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Toxic effects of sub-lethal dose of algal toxin (Microcystin-LR) on male laboratory mice *Mus muscullus* L.

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Key words: Cyanotoxins (Microcystin-LR), Algal toxins testis, Hormones, Spermatogonium, Liver enzymes

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Abstract

The present study was aimed to investigate the toxic effects (physiological and histological effects) of sub-lethal dose of microcystin-LR (0.125 µg/kg/day as i.p. injection) on male mice for long time of exposure15and 30 days .Sixty four intact adult male mice (Laboratory generations) were used in current study. Animals were divided into eight groups each one contain eight male mice. Each group treated with 0.1 ml of toxins. Results showed significant decrease in sperm account reach 37and 25× 10^4 for the two periods respectively as compared with control group. While testosterone levels increased significantly p≤0.05 after 30 days reach 0.14 ng/ml compared with control group. However non-significant increase in follicle Stimulating (FSH) and luteinizing Hormone (LH) Hormone for two exposure periods. A significant reduction in the diameter of somniferous tubules and number of spermatogonium for two exposure periods reach 45.57, 37.50 µm and 28.37, 28 µm unit respectively compared with control groups. Whereas, the diameter of germ cells was not affected significantly. Liver enzymes such as (i.e. Aspartate transaminase (AST), Alanine transaminase (ALT) were increase significantly for two periods of exposure reach 161.66 , 179 IU/L and 158.33, 180 33 IU/L respectively, While alkaline phosphatase (ALP)activity do not showed any significant increasing . Finding also appeared many histopathological changes in testis combined with exposure periods increasing and compared with control group represented by congestion, thickness of capsule wall, necrosis, excessive vacillation and closer of some somniferous tubules. In conclusion, the sub lethal dose of MC-LR may be led highly impairment in male mice testes; function and histology, sex hormones and liver enzymes when exposing for long period reach to 30 days that may be led to Infertility.

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Introduction

Microcystins (MC) are potent hepatotoxins produced by the blue-green algae of the genera *Plantothrix* , *Microcystis, Aphanizomenon*, *Nostoc*, *Anabaena* , *Calothrix* and *Hapalosiphon* and these heptapeptides have strong affinity to serine threonine protein phosphatase (PPs) by acting as an inhibitor of this group of enzymes (AL-Sultan, 2007& 2017 ; Campos and Vasconcelos,2010). Microcystins are the most frequently studied toxins because of their widely distribution among other algal toxins and high toxicity. Currently more than 80 different microcystins have been identified (Feuerstein *et al.*, 2009).

The existence of cyanobacteria blooms especially microcystins in drinking water have resulted of public health problems (Funari and Testal, 2008). Microcystins can bioaccumulation in aquatic animals and these toxins can be transferred along the food web to high trophic levels, event to human beings (Smith and Haney, 2006;Zang*et.al.*, 2007). The problem due to MC-LR gets compound by the fact that it is concentrated by boiling, this increasing risk and it is also resistant to chemical analysis or oxidation at near natural pH, Alsomicrocystin is stable even at temperature up to 300ċ in laboratory conditions (Wannermacher, 1989).

The gonads are considered as the second importance target organs of microcystinsand several studies have verified that MCs accumulated in testis and causing toxic effects on reproductive systems (Chen and Xie , 2005; Li *et al.*, 2008;Zhao *et al.*, 2012;Li and Han ,2012). Blue green algae produce wide variety of potent toxins that effect on wild domestic animals and human (Dowson,1998;Carmichael,1994;Nasri*et al.*, 2004). Therefore, chronic exposure by drinking water containing microcystinsmay induce human liver cancer (Nishiwaki-Matsushima *et al.*, 1992).

Apart from a direct effect of testis and ovaries, Microcystinsin directly affect sex hormones by damaging the hypothalamic pituitary gland (HPG) axis and liver (Chen *et al.*, 2015).

Microcystin-LR was found to be the most common algal toxins(cyanotoxins) followed by microcystin-RR and YR (Gupta *et al.*, 2003) and for reduce the risks caused by MCs ,the world health organization (WHO) has set acceptable concentration guideline of 1µg/L of MCs in water destined for human drinking water(WHO, 2008). The testis is a known target organ for injury resulting as exposure to both chemotherapeutic and toxic environmental agent (Boekelheid *et al.*, 2005). However many reports available on microcystins caused toxicity in various tissues, but very limited studies demonstration the anti-fertility effect causing by microcystinse specially on testis.

