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Immunohistochemically and semi-quantities analysis of carp gills exposed to sodium thiosulfate

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Abstract

One of the more common dechlorinated agents is Sodium thiosulfate. However, the toxicity on the aquatic animals was unknown, this study aims to determine the adverse effects of random usage and high sodium thiosulfate concentration in carp fish rearing in an earthen pond in Hawy -Al-Kanesa in Mosul city. The sodium thiosulfate used at 10 gm /L led to fish mortality of 80% through 96 days of exposure. The semi-quantities score analysis of histopathological alteration of gill represented a biological indicator for toxicity estimation, which reveals the significant alteration index ($P \leq 0.05$) in the vacuolar degeneration and hemorrhage while highly significant alteration index ($P \leq 0.01$) represented in the other vascular disturbances (edema, congestion, and aneurysm), hyperplasia, hyper trophy of cells, shortening, loss and fusion of secondary gills lamellae with abnormal cartilage morphology. Chloride and mucus cells were ++ and +++ for Periodic acid Schefft and Masson Trichrome, respectively, and apoptotic cells have been detected via caspase-3 activation, which was prominently detected mainly in the cells structure of the secondary gill lamella rather than primary filaments. This study concluded that sodium thiosulfate is toxic in high concentration to fish and leads to a pathological alteration in the vital organs such as gills. The semi-quantitative analysis is one of the modern techniques through which significant pathological changes are determined in histological sections of the gills and immunohistochemically and technique to determine programmed cell death.

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Introduction

Natural and synthetic compounds are introduced to the aquatic environment for controlling and preventing diseases as antibiotics, probiotics, additive feed supplementary, or for water and soil treatment as a disinfectant and chemotherapeutic agent (1-5). Despite the importance of these substances in increasing the productivity of the aquarium, they may have adverse effects on animals, the environment and risk to public health (6-7). Sodium thiosulfate is a compound generally used in many industrial and medical fields, as it is used in dye manufacture, photographic fixative, and food preservative, and it has therapeutic features such as anti-inflammatory and antioxidant (8). In the aquatic environment, it is used for wastewater management as dechlorinated and neutralizing agent at a concentration of 6.7 mg/l and reduces metal toxicity. Also used as a fish embryo disinfectant (9-11), Rose-Janes and Playle (12) reported the ability of thiosulfate to reduce the silver toxicity in rainbow trout (*Oncorhynchus mykiss*). Although thiosulfate is used to remove chlorine from the aquatic environment at low concentrations, it may lead to increased toxicity in the case of contamination of the aquatic environment with metal and other pollutants (13). In Iraq, carp fish is the main species cultivated in the earthen pond, small tanks, and cages (14,15); many antibiotics and chemical compounds are used to eliminate and control diseases, but the randomly and wrong usage or high concentrations and doses lead to toxicity and mortality may occur, and because there is no information of previous study

or literature about effects of sodium thiosulfate to induce programmed cell death.

Therefore, this study aimed to evaluate the histopathological alteration and semi-quantitative analysis in the gills of fish exposed to overconcentration of sodium thiosulfate and evaluate the toxic effects of sodium thiosulfate to induce apoptosis in the gills of carp fish.

Materials and methods

Ethical approve

The recent work was approved by the Scientific Committee of the Department of Pathology and Poultry Diseases, University of Mosul on 24/10/2022.

Fish

A total of fifty *Cyprinus carpio* weight 150±10 g was collected from two earthen ponds with standard measurement for each one 2500 m^2 with high water column 1.5 m in Hawy Al-kanesa in Mosul city in Iraq, fish in first pond were bathing in sodium thiosulfate as disinfectant at concentration ten g/L for sex hour (treated fish), in second pond fish were untreated.

Microscopic examination

Lethargy and restlessness fish after 96 hours from exposure to sodium thiosulfate overconcentration were exposed to general anesthesia MS-222 (150 mg/L) (16), gills were desecrated, collected, and fixed in neutral buffer formalin 10% at least 48 hours for routine (H&E) and histochemical stains (Periodic acid chief PAS and Masson Trichrome) (17). According to the Flores-Lopes and Thomaz (18), the severity of the microscopic lesions was graded into three grades (Table 1).

