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Effect of Using Sodium Carbonate Peroxyhydrate (OXY-SOS™) as an Alternative Source of Oxygen on Different Parameters in Nile Tilapia (*Oreochromis Niloticus* L.)

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Key words:

Oxygen, Nile Tilapia, Sodium carbonate peroxyhydrate, DNA fragmentation.

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ABSTRACT

In the present study sodium carbonate peroxyhydrate ($2Na_2CO_3.3H_2O_2$) which is considered as eligible algaecide and fungicide, was used as an alternative source of oxygen in fish ponds with concentration of 50 and 100 mg/L for 10 days. To approach our target hematological (RBCs, Hb,PCV, total and differential leukocytic count) together with several serum biochemical parameters as (glucose ,total proteins ,albumin, globulins, A/G, amino transferases (ALT,AST),urea and creatinine) were estimated. DNA fragmentation and a mild degree of DNA degradation as shown by DNA smear and a hallmark apoptosis were observed in samples from gills, brain and liver after 6 days of the experiment. Pathological changes started to appear first in the brain tissue then other organs (liver and kidney) were also affected after 6 days of the experiment with 100mg/L treated groups indicating the sensitivity of the brain and the direct effect of hypoxia on this organ. These results suggest the resistance of fish to oxygen depletion in case of using sodium carbonate peroxyhydrate (OXY-SOS) and the appearance of pathological changes after 6 days of use indicate its efficiency as alternative oxygen source. Thus, sodium carbonate peroxyhydrate (OXY-SOS) can be used as a stable oxygen releaser and as a suitable alternative to artificial aeration in fish farms for a period up to 6 days without adverse effect on treated fish.

1. INTRODUCTION

Environmental hypoxia is an increasing problem worldwide with severe consequences for aquatic ecosystems. Oxygen supply in water bodies is generally mediated by the diffusion of atmospheric oxygen and by the production of the photoautotrophic plants, phytoplankton and algae (Valiela et al., 1997). The concentration of dissolved oxygen (DO) in aquatic environment depends on temperature, salinity and pressure (Diaz, 2001).

Hypoxia is a state of oxygen deficiency that is sufficient to cause impairment of organismal function or in extreme cases, death. Hypoxia has received extensive attention in biomedical research (Liu and Simon, 2004, Scott and Greco, 2004) and is becoming an increasingly important environmental concern (Wu *et al.*, 2003,Shang and Wu, 2004).

Hypoxia is considered as the most important factor that has altered ecosystems of many coastal countries. Such

changes in larger ecosystems may not recover from the development of persistent hypoxia (Ju et al., 2007).

Typically, environmental hypoxia is defined when the DO is below 2 mg O_2/l. Under these conditions, the first effects appear in most aquatic organisms (Diaz, 2001 and Diaz and Rosenberg, 2008). Hypoxia mainly occurs during the summer months when the solubility of oxygen in water decreases due to the rising temperature. This effect is further enhanced by the stratification of the water column by formation of thermo- and haloclines (Conley *et al.,* 2008).

On the morphological and physiological levels, some species have evolved specific adaptations that help them cope with hypoxic periods. These adaptations include, for example, the apoptosis-based expansion of the gill surface, the counter-current oxygen exchange and the increased oxygen affinity of fish haemoglobin, resulting in oxygen extraction efficiencies of 50–90% (Sollid *et al.,* 2003,Sollid and Nilsson, 2006 and Nilsson, 2007). On the molecular level, various fish species show the typical cellular responses which depend on the activation of the hypoxia-inducible transcription factor (Hif-1) or are part of the conserved cellular stress response (Tiedke *et al.,* 2014).

Hypoxia also triggers the transcription of regulatory genes that promote O2 delivery and anaerobic metabolism, suppress major energy-requiring processes, and inhibit growth and development in animals ranging from invertebrates to mammals (Kajimura *et al.,* 2006).

A recent report on channel catfish showed that a reduction in oxygen from 6 mg/l to less than 2 mg/l resulted in a drop of complement hemolytic activity (Welker *et al.,* 2007). Another report using seabream induced hypoxia by air exposure found no change in alternative complement activity (Ortuno *et al.,* 2002).

Sodium percarbonate ($2Na_2CO_3.3H_2O_2$) was identified as a potential source of oxygen. Where it releases hydrogen peroxide which in turn decomposes to oxygen (Vesper *et al.,* 1994). The mode of action of sodium carbonate peroxyhydrate in competing micro-organisms in water, is to kill the target organisms by oxidizing critical components, such as the cellular structure of the target organism. The Environmental Protection Agency, USA, has classified sodium carbonate peroxyhydrate ($2Na_2CO_3.3H_2O_2$) as eligible as algaecide and fungicide. It is a non-complex chemical and its physical and chemical characteristics are well understood. In the presence of water, the granules or crystals of sodium carbonate peroxyhydrate are dissolved and transformed into hydrogen peroxide and sodium carbonate. Upon contact the hydrogen peroxide oxidizes its targets and kill them, then breaks down into water and oxygen, neither of which engender toxicological concern.

