase, hydrochloric acid (HCl), trichloroacetic acid (TCA), thiobarbituric acid (TBA), hydrogen peroxide, sodium azide and EDTAwere supplied by J.T.Baker Chemical Co. Phillipsburg, N. J. 08865. Lactic dehydrogenase Kits for colorimetric assay were obtained from Salucea Co. -Dutch technology in life science, India.

2.2. Fish.

Mosquito fish *G. affinis* were collected from an aquaculture unit in El-Behira governorate as unwanted fish in its cultural program. The animals were daily fed *adlibitum* with commercial fish food (supplied by El-Dalea import Co, China). They were acclimated for 2 weeks in 20-L glass aquarium containing dechlorinated water (pH=7.5-7.9) and continuously aerated. The selected fish were at length of 2.02±0.25 cm and three replicates were used for each treatment. No food was provided during the experimental period.

2.3. Toxicity tests.

2.3.1. Acute toxicity.

The bioassay experiment was done according to EPA protocol[10]. Moreover, the animals were exposed to series of thiobencarb concentration 0.01, 0.1, 0.3 and 1.5 mg. L^{-1}. The animals were divided into 3 replicates for each concentration level (10individual for each). Herbicidal solutions were prepared in distilled water and the exposure period was 96 hrs[11]. Fish mortality was observed and recorded at 24, 48, 72 and 96 hrs from stocking. Dead fishes were removed immediately. The percent of mortality was estimated on computer probit program[12].

2.3.2. Sublethal treatment.

To assay the effect of herbicide; thiobencarb and O_3 and their mixtures on some biochemical aspects of mosquito fish, animals were exposed for 96 hrs to sublethal concentrations of examined herbicide (0.0185 and 0.0074 mg. L^{-1}) which account for 1/5 and 1/10 LC_{50}, respectively, 1 and 5 ppm of O_3. Three replicates were used for each level and 10 fishes were distributed for each replicate. In addition, control group was used without herbicide. All groups were daily observed and the dead animals were removed immediately. At the end, live fishes were dissected and stored at -20 $^{\circ}$C until analysis.

2.4. Decomposition experiment.

Persistence of thiobencarb was examined under ozonation treated water for experimental exposure period (96 hrs). At the end, one liter of water for each treatment was passed through a C_{18} SEP-PAK cartridge with slightly vacuum. The cartridge was washed with 10 ml methanol before used. Then, it was dried under

stream of N_2, eluted with 2 ml of acetone and subjected on liquid chromatographic analysis[13]. High performance liquid chromatography (HPLC)(HP-Agilent 1100-Japan) equipped with an analytical column (C_{18}, 30×0.8 cm) was used. Solvent system of CH_3CN: CH_3OH (65:35 v/v) was used at a flow rate of 1 ml. min^{-1}. The herbicide was examined against UV absorbance at 230nm. All used solvents were at analytical grade and thiobencarb was measured upper a limit of detection of 0.01 ng. The treated samples were examined against blank and the data were correctedto recovery percent (95.2%).

2.5. Biochemical analysis.

2.5.1. Sample preparation

The stored fishes were polled and divided into replicates. For each, the tissues of whole body were weighed, homogenized with 10 volumes (w/v) of ice-cold saline for 30sec and centrifuged at 5000xg for 30 min at 4 $^{\circ}$C. The supernatant was used as a source of catalase (CAT) and glutathione peroxidase (GP_x) as well as protein content. However, homogenate was used as a source of lipid peroxidase (LPO) and lactic dehydrogenase (LDH). Among cholinesterase, both head and whole body tissues were weighted and homogenized with 10 volumes (w/v) of 0.1M phosphate buffer, pH 8.0 and the procedures were done as described before. The supernatant was used as a source for enzyme assay.

2.5.2. Cholinesterase assay

Acetylcholine and butylcholinesterase activities were determined according toEllman*et al.*[14]with slight modification using acetyl or butylthiocholine iodide, respectively, as substrates. The activities were expressed as μmole of substrate hydrolyzed per minute per mg protein.