Semi-quantities score system

The Histological pathological alterations in the gill tissue were estimated semi- a quantitative analysis system that involved the severity code explained by Darwish *et al*. (19) as in table 2; the semi-quantitatively analysis depended on the importance factor (IF), which classified the lesions to the reversible (IF= 1) or irreversible (IF= 3), each histopathological categories (cell injury, vascular, cell growth and architecture disturbances) were taken score value 0, 2, 4, and 6 (20).

Table 1: Grade for gills histopathological alteration in fish

Grading	Histopathological categories	Description
Grade I	Vascular disturbances	Infiltration of inflammatory cells, edema, congestion, and vascular dilatation
	Cell growth disturbances	Hyper trophy and hyperplasia of epithelium cells lining secondary gills
	Cell injury	Vacuolar degeneration
	Architecture disturbances	Loss of normal straightening secondary gills filaments, shortening, organization, and lamellar fusion
Grade II	Vascular disturbances	Vascular injury and hemorrhage
	Cell growth disturbances	Hyper trophy and hyperplasia of mucus and chloride cells
	Cell injury	Vacuolar degeneration
	Architecture disturbances	Lifting and rupturing epithelium cells lining secondary gills
Grade III	Vascular disturbances	Aneurysm
	Cell growth disturbances	Telangiectasia in the secondary gill lamellae
	Cell injury	Apoptosis and necrosis
	Architecture disturbances	Cartilage disintegration

Table 2: Severity code of gill filament integrity

Score	Lamellar fusion (LF)	Epithelial hyperplasia (EH)	Epithelial lifting (EL)%
0	Normal lamellar	Normal epithelial cells number	30%
I	LF about 1/3	Multifocal increase number 30%	31%
II	LF about 1/3 - 2/3	Multifocal increase number 31 -60%	60%
III	LF > 2/3	Diffuse increase number >61%	> 60%

Immunohistochemically analysis

TUNEL assay is immunohistochemically based on the detection of labeled DNA fragments or caspase-3 activation, which release during apoptosis (21); the semi-quantification (score and grade) of apoptotic cells depended upon the percentage of apoptotic and dying cells (22) as in table 3.

Table 3: Score and grade of Apoptotic and dying cells in gills

TUNEL semi quantification	Apoptotic and dying cells			
Score	-	+	++	+++
Grade	Normal	Mild	moderate	severe

Statistical analysis

The data were expressed as (mean ± SD), which were statistically analyzed by t-test at values $P<0.05$ and $P<0.01$ (23).

Results

The mortality rate reach to 80% through 96 hour, the microscopic examination of gill fish exposed to overconcentration of sodium thiosulfate for 96 hour revealed three stages of variable histopathological alteration ranging from vascular disturbances (stage I) represented by congestion of capillary in the secondary gills filaments to architecture disturbances characterized by LF about 1/3 which represented code I, as in (Figure 1) and more significant than 2/3 of LF and 30% EH codes III and 0 respectively, with vacuolar degeneration of non -specific cells (Figure 2).

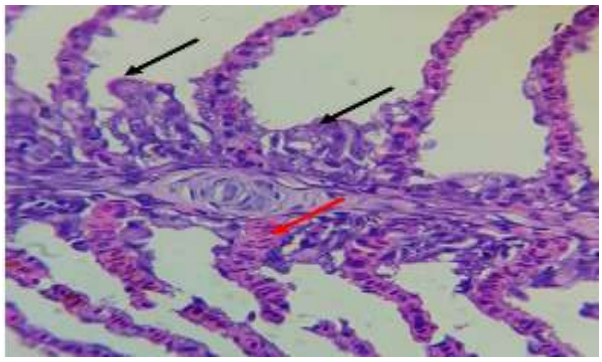

Figure 1: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by congestion of capillary in secondary gills filament (red row) with LF about 1/3 (black row), H&E, 40 × 3.2X.