Because of the instability of the sodium carbonate peroxyhydrate molecule in the presence of water, there is negligible risk that municipal drinking water will be affected, or that any possible runoff to surface or groundwater could result in any harmful (toxicological) effect.

The current research was conducted to study the ability of sodium carbonate peroxyhydrate to be used an alternative oxygen releaser and evaluating the comparative effects of different doses of sodium carbonate peroxide on the blood & serum parameters, histopathological changes and DNA changes of *Oreochromis niloticus*

2. MATERIALS AND METHODS

2.1. Animals used

The experiment was conducted in the laboratory of Fish processing and biotechnology department, Faculty of Aquatic and Fisheries Sciences, Kafrelsheikh University. Nile tilapia (*Oreochromis niloticus* L.) were obtained from a local fish farm in Kafrelsheikh governorate.

2.2. Experimental design

All of the experimental procedures complied with the Egyptian ethical codes for studies on experimental animals and approved by the Animal Ethical Committee, Kafrelsheikh University.

2.2.1. Experimental groups

Fish were randomly distributed to glass aquaria (40x80x60) with density of 15 fish per aquarium with mean body weight of 130±10 gm. (mean±SD). Fish were maintained in aerated aquaria at 20°C in a recirculating water system with mechanical filters and fed fish pellets *ad libitum*. The fish groups included: control group with artificial oxygen supply, control group without oxygen supply, group received sodium carbonate peroxyhydrate (OXY-SOS™, ANOVA Pharma) with concentration of 50mg/liter of water and group with 100mg/liter for 10 days. All treatment was performed in triplicates. Feed intake and mortality were monitored along the course of the experiment.

2.2.2. Blood and tissue sampling

Each 3days blood samples were taken gently from caudal vein of sampled fish from all groups under the experiment. Two different aliquots of blood were used for different analyses. The first aliquot was transferred

to a plastic tube coated with EDTA as anticoagulant and was used for hematological determination. The second aliquot was transferred to a plastic tube without EDTA, clot at 4 ^{o}C, centrifuged at 3000 g for 10 min, and then stored at - 20 ^{o} C for subsequent biochemical analysis. Tissue samples (liver, kidney and brain) were collected on formalin (10%) for histopathological examination. Tissue samples (gills, brain and liver) were also collected in 2ml eppendorf tubes and stored frozen at - 80 ^{o} C until DNA extraction could be completed.

2.3. DNA extraction and laddering

The degree of DNA damage in different tissues of the fish under experiment was tested as follows:

Tissue samples were collected from brain, gills and liver of all fish under experiment and DNA extraction was performed using Quick-gDNA MiniPrep (Zymo Research, USA) according to the manufacturer protocol. After extraction, DNA was separated by electrophoresis on a 1.5% ethidium bromide-incorporated agarose gel. The DNA pattern was then examined by UV transillumination

2.4. Hematological and biochemical assays

Hematological parameters including (red blood cell count (RBCs), packed cell volume (PCV), hemoglobin (Hb), total white blood cell (WBCs) and differential leukocyte counts were assessed according to the routine hematological procedures for fish according to Feldman et al. (2000). Freshly separated sera were used for estimation of serum hepatorenal injury biomarkers according to manufacturer's protocol. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were evaluated using a colorimetric method according to Reitman and Frankel (1957). Serum total protein was measured according to Lowry et al. (1951) and albumin according to Doumas et al. (1971). The renal product creatinine was determined according to Larsen (1972), urea according to Coulombe and Favreau (1963).

2.5. Histopathological examination

Sections of the organs samples (brain, kidney and liver) from all fish groups were fixed immediately in 10% formalin and processed for histopathological evaluation, using routine paraffin embedding method. Sections of 3µm thick were cut and stained using hematoxylin and eosin (HE) for light microscopic examination as mentioned by Bancroft and Gamble, (2007).

2.6. Statistical analysis

The results were expressed as mean±SD. Data were statistically analyzed using t-test. A value of ($P < 0.05$) was considered significant.

3. RESULTS

1. Oxygen level measurements

Table (1): Comparison of mean dissolved oxygen concentration (mg/L) in water of different treatments.

Treatment	The first 3days*	Second 3days	The last 3 days
OXI-SOS 100mg/L	2.1	2.46	2.06
OXI-SOS 50mg/L	1.05	0.98	0.37
Control with aereation	8.48	7.98	7.01
Control without aereation	1.3	1.61	0.96
Control water	10.9	9.77	11.2
Water with OXI-SOS tablets	11.7	10.8	10.1

*Mean oxygen concentration for each three successive days.

The oxygen concentration was almost constant in case of 100mg/L OXI-SOS along the period of the experiment. While in case of 50mg/L the oxygen level started to decline after 3days.