2.5.3. Lipid peroxidase (LPO)

The barbituric acid reactive substances ($TBAR_s$) were used as an index of lipid peroxidation according to Rice-Evans *etal.*[15]with modification. $TBAR_s$ were determined using spectrophotometric quantification of malondialdhyde (MDA) content in the tissue. A sample of 250 μl of tissue homogenate was mixed with 1 ml of 15% (w/v) trichloroacetic acid (TCA) in 25mM HCl and 0.37% (w/v) thiobarbituric acid (TBA) in 25mM HCl. Next, the samples were boiled for 10 min, then quickly cooled, and immediately centrifuged at 6500xg for 5 min. The measurement was maintained at 535nm. MDA was quantified using an extinction coefficient of 156mM and its concentration was expressed as $mM.g^{-1}$ wet mass.

2.5.4. Catalase (CAT)

The enzyme activity was measured following the decrease of absorbance at 240nm due to hydrogen peroxide (H_2O_2) consumption[16]. The reaction mixture consisted of 1 ml of 12.5mM H_2O_2 (substrate), 2 ml of 66.7mM phosphate buffer, pH 7.0 and an aliquot of enzyme source. The activity was expressed as U. g^{-1} wet mass. The unit of CAT is the amount of enzyme which liberates half the peroxide oxygen from hydrogen peroxide solution of any concentration in 100sec at 25 $^{\circ}$C.

2.5.5. Glutathione peroxidase (GP_x)

The enzyme activity was measured according toFlohe and Gunzler[17] by mixing phosphate buffer solution (100mM), EDTA (50mM), sodium azide (250mM), H_2O_2 (10mM) and enzyme in a cuvette. The change in absorbance at 340nm was recorded every 3sec for 40sec. The activity was expressed as $mUGP_x.mg^{-1}$ protein. One unit of GP_x is defined as the amount of enzyme necessary to oxidize 1μmole of NADPH per min.

2.5.6. Lactic dehydrogenase (LDH)

The lactic dehydrogenase activity of tissue homogenate was measured by the method of Anonymous[18]. Sodium pyruvate was used as a standard. The enzyme activity was expressed as U. L^{-1}.

2.6. Protein determination

Protein was determined according to the method of Lowry*et al.*[19]with bovine serum albumin as a standard.

2.7. Statistical analysis.

Value of 50% lethal concentration (LC_{50}) was calculated using the EPA Probit Analysis Program (Version 1.5). All data were presented as a mean ± SE and subjected to analysis of variance (ANOVA). The statistical analysis was performed using the Costat program[20].

3. Results.

3.1. Toxicity of herbicide

The toxicity of thiobencarb on mosquito fish, *G. affinis* was assayed using methods of ASTM[11] as a simple, cheap and easy approach. The results of lethality at various exposure times are presented in Table 1. The obtained data showed an increase in lethality at higher concentrations compared to lower ones. Also, higher toxicity of thiobencarb occurred at the end of the exposure period (96 hrs) compared with untreated group.

3.2. Decomposition rate

The potential effect of O3on thiobencarb degradation was established under two levels of examined compound as presented in Table 2 and illustrated in Figure 1. Moreover, all treatments showed significantly decomposition rates compared with positive control (herbicide alone). The treated case [1/10LC_{50}+ O_3 (5 ppm)] accounted for the highest value (71.12%) followed by [1/5LC_{50}+ O_3 (5 ppm)]treatment arising a value (65.27%). In case of O_3 treatment at 1 ppm level, no significantly difference obtained between the two examined levels.

Table 1: LC_{50} of thiobencarb for *G. affinis* fish at acute toxicity[10] for 96 hrs.

Concentration (mg. L^{-1})	Values		Slope
	% mortality	LC_{50} (mg. L^{-1})	
0.01	20	0.185 (0.123-0.283)	0.74±0.09
0.1	40		
0.3	60		
1.5	80		

- The experimental period=96 hrs.
-Thirty animals were used for each treatment

Table 2: Decomposition rate of herbicide, thiobencarb in aqueous ozonized media under two examined levels.

Treatment	fortified level (µg)	remaining level (µg)	decomposition rate (%)
1/10LC_{50} thiobencarb+O_3 (1 ppm)	14	6.82^{d}±0.12	50.48^{c}±0.02
1/5LC_{50}thiobencarb+ O_3 (1 ppm)	28	12.34^{b}±0.07	55.81^{b}±0.02
1/10LC_{50} thiobencarb+O_3 (5 ppm)	14	8.08^{d}±0.11	71.12^{a}±0.01
1/5LC_{50}thiobencarb+ O_3 (5 ppm)	28	6.81^{d}±0.12	65.27^{a}±0.02
1/10LC_{50} thiobencarb	14	10.84^{c}±0.05	22.55^{e}±0.09
1/5LC_{50}thiobencarb	28	17.33^{a}±0.05	37.45^{d}±0.05
Untreated	–	–	–
LSD (0.05)	–	1.28	1.44

-Each value is the mean of three samples±SE.
-No significant indicates in the same letters at 0.05 levels.