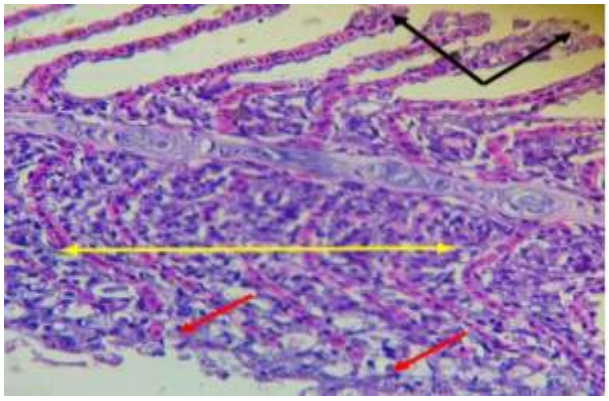

Figure 2: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by vacuolar degeneration of non-specific cells (red row), LF more than 2/3 (two head yellow row) with EH about 30% (black row), H&E, 40 × 4X.

Categories of histopathological alteration in the gills occupy in stage I and II characterized by infiltration of inflammatory cells, edema and hemorrhage with inflammatory exudate (Figure 3), and vacuolar degeneration in pillar cells with 2/3 LF which represented code II, as in (Figure 4). Stages I and II represented in (Figure 5) which characterized by shortening of secondary gills filaments with code I of the EL about 31%, while stage III histopathological categories represented by aneurysm and telangiectasia in the secondary gill lamellae and drumstick appearance correlated to 30% EH code I and 31% for EL as in (Figure 6).

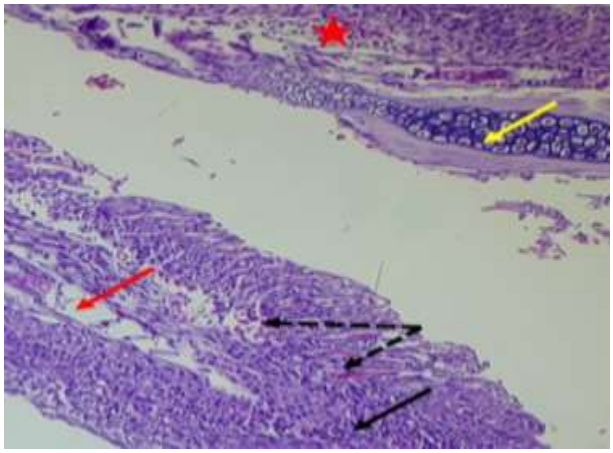

Figure 3: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by infiltration of inflammatory cells (black row), edema (red row), hemorrhage (dot black row), with abnormal cartilage shape (yellow row) and inflammatory exudate (red star), H&E, 10 × 4.7X.

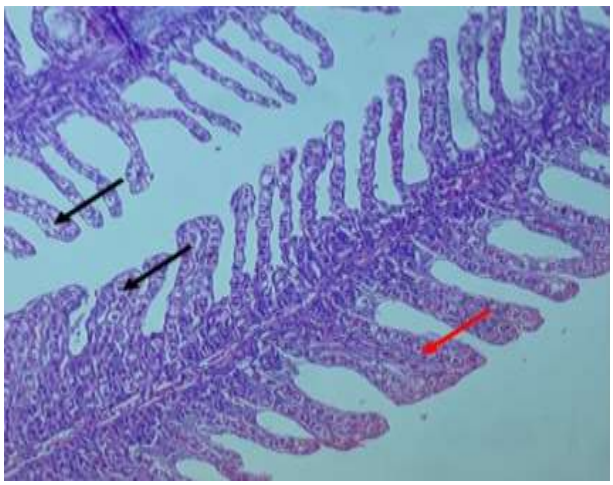

Figure 4: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by vacuolar degeneration of pillar cells (black row) and LF about 2/3 (red row), H&E, 10 × 4.1X.