2. Blood parameters

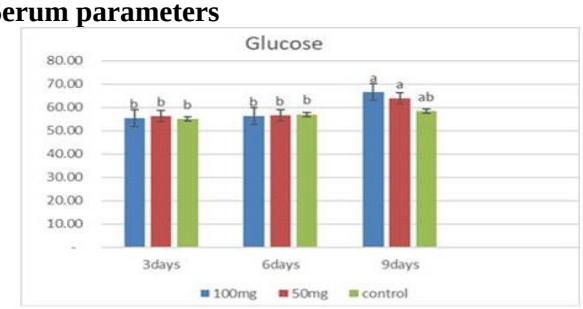
Hematological parameters of Nile tilapia O. niloticus in different treated groups are shown as Means ± SEM.

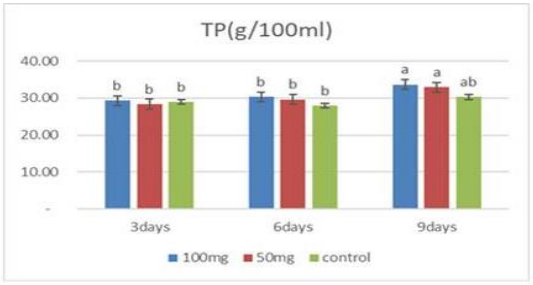
Sodium carbonate peroxyhydrate at both examined concentrations did not significantly alter the number of RBCs among treatments or days until day 6, where the abundance of red blood cell increased as the fish reached to day 9 at a lower levels of dissolved oxygen.

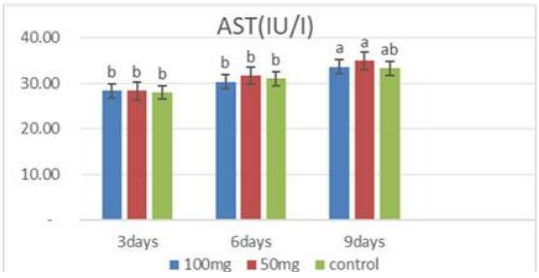
As well as, no significant increase in total leukocytic count or differential leukocytic count up to day 6 of treatments with both concentrations of sodium carbonate peroxyhydrate (50 and 100 mg/L), While, at

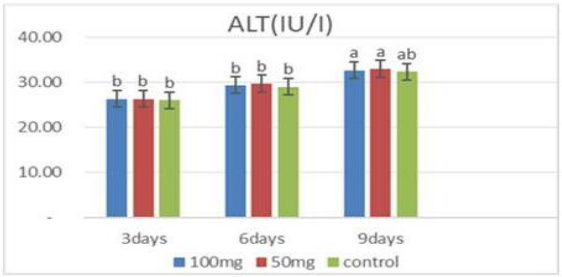
day 9 , leukogram finding revealed leukocytosis , heterophilia, lymphopenia, eosinopenia and monocytosis.

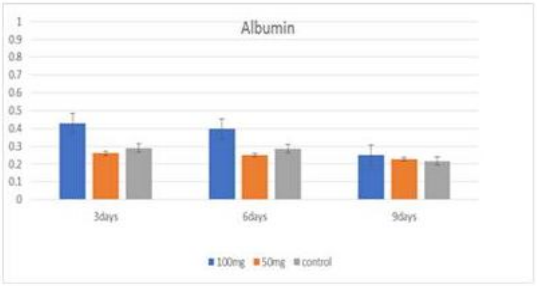
3. Serum parameters

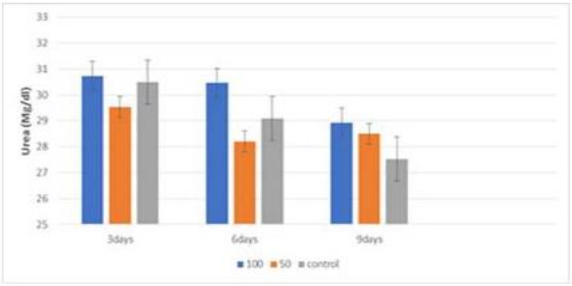


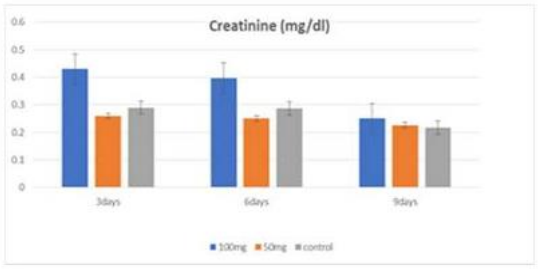












Biochemical parameters of Nile tilapia O. niloticus of different treated groups are shown as Means± SEM.
There were no significant differences in blood glucose, either between treatments or days. While at day 9 of the experiment, sodium carbonate peroxide exposure groups (100 mg/L) revealed hyperglycemia but statistically in significant compared to the control group. Fish after 9 days showed a significant increase in the levels of the serum total protein. There was significant increase in ALT and AST levels after day 9 in comparing to other treated groups.