3.3. Biochemical quantification

3.3.1. ChE activity.

The activities of acetyl or butyl cholinesterase were presented in Table 3. The specific activity of AChE was higher than those of BuChE. The treatment of [1/5LC_{50} thiobencarb+O_3 (5 ppm)] accounted for the highest

value of AChE activity (0.186 µmole. mg^{-1}. min^{-1}) followed by O_3 (1 ppm) treatment at a value (0.146 µmole. mg^{-1}. min^{-1}). However, the lowest value (0.047 µmole. mg^{-1}. min^{-1}) was recorded for treatment of $1/10LC_{50}$ thiobencarb. In case of BuChE activity, treatment with O_3 at level of 1 ppm revealed the highest value of enzyme activity (0.032 µmole. mg^{-1}. min^{-1}), while the lowest one (0.010 µmole. mg^{-1}. min^{-1}) was recorded for treatment [$1/10LC_{50}$ thiobencarb+O_3 (1 ppm)], compared with untreated group which not exceeded than 0.006 µmole. mg^{-1}. min^{-1}.

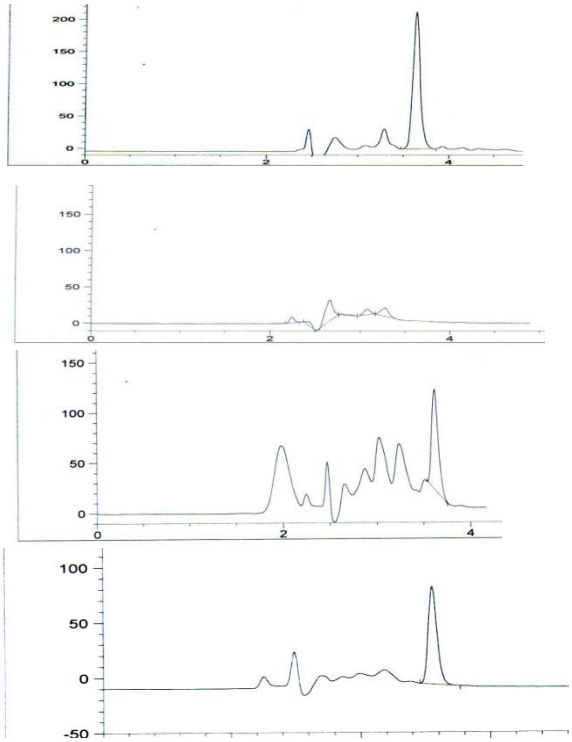

Figure 1 : illustrates HPLC chromatograms of herbicide; thiobencarb after different treatments, (a) standard of thiobencarb (99.5%), (b) untreated water, (c) ozonized water + thiobencarb revealing degradation products and (d) water treated with technical thiobencarb. Arrow represents the parent compounds

Table 3: Specific activity of cholinesterase in *G. affinis* fishafter exposure to sublethalconcentrations of thiobencarb in aqueous ozonation media.

Treatment	Exposure time(hr)	Activity (µmole. mg^{-1}. min^{-1})	
		AChE	BuChE
$1/10LC_{50}$ thiobencarb+O_3 (1 ppm)	96	0.105^{bc}±0.60	0.010^{cd}±0.50
$1/5LC_{50}$thiobencarb+ O_3 (1 ppm)	96	0.112^{bc}±0.30	0.012^{bcd}±0.40
$1/10LC_{50}$ thiobencarb+O_3 (5 ppm)	96	0.060^{c}±0.60	0.017^{bc}±0.30
$1/5LC_{50}$thiobencarb+ O_3 (5 ppm)	96	0.186^{a}±0.20	0.013^{bcd}±0.40
$1/10LC_{50}$ thiobencarb	96	0.047^{c}±0.80	0.013^{bcd}±0.40
$1/5LC_{50}$thiobencarb	96	0.071^{c}±0.50	0.019^{b}±0.20
O_3 (1 ppm)	96	0.146^{ab}±0.30	0.032^{a}±0.10
O_3 (5 ppm)	96	0.071^{c}±0.50	0.013^{bcd}±0.40
Untreated	96	0.086^{bc}±0.50	0.006^{d}±0.80
LSD (0.05)	-	0.067	0.008

-Each value is the mean of three samples±SE.