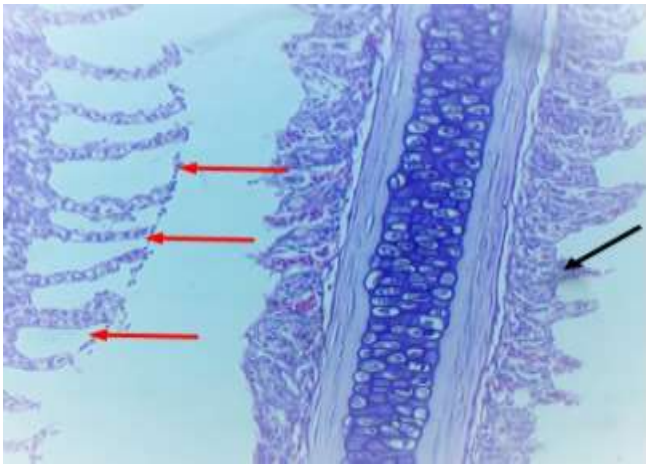

Figure 5: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by shortening of the secondary gill's filaments (black row) and EL about 31% (red row), H&E, 10 × 4.8X.

The histopathological alteration can be classified into four categories (vascular, cell growth, architecture disturbances, and cells injury); the statistical analysis of the alteration index for each category is highly significant (P≤0.01) except the hemorrhage in vascular disturbances and vacuolar degeneration in the cell injury these both alteration indexes was significant at (P≤0.05) in contrast to the untreated group (Table 4).

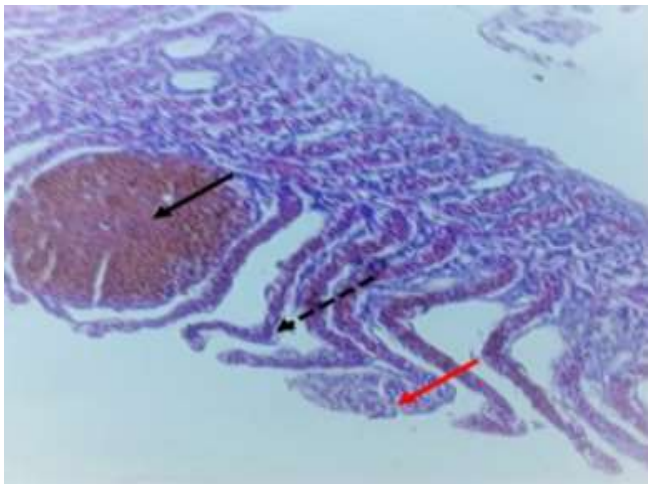

Figure 6: Histopathological alteration of gills in fish exposed to 10g/l of sodium thiosulfate represented by aneurysm and telangiectasia (black row), drumstick appearance of the apex of secondary gill lamellae (red row) and 31% EL (dot black row), H&E, 40 ×2X.

Table 4: Semi quantities statistical analysis of histopathological lesions in the gill of fish exposed to sodium thiosulfate

Categories	Histopathological lesions	Groups	Mean	SD	t value	Sig.
Vascular disturbances	Hemorrhage	Treatment	9.60	5.37	2.56*	0.03
		Untreated	2.40	3.29		
	Congestion	Treatment	4.00	0.00	6.53**	0.00
		Untreated	0.80	1.10		
	Aneurysm	Treatment	3.60	0.89	9.00**	0.00
		Untreated	0.00	0.00		
	Edema	Treatment	3.60	1.67	3.77**	0.01
		Untreated	0.40	0.89		
	Infiltration of inflammatory cells	Treatment	3.60	1.67	4.81**	0.00
		Untreated	0.00	0.00		
Cell growth disturbances	Hyperplasia	Treatment	4.80	1.10	9.80**	0.00
		Untreated	0.00	0.00		
	Hypertrophy	Treatment	2.80	1.10	5.72**	0.00
		Untreated	0.00	0.00		
Cell injury	Vacuolar Degeneration	Treatment	2.80	1.10	2.31*	0.05
		Untreated	1.20	1.10		
Architecture disturbances	Shortening and loss of lamella	Treatment	14.40	3.29	9.80**	0.00
		Untreated	0.00	0.00		
	Lamella fusion	Treatment	15.60	3.29	10.61**	0.00
		Untreated	0.00	0.00		
	Abnormal cartilage morphology	Treatment	14.40	3.29	9.80**	0.00
		Untreated	0.00	0.00		

* Refer to significant differences between groups at P<0.05. ** Refer to significant differences between groups at P<0.01.