4. Tissue damage after hypoxia and DNA fragmentation

To determine whether hypoxia lead to apoptotic cell death, genomic DNA was isolated from different tissues including brain, liver and gills and DNA

fragmentation was assessed. A laddering pattern of DNA fragmentation was observed in the gill samples at 6 days of the experiment. A mild degree of DNA degradation as shown by DNA smear and a hallmark apoptosis were observed in samples from brain at 6 days and liver at 6 and 9 days of the experiment. However, there was a mild degree of DNA degradation in the control groups as well

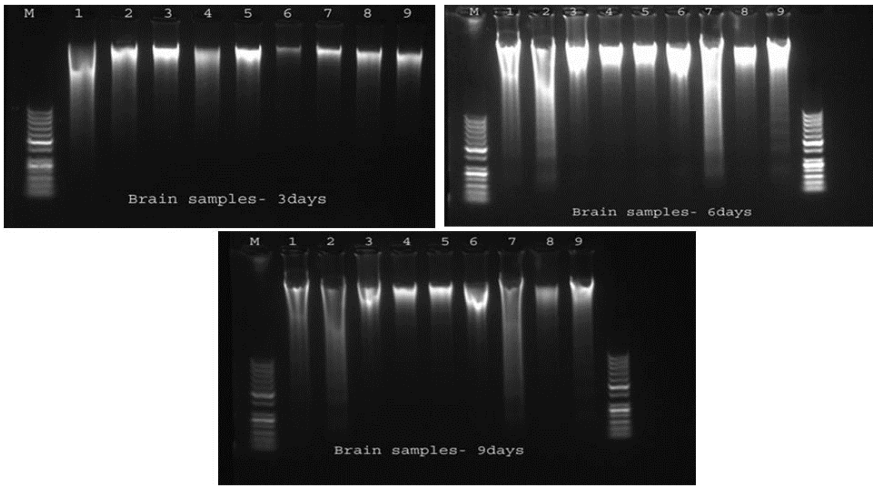


Figure (1): Gel electrophoresis of DNA samples from brain of different fish groups in days 3, 6 and 9 of the experiment. Where: M: molecular marker (DNA ladder), 1-2-3 are samples from the control group, 4-5-6 are the samples from 50mg/L and the 7-8-9 are the samples from 100mg/L.

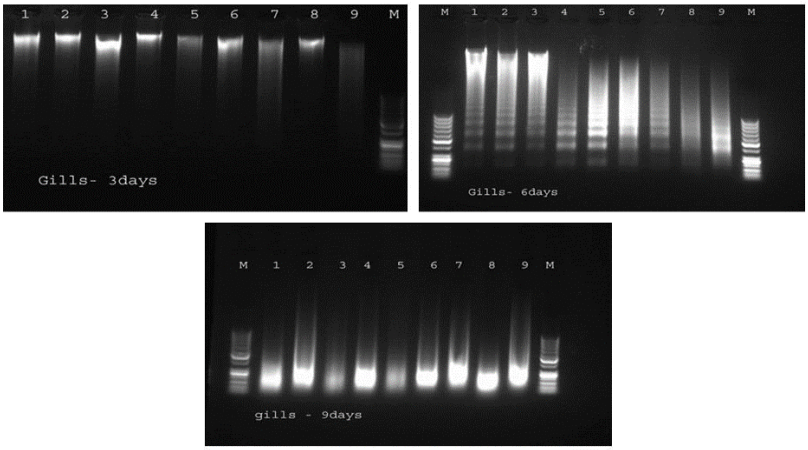


Figure (2): Gel electrophoresis of DNA samples from gills of different fish groups in days 3, 6 and 9 of the experiment. Where: M: molecular marker (DNA ladder), 1-2-3 are samples from the control group, 4-5-6 are the samples from 50mg/L and the 7-8-9 are the samples from 100mg/L.

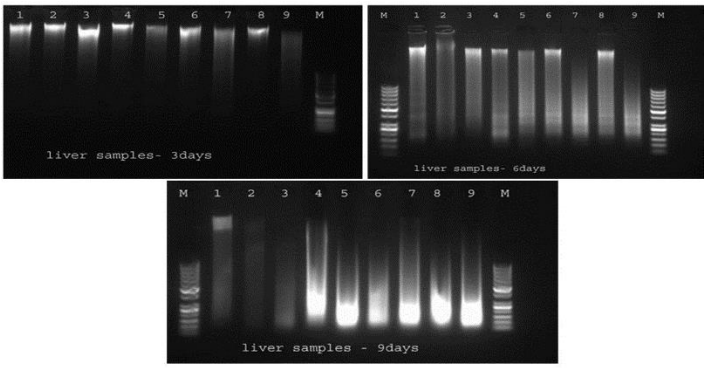


Figure (3): Gel electrophoresis of DNA samples from liver of different fish groups in days 3, 6 and 9 of the experiment. Where: M: molecular marker (DNA ladder), 1-2-3 are samples from the control group, 4-5-6 are the samples from 50mg/L and the 7-8-9 are the samples from 100mg/L.