-No significant indicates in the same letters at 0.05 levels

In case of total protein, the data were tabulated in Table 4, where the treatment with thiobencarb of two levels stimulated protein content more than other treatments in head and whole body tissues, respectively. On the other hand, O_3 contained treatments observed the lowest value of total protein contents. Moreover, treatment of [1/5LC_{50} thiobencarb+O_3 (1 ppm)] induced value (457.8 mg. L^{-1}) in head tissue compared with untreated animals which not exceeded than 816.7 mg. L^{-1}.

Table 4: Variation in protein profile in *G. affinis* fishafter exposure to sublethal concentrations of thiobencarb in aqueous ozonation media.

Treatment	Exposure time (hr)	Total protein (mg. L^{-1})	
		head	Whole body
1/10LC_{50} thiobencarb+O_3 (1 ppm)	96	590.0^{cd} ± 0.5	1083.3^{c} ± 0.4
1/5LC_{50}thiobencarb+ O_3 (1 ppm)	96	457.8^{d} ± 0.6	1320.0^{bc} ± 0.3
1/10LC_{50} thiobencarb+O_3 (5 ppm)	96	1782.4^{b} ± 0.2	982.3^{c} ± 0.4
1/5LC_{50}thiobencarb+ O_3 (5 ppm)	96	787.8^{cd} ± 0.4	1056.6^{c} ± 0.4
1/10LC_{50}thiobencarb	96	2915.6^{c} ± 0.1	1858.7^{ab} ± 0.2
1/5LC_{50}thiobencarb	96	1772.2^{b} ± 0.2	2451.1^{a} ± 0.2
O_3 (1 ppm)	96	984.5^{c} ± 0.3	2396.7^{a} ± 0.2
O_3 (5 ppm)	96	1556.7^{b} ± 0.2	986.7^{c}± 0.4
untreated	96	816.70^{d} ± 0.3	980.2^{c} ± 0.4
LSD (0.05)	-	484.32	719.7

- Each value is the mean of three samples±SE.
-No significant indicates in the same letters at 0.05 levels

3.3.2. Antioxidant defense enzymes.

Antioxidant defense enzyme activities in whole body tissues of fish were assayed under different treatments as shown in Table 5 for LPO,CAT and GP_x, respectively. All treatments caused increase in MDA levels compared with untreated group which not exceeded than 1.52 mM.g^{-1} tissue. Additionally, treatment of 1/5 LC_{50} thiobencarb observed the highest value (4.65 mM. g^{-1} tissue) followed by treatment of [1/10LC_{50}thiobencarb+O_3 (1 ppm)] after exposure period (96 hrs). In case of CAT, the activity reached the highest value (913.56 U. mg^{-1} protein) after exposure to 5 ppm of O_3 followed by treatment of [1/5LC_{50} thiobencarb+O_3 (1 ppm)] accounting for 11.52 U. mg^{-1}protein. In contrast, other treatments attributed to O_3 mixing revealed values lower than untreated group as follows: 4.14, 4.24 and 3.39 U. mg^{-1} protein for treatments; [1/5LC_{50} thiobencarb+O_3 (5 ppm)], [1/10LC_{50} thiobencarb+O_3 (1 ppm)] and [1/10LC_{50} thiobencarb+O_3 (5 ppm)], respectively. Most of the treatments decreased GP_x activity compared with untreated group which not exceeded than 1.15 nmole. mg^{-1} protein. The lowest value of GP_xactivity(0.05 nmole. mg^{-1} protein) was recorded for treatment of [1/5LC_{50} thiobencarn+O_3 (5 ppm)]. On the other hand, the highest one (0.52 nmole. mg^{-1} protein) was recorded for O_3 treatment at level of 1 ppm.