Androgens play an important role to initiating and maintenance of male reproductive function or testicular function which includes spermatozoa production and the main testicular androgenis testosterone which is produced by leyding cells under the stimulation of pituitary LH hormone. Testosterone is essential for spermatogenesis, fertility and maintenance of the male phenotype spermatogenesis (Sharpe *et al.,* 1988). Microcystin have shown to interact with the mitochondria, the consequences are the dysfunction of organelle, induction of reactive oxygen species (ROS) , apoptosis and microcystin activity leads to the differential expression/activity of transcriptional factors and protein kinases involved in the pathways of cellular differentiation, proliferation and tumor promotion activity and this activity may result from the direct inhibition of protein phosphatase

S PP1 and PP2A (Campos and Vasconcelos,2010). However, there is a little information about toxicity effects of microcystin under low concentrations in the structure of testis, sex hormones and liver enzymes, histological effects in adult male mice.

Therefore, the main objective of this study was to investigate the effects of sub-lethal dose (effective dose) of cyanotoxins MC-LR (hepatotoxins) on testes, sex hormones, enzymes and histopathological effects on adult laboratory mice after long period exposure.

Material and methods

Preparation sub lethal dose (effective dose) of algal toxins (MC-LR) on mice

The sub-lethal dose of cyanotoxin MC-LR was prepared from standard toxins brought from Alexis Company (USA). These toxins were dissolved in 10 ml distal water and then the sub lethal dose of hepatotoxins (MC-LR) was made according to the mice body weight (Table 1).

Animals

Male mice *Mus musculus* L. Strain Balb/C weigh 23-25 gwere used in this study. The mice were housed in standard plastic cages with saw dust cover on the floor in animal house of the Biology Department/College of Education for Pure Sciences/ Basra University/Iraq. Mice were maintained on a 12:12 light-dark cycle at laboratory condition with free access chow and tap water.

The pellets were prepared in the laboratory by mixing crude protein, milk powder, mineral and vitamins, ground soya bean. Wheat flour and wheat brain (Jawad, 1996).

Toxicity experiments –dosing and period of exposure

Sixty four intact adult male mice (Laboratory generation) were used in present study. Animals were divided into eight groups (each group contain eight male mice). Each group of mice treated with 0.1 ml (i.p. injection) of sub-lethal dose of algal toxins MC-LR (0.125 µg/kg/day) after dividing for two exposure periods 15 and 30 days. The control group was injected with 0.1 ml of normal saline (i.p. injection).

Sperm count

After 15 and 30 days of MC-LR exposure, the male mice were scarified by using chloroform. The epididymis were collected and rinse in phosphate buffered saline (pH=7.2). The suspension was mixed with 1% of aqueous eosin Y (10:1) and kept for 30 minute to staining the sperms.

The an aliquot of stained the filtrate was taken in white blood cell pipette and diluted by phosphate buffer solution (PBS).

The mixture was stirred and charged into slide count (Neubauer chamber) and sperms counted in 8 squares of $1mm^2$ for WBC count and multiplied by 5×10^4 factor to calculate total number of sperms (Vega *et al*., 1988).

Morphometrical analysis of the testis

Testes were examined for structural changes. The following parameters were recorded: Spermatogonium, Sperm count, seminiferous tubules diameter (STD) and diameter of germ cells which was measured by light microscope according to (Klassem and Persaud, 1978).

Hormones assay

Stimulating hormone (FSH), luteinizing hormone (LH) and testosterone were measured by using enzyme linked immunosorbent assay (ELISA-kits)as described in the instructions produced by manufacturing kits (Human Co. Germany).

Enzymes assay

The liver enzymes aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were measured in serum blood of male mice using ELISA- kits as described in the instructions produced by manufactures kits (Biondox Company).

Histopathological examinations

After 15 and 30 days the last sub-lethal dose of algal toxin (MC-LR) injection, testes were immediately fixed in Bonus solution. For each testis of each period exposure, serial sections (7µm thickness) were made. These sections were stained by hematoxylin-eosin (H & E) and morphometrically examined by light microscopy (Humason, 1972).

Statistical analysis

The SPSS program version -20 were used. ONE-WAY ANOVA analysis was applied under significant level $p\leq0.05$ with calculate the revised least significant differences (R.L.S.D) to compare between means of treatments.

Results

Effects of sub-lethal dose of algal toxins (MC-LR) on sperm account and sexual hormones in adult male mice

Results showed significant decrease in sperm account after two periods exposure to sub-lethal dose of MC-LR reach to 37and 25 $\times 10^4$ compared with control group 70 $\times 10^4$ (Fig. 1).