5. Histopathological examination

The results of the histopathological examination showing normal morphological appearance in the control group as well as groups treated with 50mg/L (fig.4,5 and 6) and 100mg/L OXY-SOS after 3 days of the experiment, except for the brain of 100mg treated fish which (fig.6B3) started to show a mild degree of molecular layer vacuolation. However, after 6 days of the experimet groups with 50mg/L showed no pathological changes in the liver and kidney samples (fig.7D2 and 8E2), while in brain samples a mild degree molecular and granular layers vacuolation appeared (fig.9), groups treated with 100mg/L revealed degenerated hepatocytes mostly fatty changes and both degeneration and necrosis of hepatopancreas (fig.7D3), tubule-interstitial nephritis, interstitial inflammatory cell infiltration (fig.8E3), as well as marked vacuolation of the molecular layer of the brain and pathological satellitosis associated with gliosis (fig.9F3).

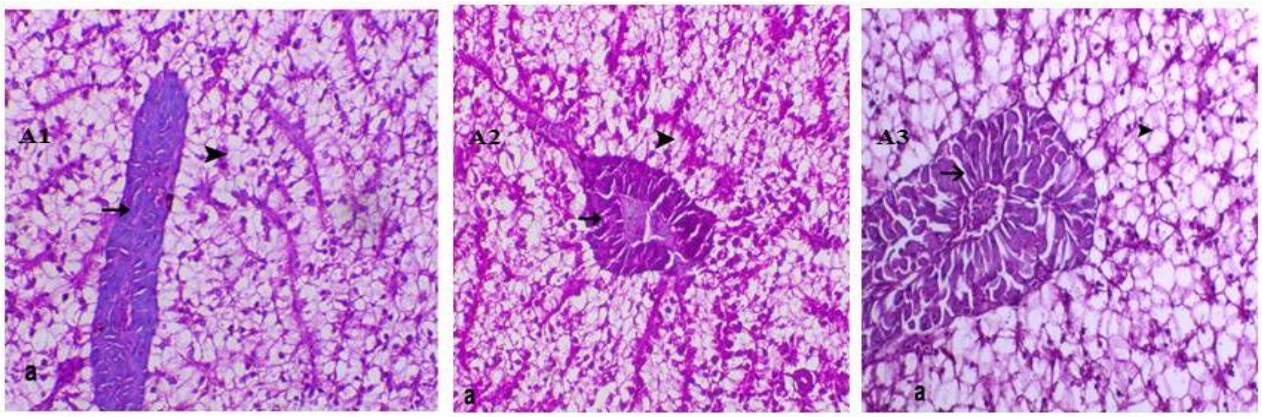


Fig. 4. Cross sections of histopathological examination from **liver** of (**control group**): (**A1**) showing normal hepatocytes and hepatopancreas (arrowhead and arrowhead respectively), of fish treated with **(50mg/L** OXI-SOS **after 3 days of the experimen**t: (**A2**) showing normal hepatocytes and hepatopancreas (arrowhead and arrow respectively and of fish treated with **(100mg/L OXI-SOS, after 3 days of the experiment)**: (**A3**) showing normal hepatocytes and hepatopancreas (arrowhead and arrow respectively), H&E, X200.