Histochemical and immunohistochemical analysis

The histochemical examination of gills in carp fish exposed to overconcentration of sodium thiosulfate reveals a positive reaction for PAS stain represented as hyperplasia of chloride cells which was semi-quantitatively and score assessed as ++ as in (Figure 7), also typical hyperplasia of mucus cells was detected under light microscope and scoring for Masson Trichrome as positive reaction +++ as in (Figure 8).

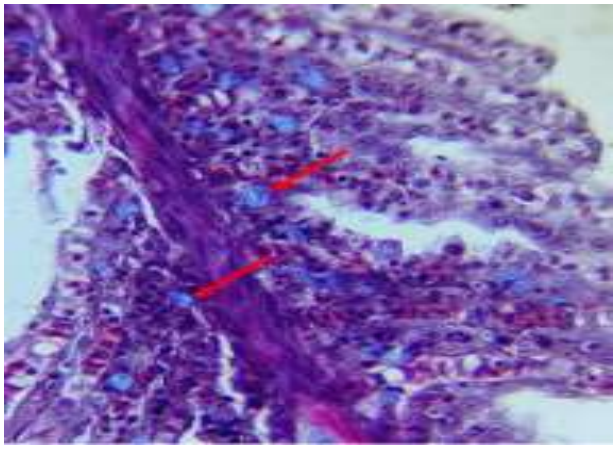

Figure 7: Histochemistry examination for gills in fish exposed to 10g/l of sodium thiosulfate represented by hyperplasia of chloride cells (red row), PAS-positive reaction (++), 40×3X.

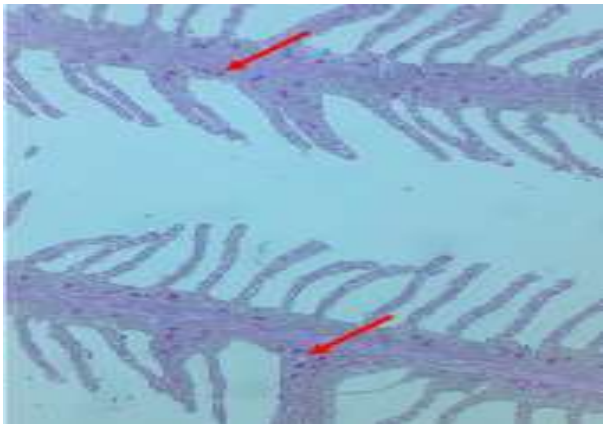

Figure 8: Histochemistry examination for gills in fish exposed to 10g/l of sodium thiosulfate represented by hyperplasia of mucus cells (red row), Masson Trichrome positive reaction (+++), 40×1.6X.

The semi-quantitatively score system for caspase -3 activity was detected in the gills of fish that were exposed to toxicity with sodium thiosulfate (10g /L); the microscopic examination revealed that the severity of caspase -3 activity reaction is variable in the grade and score in the secondary gills filaments in which apoptotic alteration grade as moderate (score ++) in contrast to the grade of apoptotic alteration in primary gills filaments which are mild with score (+) in contrast to negative reaction grade normal and score (-) as in (Figure 9 and 10).

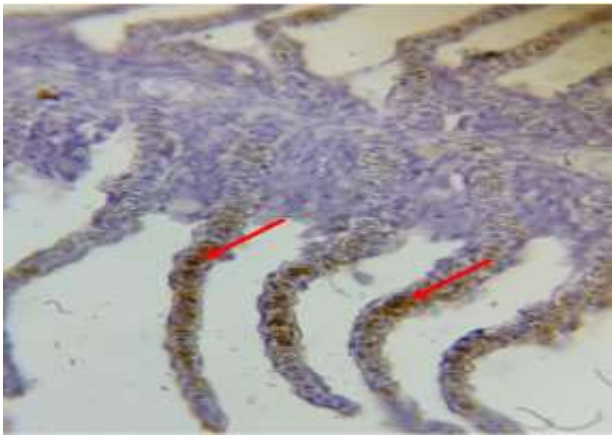

Figure 9: Immunohistochemistry examination for gills in fish exposed to 10g/l of sodium thiosulfate represented by apoptotic alteration (red row), moderate TUNEL reaction (++) in secondary gills filaments, 40×1.6X.