3.3.3. LDH.

The activity of LDH enzyme is illustrated in Figure 2. All treatments stimulated enzyme activity compared with untreated group. The highly effects observed at 1/10LC_{50}thiobencarb treatment accounting for 512.7 U. L^{-1} followed by treatment of [1/5LC_{50}thiobencarb+O_3 (1 ppm)] inducing activity(418.2 U. L^{-1})($P<0.05$).The lowest one was recorded for [1/10LC_{50} thiobencarb+O_3 (1 ppm)] treatment to be 129.5 U. L^{-1}.

Table 5: Variations in activity of antioxidant biomarkers (MDA, CAT and GP_x) in *G. affinis*fish after exposure to sublethal concentrations of thiobencarb in aqueous ozonation media.

Treatment	Exposure time (hr)	Biomarker		
		MDA ($mM.g^{-1}$ tissue)	CAT ($U.\ mg^{-1}$protein)	GP_x (nmole. mg^{-1}protein)
$1/10LC_{50}$ thiobencarb+O_3 (1 ppm)	96	$4.01^{a} \pm 0.2$	$4.24^{c} \pm 0.6$	$0.55^{ab} \pm 7.8$
$1/5LC_{50}$thiobencarb+ O_3 (1 ppm)	96	$2.18^{bc} \pm 0.3$	$11.52^{a} \pm 0.2$	$0.50^{ab} \pm 8.6$
$1/10LC_{50}$ thiobencarb+O_3 (5 ppm)	96	$2.50^{bc} \pm 0.3$	$3.39^{bc} \pm 0.7$	$0.82^{ab} \pm 5.2$
$1/5LC_{50}$thiobencarb+ O_3 (5 ppm)	96	$2.29^{bc} \pm 0.3$	$4.14^{c} \pm 0.6$	$0.05^{b} \pm 85.5$
$1/10LC_{50}$thiobencarb	96	$2.79^{b} \pm 0.2$	$6.30^{bc} \pm 0.4$	$0.61^{ab} \pm 7.0$
$1/5LC_{50}$thiobencarb	96	$4.65^{a} \pm 0.1$	$10.54^{ab} \pm 0.2$	$1.45^{ab} \pm 2.9$
O_3 (1 ppm)	96	$2.29^{bc} \pm 0.3$	$6.42^{bc} \pm 0.4$	$7.52^{a} \pm 0.6$
O_3 (5 ppm)	96	$2.41^{bc} \pm 0.3$	$13.56^{a} \pm 0.2$	$0.29^{ab} \pm 14.7$
Untreated	96	$1.52^{c} \pm 0.4$	$4.96^{c} \pm 0.5$	$1.15^{ab} \pm 3.7$
LSD (0.05)	-	1.18	4.360	7.400

- Each value is the mean of three samples±SE.
-No significant indicates in the same letters at 0.05 levels

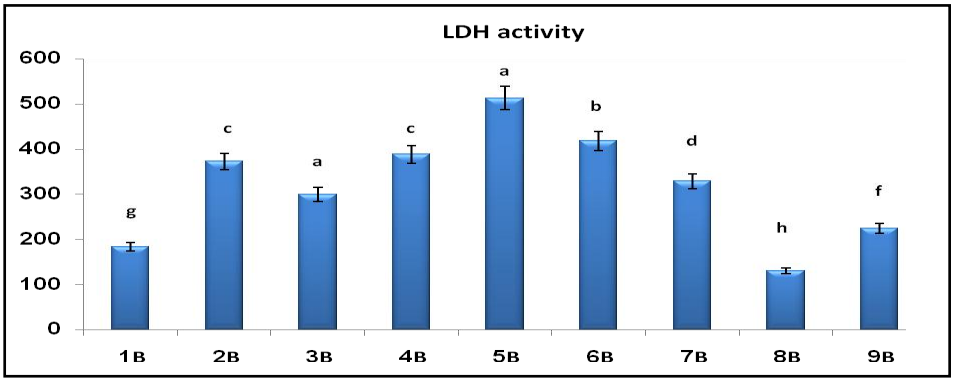

Fig 2: The activity of lactate dehydrogenase ($U.\ L^{-1}$) in *G. affinis* fish after exposure to sublethal concentrations of thiobencarb in aqueous ozonation media; (B1) untreated, (B2) O_3 (1 ppm), (B3) O_3(5 ppm), (B4) $1/5LC_{50}$thiobencarb, (B5) $1/10LC_{50}$thiobencarb, (B6) $1/5LC_{50}$thiobencarb+ O_3 (1 ppm), (B7) $1/5LC_{50}$thiobencarb+ O_3 (5 ppm), (B8) $1/10LC_{50}$ thiobencarb+O_3 (1 ppm), and (B9) $1/10LC_{50}$ thiobencarb+O_3 (5 ppm). Each value represents three samples±SE and vertical bars indicate no significant difference at the same letters (P<0.05).