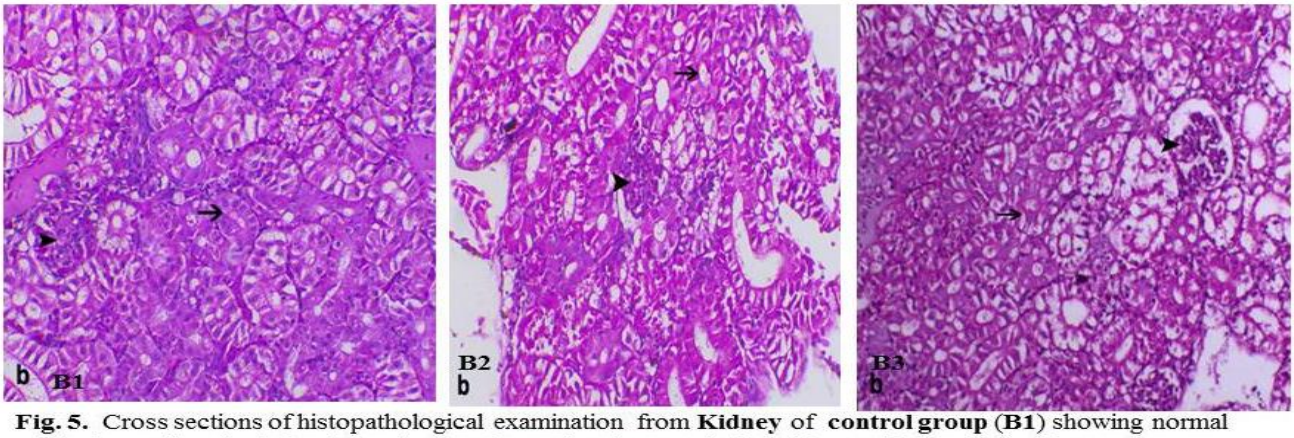


Fig. 5. Cross sections of histopathological examination from **Kidney** of **control group** (**B1**) showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively .Kidney of fish treated with **(50mg/L** OXI-SOS **after 3 days of the experimen**t: (**B2)** showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively .kidney of fish treated with (**100mg/L** OXI-SOS, after **3 days of the experiment**: (**B3**) ,showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively (H&E, X200)

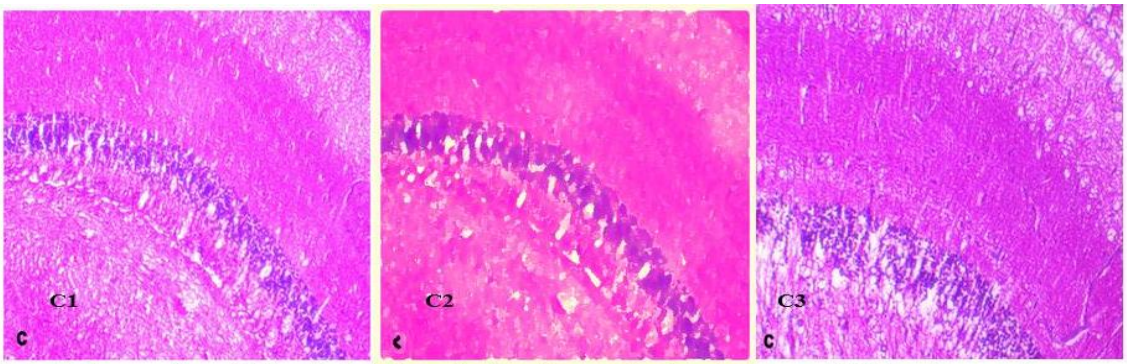


Fig. 6. Cross sections of histopathological examinations from Brain of **control group**: (**C1**) showing normal molecular and granular layers, H&E, X100. Brain of fish treated with **(50mg/L** OXI-SOS **after 3 days of the experimen**t: (**C2)** showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively. Brain of treated fish treated with (**100mg/L** OXI-SOS, after **3 days of the experiment**: (**B3**) showing mild degree of molecular layer vacuolation, H&E, X100.

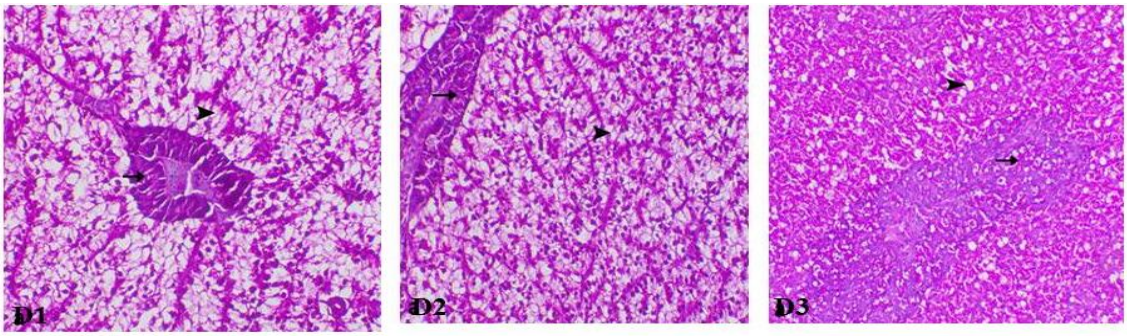


Fig. 7: Cross sections of histopathological examination from **liver** of (**control group)**: (**D1**) showing normal hepatocytes and hepatopancreas (arrowhead and arrowhead respectively),of fish treated with **(50mg/L** OXI-SOS **after 6 days of the experimen**t: (**D2)** showing normal hepatocytes and hepatopancreas (arrowhead and arrow respectively and of fish treated with **(100mg/L OXI-SOS, after 6 days of the experiment)**: (**D3**) showing degenerated hepatocytes mostly fatty changes (arrowhead) and both degeneration and necrosis of hepatopancreas (arrow), H&E, X200.