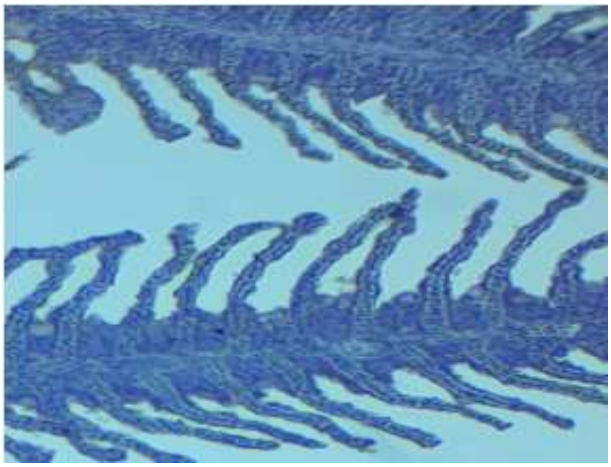

Figure 10: Immunohistochemistry examination for gills in un-toxic fish, TUNEL reaction (-), 10×6.8X.

Discussion

Histopathological alteration has been generally used as bioindicators in the assessment of the health and activity of the fish exposed to pollutants and infection, both in the field and laboratory studies (24-27); although sodium thiosulfate is used sparsely as a fish embryonic disinfectant and its one of the more general dechlorinated agents because its low toxicity for the aquatic organism (28-30).

Sulfuric compounds and their derivatives are potential risks to aquatic organisms' health; sodium thiosulfate is one of these chemical compounds; oh *et al*. (13) reported that the effective concentration of sodium thiosulfate in Daphnia *magna* is 1334 mg/l, and teratogenic effects on zebrafish embryonic development at concentration 0.1-1 mol/l and may kill fish at 34-40 mg/l (29,31). However, it was observed through this study that fish exposure to a high concentration of sodium thiosulfate led to a histopathological alteration in the gills that variable from vascular changes such as hemorrhage, congestion, edema, aneurysms, and vacuolar degeneration. to abnormal architecture with cellular alteration as infiltration of inflammatory cells with variable degree of EL and EH and LE. The reason for these histopathological alterations is due to the indirect effect of sodium thiosulfate on the fish through the activity of a type of bacteria present in an ecosystem that can metabolize precipitated sulfate compounds and produce sulfuric acid, which leads to a decrease in pH, as well as depletion dissolved oxygen (29), the histopathological alteration in the gills of the recent study agreement with results of both studies of Matey *et al.* (32) and Zhou *et al.* (33) who reported the effects of hypoxia on gills tissue architecture in crucian carp, Goldfish (*Carassius carassius*) and Tilapia (*Oreochromis niloticus*). Gills are vital organs for respiration and osmoregulation. Oxygen is essential for the aerobic generation of adenosine triphosphate (ATP) in the mitochondria through oxidative phosphorylation (34).

Gills hypoxia under the toxic pollution as sodium thiosulfate cause stimulation to the anaerobic metabolism, which leads to cellular oxidative stress (OS) and produces reactive oxygen species (ROS) that cause lipid peroxidation and loss cell membrane permeability and imbalance ionic regulation, leading to cell injury and vacuolar degeneration, with increase glycolysis to supply energy but leads to decline cellular pH, glycogen and protein synthesis and activation lysosomal enzymes lead to dead tissue and necrosis with alter cytoskeleton (35). In gill sections, typical alteration in both Masson Trichrome and PAS stains were detected as positive cells only for chloride and mucus as hypertrophy and hyperplasia as well as other pathological alteration in the other gill cellular complexes (pavement, epithelial, and pillar cells), increased mucus secretion and lamellae fusion represented adaptive, defense and compensatory mechanisms to reduce the pathological effects of toxicant but at the same time decrease oxygen intake and this consider a pathway for hypoxia (36,37).