4. Discussion

The present study investigated attempts to evaluate the role of O_3 to destruct thiobencarb and enhance its oxidative stress among mosquito fish in aqueous media. The obtained data represented that, by-products of aqueous ozonation (ROS) and herbicide increased oxidative stress on fish tissue components. Moreover, experimental conditions affected on potential toxic effects of examined herbicide. The ozonation reactions of pesticides in aqueous media had been reviewed under different conditions depending on pesticide concentration, ozone dose, current and pH of reaction media[6].

In literature, many investigations obtained the degradation of pesticides by aqueous ozonation. In case of chlorinated hydrocarbon pesticides, it was demonstrated reactivity with ozone ranged from slight to substantial. Some evidence observed epoxide formation following ozone attack to double bound of cyclodienes such as aldrin and heptachlor[21].Another group, organophosphrous pesticides are less stable upon ozonation compared to organochlorine pesticides. Products of reaction were identified as oxons, especially for malathion, parathion, methyl parathion and phosal. The cleavage of the P-S, O bond results in production of phosphoric acid, succinic acid derivatives and nitrophenols[22].

In case of herbicides, some studies demonstrated the destructive of 2,4-D, MCPA and MCPBcompounds upon ozonation. The reaction products identified tended to be aliphatic acids of short carbon chain length, CO_2 and chlorine ion[23]. Another study byKidak andDogon[24] stated that, ozone provided 95% removal of herbicide, atrazine in aqueous media. Moreover, integration of ultrasonic technique with ozonation enhanced 100% removal of atrazine at shorter time and higher reaction rate compared to single process.In this study, we can understand that, the main products upon ozone reaction may be 4-chlorobenzoic acid and CO_2 as observed in liquid chromatographic chromatograms (Figure 1) compared with positive control.

The change in ozonation efficiency associated with variation in pH results in variable ozone decomposition rate. As example, acidic media (pH, 3-5) is most suitable for dye removal depending on dechlorination of heterocyclic compounds. Moreover, using granular activated carbon (GAC) imposed removal of by-products resulted from ozonation reactions, such as aldehydes and ketones[25].

The obtained data showed information on oxidative stress on tissues of *G. affinis* upon ozonation reaction with herbicide, thiobencarb. This fact may due to the combination of potential toxic effect of O_3 and herbicide reaction by-products. They may be $O_2^{\cdot-}$, OH^- and H_2O_2 for O_3, while 4-chlorobenzoic acid is the main by-product of thiobencarb.

The measured components of tissue homogenates represented biomarkers of oxidative stress role in this work. Recently, lipid peroxidation represented the risk among this reaction, where cell membranes of organisms are potential targets of attack by ROS. The attack of membranelipids by these species initiates oxidation process resulting in generating lipid hydroperoxides (LOOH) ending to intracellular excess of MDA which disrupts the normal cellular metabolism causing cell death[26]. Other parameters such as GP_x is generally believed to be up-regulated by oxidants[27], where increased of GP_x in tissues submitted to oxidative stress. CAT has a minor role in H_2O_2 decomposition and detoxifies it in cells. In the present study CAT and GP_x showed activities are not in agreement with that obtained by Abdel-Halim and Massoud[28] on this species, where thiobencarb at levels of 1/10 and 1/25LC_{50} induced decrease in CAT activity, while stimulated GP_x activity at values of 64.31 and 36.56 nM. mg^{-1} protein compared with control which not exceeded than 15.42 nM. mg^{-1} protein. Lacticdehydrogenaseplays the main role among conversion of lactate to pyruvate depending on the availability of NAD coenzyme[29]. Increase of LDH activity compared with control provides an index of physiological stress[30].

5. Conclusion

In fact, we can summarize this work as benefited usage ofO3 disinfection or removal of pesticides and wastewater treatment. The processes may be done under especial conditions and down international regulations for safe water ending to beneficial status to protect consumers and organisms avoiding health hazards. Moreover, usage of catalytic agents, ultrasonic and GAC filtration are considered important factors during ozonation reaction.

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