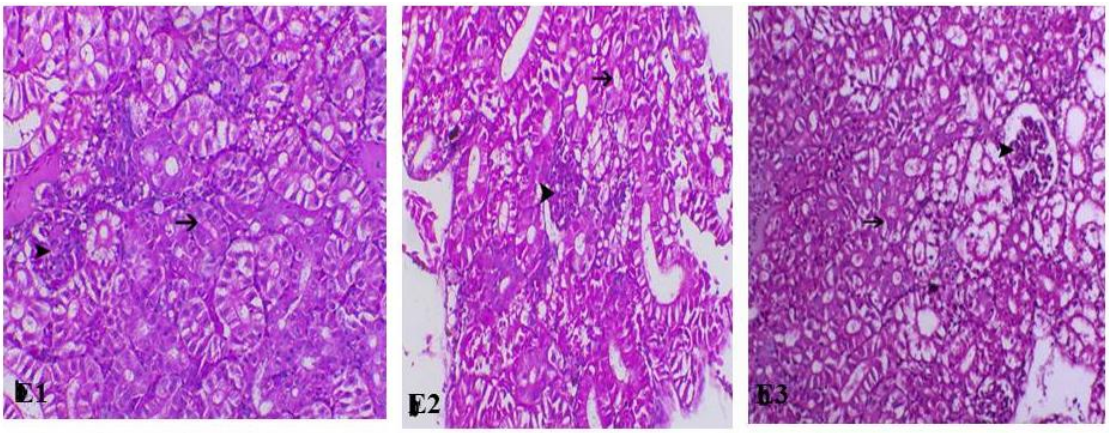


Fig. 8: Cross sections of histopathological examination from **Kidney** of **control group** (**E1**) showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively .Kidney of fish treated with **(50mg/L** OXI-SOS **after 6 days of the experimen**t: (**E2)** showing normal renal glomeruli and tubules (arrowhead and arrowhead respectively .kidney of fish treated with (**100mg/L** OXI-SOS, after **6 days of the experiment**: (**E3**) , showing tubule-interstitial nephritis (arrow indicates tubular necrosis and arrowhead indicates interstitial inflammatory cell infiltration), H&E, X200.

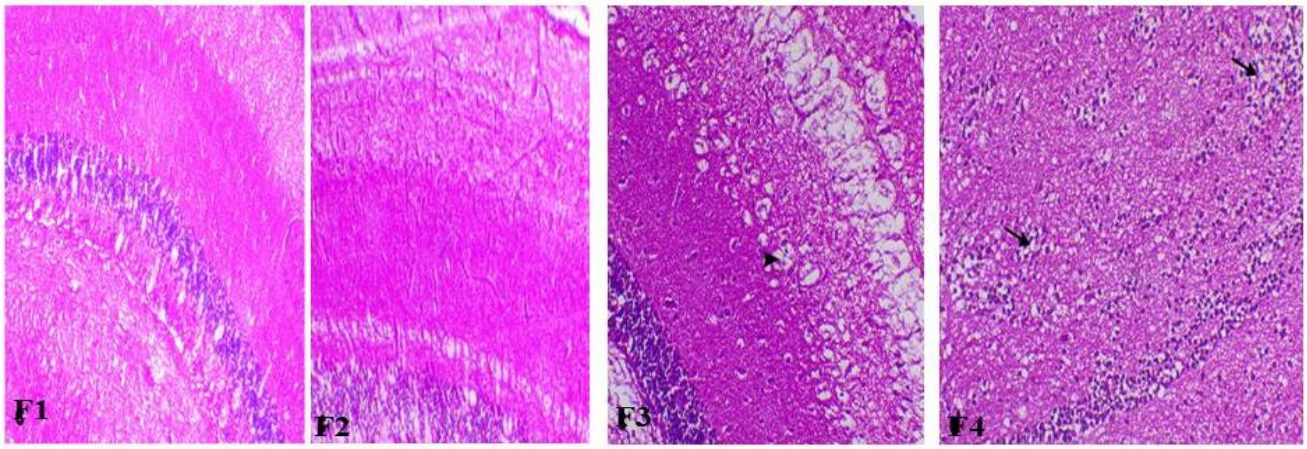


Fig. 9: Cross sections of histopathological examinations from Brain of **control group**: (**F1**) showing normal molecular and granular layers, H&E, X100. Brain of fish treated with **(50mg/L** OXI-SOS **after 6 days of the experimen**t: **(F2)** showing mild degree molecular and granular layers vacuolation, H&E, X100. . Brain of treated fish treated with **(100mg/L** OXI-SOS, **after 6 days of the experiment**: (**F3**) showing marked vacuolation of the molecular layer (arrowhead), H&E, X100**. (F4)** Brain showing pathological satellitosis associated with gliosis (arrows), H&E, X200.

4. DISCUSSION

Adequate concentrations of oxygen in water are vital to intensive fish farming (Ritola et al., 2002). The number of red blood cells (RBC) is an important parameter as these are associated with the oxygen carrying capacity of the blood.

Concerning the erythrogram, the current study declared that, sodium carbonate peroxyhydrate at both examined concentrations did not significantly alter the number of RBCs in examined fish among treatments or days until day 6 ,where the abundance of red blood cell increased as the fish reached to day 9 at a lower levels of dissolved oxygen during this period compared to the control indicated the time-dependent variation in RBC number with response to oxygen stress. Under hypoxic conditions, increasing the RBC level may help to acquire more oxygen and increase gas transport capacity of the fish blood (Wu et al. 2014). Recent studies showed that some hematological parameters including red and white blood cell count, hematocrit and hemoglobin concentrations are significantly increased after hypoxia treatment in fish (Petersen and Gamperl, 2011, Richards, 2011,Ni et al., 2014).