The semi-quantities score system is the modern method methods in assessing histopathological lesions (38), so this study shows some microscopic lesions are significant, which may be reversible alterations others are highly significant, such as in architecture and circulatory disturbances, which are classified as irreversible alterations, Hu *et al.* (31) reported the toxicity of sodium thiosulfate in the cytoskeleton.

Caspase-3 activity has been detected in the gills tissue of the fish in this study, and the severity of TUNEL stain is more predominant in the secondary gill lamellae rather than primary filaments; this result was reported by Topal (39), who reported that the caspase-3 expression is more detected in the secondary lamellae in gills of Rainbow trout exposed to chlorpyrifos toxicity, there are previous studies suggested that aquatic pollution and environmental stress can be induction apoptosis (40) as polyethylene microplastic in carp fish (41) and pesticide as Lambda toxicity *Oreochromis niloticus* (42) and cypermethrin in the zebrafish (43). The pathway mechanisms of sodium thiosulfate to induce apoptosis are still unknown. However, in general, the mechanisms of programmed cell death may be related to the activity of reactive oxygen species, which leads to activation of caspase-3 expression or may be related to the imbalance of ionic osmoregulation, mainly Ca^{+2} (44).

Conclusion

It was concluded from this study that the application concentration of chemicals compound is essential as lower concentrate does not improve chemical and physical features of the aquatic environment while higher concentrate cause abnormal water quality and kills fish, so the use of sodium thiosulfate has a consider ecotoxicological hazard for fish when it used randomly and in high concentration lead to histopathological alterations in the gills, these alterations were detected by using and for the first time, Immunohistochemistry and immunohistochemical stains, in addition to semi-quantitative score analysis. Further immunohistochemical and biochemical studies are essential to elucidate the role of chemotherapeutic agents during treatment and controlling fish diseases.

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Conflict of interest

No conflict interests

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الكيمياء المناعية النسجية والتحليل شبه الكمي لغلاصم اسماك الكارب المعرضة لثايوسلفات الصوديوم

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الخلاصة

واحد من أكثر المواد إزالة الكلور هو الصوديوم ثايوسلفيت ولكن سميته على الأحياء المائية غير معروفة، ولذا هدفت هذه الدراسة لتحديد الأثار الضارة للاستخدام العشوائي وبتركيز عالي للثايوسلفات الصوديوم في اسماك الكارب والمستزرعة في حوض ارضي ترابي في حاوي الكنيسة من مدينة الموصل. أدى استخدام الثايوسلفات الصوديوم بتركيز ١٠غم/لتر الى نفوق الأسماك بنسبة ٨٠% وخلال ٩٦ ساعة من التعرض. يمثل التحليل شبه الكمي للتغيرات المرضية النسجية في الغلاصم مؤشر حيوي لتقييم السمية والتي كانت معنوية في مؤشر التغييرات الفجوية والنزف وكانت عالية المعنوية بالتغييرات الأخرى والمتمثلة بالاضطرابات الوعائية (الوذمة والاحتقان وأمهات الدم) وفرط تنسج وضخامة الخلايا مع قصر وفقدان استقامة والتحام الصفائح الغلصمية الثانوية مع الغضروف غير الطبيعي. وكانت شدة خلايا الكلوريد والمخاطية ++ و +++ لصبغة بيرودك الحامضية وملون ماسون ثلاثي الصبغ وعلى التوالي وتم الكشف عن موت الخلايا المبرمج من خلال تنشيط تعاقب -٣ والذي تم تحديده بشكل بارز في التركيب الخلوي للصفائح الغلصمية الثانوية. استنتج من هذه الدراسة أن استخدام الثايوسلفات الصوديوم وبتراكيز عالية يؤدي الى تغييرات مرضية نسجية في الأعضاء الحيوية ومنها الغلاصم ويعد التحليل شبه الكمي من الطرق الحديثة والتي من خلالها يتم تحديد معنوية التغييرات النسجية في مقاطع الغلاصم بالإضافة الى كيمياء النسيج المناعية وتقنية موت الخلايا المبرمج.

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