Concerning leukogram the results revealed no significant increase in total leukocytic count or differential leukocytic count up to day 6 of treatments with both concentrations of sodium carbonate peroxyhydrate (50 and 100 mg/L), While, at day 9 , leukogram finding revealed a stress picture of leukogram represented the increase of WBCs (leukocytosis) , increased number of heterophils (heterophilia), reduced number of lymphocytes (lymphopenia) , reduced number of eosinophil (eosinopenia) and increased number of monocytes (monocytosis). This can be related to hypoxic condition. Many reports suggested that cortisol is an important stress hormone secreted from the hypothalamus- pituitary internal axis (HPI) when fish is subjected to stress situation. The increase of cortisol in serum can be seen as the sensitive signal of fish stress (Mommsen et al., 1999).

Blood glucose in response to cortisol can be as stress indicators. There were no significant differences in blood glucose, either between treatments or days could be attributed to the stressor were found.

While at day 9 of the experiment, sodium carbonate peroxide exposure groups (100 mg/L) revealed elevated blood glucose concentration but statistically in significant compared to the control group. During the hypoxia treatment, glucose increased while the hepatic glycogen content decreased, which has also been reported in fish and other species (Douxfils et al., 2012, Polak et al., 2013). It is suspected that hepatic glycogen is the primary source of glucose, when animals are subjected to acute hypoxia, glycogenolysis is enhanced and hepatic glucose is released to blood to provide enough blood glucose and energy (through anaerobic glycolysis), thus ensure adequate basal metabolism for organism survival (Chen et al., 2007). Therefore, glucose homeostasis during the hypoxia treatment depends primarily on hepatic glucose output (Wahren and Ekberg, 2007). Exposure of fish to common stressors activated the hypothalamic pituitary-interrenal axis leading to increased plasma cortisol (Bertotto et al., 2011). It has been reported that serum glucose level might be up-regulated with the increasing of the level of cortisol (Van Raaij et al., 1996). The reason may be that cortisol can promote gluconeogenesis and glycogenolysis to meet the increasing demand of energy from fish under stress (Mommsen et al., 1999). Serum total protein reflects the level of non-specific immunity (Ortuño et al., 2001).

In our study, fish after 9 days showed a significant increase in the levels of the serum total protein. It is speculated that fish may increase specific proteins such as lysozyme or complement to enhance the immunity level to cope with stress (Ming et al., 2012). The changes of serum total protein and glucose are the secondary response to stress environment.

Serum enzyme activities, which reflect changes in the metabolism of different substances and altered functions of various tissues and organs responding to environmental stress, are important signs of integrity of cellular membrane function (Ou et al., 2014).

It is known that following prolonged hypoxia, the liver becomes necrotic and shows multifocal areas of moderate to severe lipid hepatocyte vacuolation (Mustafa et al., 2012). Similar findings have been reported for Cyprinus carpio (Mustafa et al., 2012). Considering the changes in ALT and AST levels after day 9, we can conclude that the liver was damaged by day 9 of experiment under hypoxic stress condition. In our study, the most apparent histopathological alterations in the kidneys in the hypoxic group were abnormally swollen and degenerated renal tubules with necrotic epithelia and depolymerized glomeruli with destroyed renal capsules. These features were not observed in control samples, and their presence indicated that hypoxia impaired the osmotic balance in the fish. The gills, which are directly exposed to the external environment, are important respiratory organs involved in various homeostatic activities, such as filter feeding, gas exchange, and ion regulation. The gills can adapt to changes in ambient oxygen levels by exhibiting potentially widespread morphological variations, as reported for carp (Huang et al., 2015).

Measurements of hematological, serum biochemical parameters histological analysis as well as DNA smear all revealed that sodium carbonate peroxide (OXI-SOS™) can be used an alternative source for oxygen without adverse effect on Nile tilapia (Oreochromis niloticus L.). up to 6 days.

Further work on the expression levels of fish cytokines will be important to increase explanatory potential for investigating alternative source for oxygen (sodium carbonate peroxide (OXI-SOS™) effects on the

immune system of Nile tilapia (Oreochromis niloticus L.).

5. CONCLUSION

The present study revealed that sodium carbonate peroxyhydrate (OXI-SOS) can be used as a stable oxygen releaser and as a suitable alternative to artificial aeration in fish farms for a period up to 6 days where pathological changes started to appear in different organs of the examined fish. This oxygen supplier showed stability of dissolved oxygen level in the water and had no adverse effect on both blood and serum parameters of examined fish at concentration of 50mg/L, as well as integrity of DNA and histopathological changes in examined organs.

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