Non-significant decreasing ($p \leq 0.05$) in the levels of testosterone hormone after 15 days of exposure reach to 2.46 ng/ml, while with increase of exposure period to (30 days) a significant decreasing were showed in the levels of this hormone reach to 0.14 ng/ml compared with control group 5.75 ng/ml(Fig. 2).

Table 1. Preparation the sub lethal dose of algal toxin (Microcystin-LR).

Standard toxin MC-LR Conc.	Lethal dose conc. Of MC-LR (WHO, 1999)	Lethal dose of MC-LR for mice weighing (20-25)g	Sub lethal concentration LD50 of MC-LR /10
100µg	50 µg/kg	1.25 µg/kg	**0.125** µg/kg/day

Table 2. Toxic effect of IP injection of sub lethal dose of algal toxin (MC-LR) on testicular tissue of laboratory mice after 15 and 30 days of exposure.

parameters	Exposure time to (0.125 µg/kg) of algal toxins MC-LR)			R.L.S.D
	Control Mean±SD	15 days Mean±SD	30 days Mean±SD	
Diameter of seminiferous tubules (µm)	54±1.21a	45.57±2.25b	37.50±1.88b	5.34
Spermatogonium	35±1.125a	28.37±1.253b	28±1.10b	3.17
Diameter of germ cells (µm)	16.75±1.34	14.37±2.20	11.25±1.28	--

R.L.S.D= revised least significant differences ($p \leq 0.05$), (-): non-significant differences.

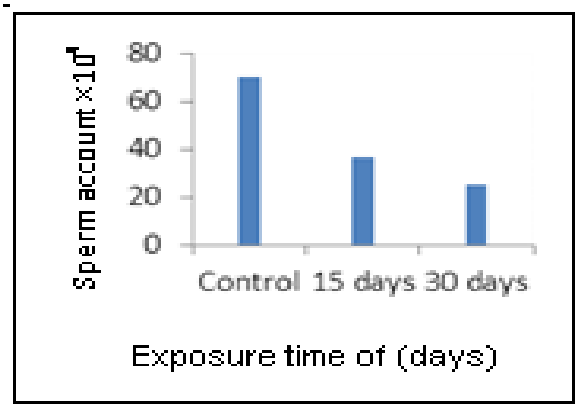

Fig. 1. Sperm account in male mice after exposure to sub- lethal dose of algal toxins (MC-LR).

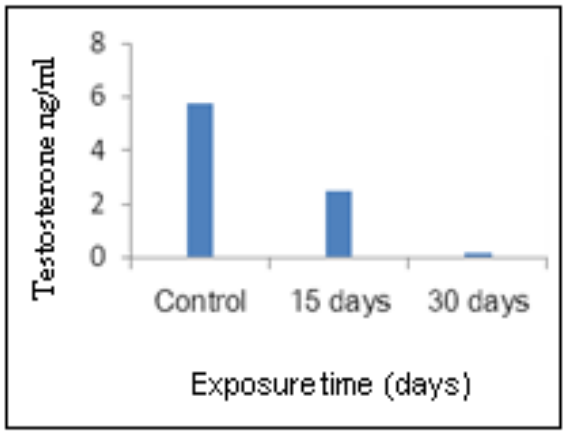

Fig. 2. Testosterone concentration in male mice after exposure to sub-lethal dose of algal toxins (MC-LR).

However, FSH and LH did not appear any significant increasing in their values for two exposure period (15 and 30 days) which reach to 3.05 , 3.03 and 0.27, 0.40 IU/L for both hormones in both periods respectively that in comparing with control group 3.02 and 0.40 IU/L (Fig. 3 and Fig. 4.).

Effects of sub lethal dose of algal toxins (MC-LR) on testes structure

The normal spermatogenesis could be seen in the testis sections of the control group (Fig. 8).

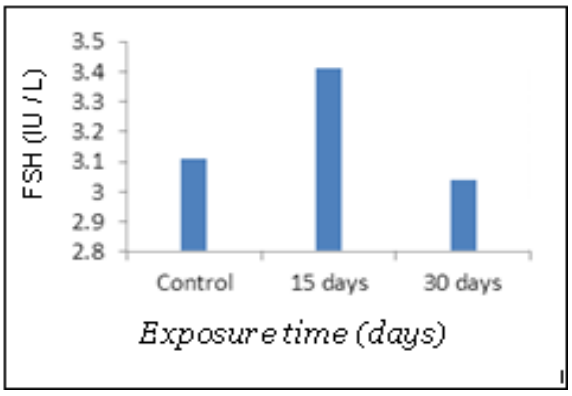

Fig. 3. The FSH hormone in mice after exposure to sub-lethal dose of algal toxins (MC-LR).

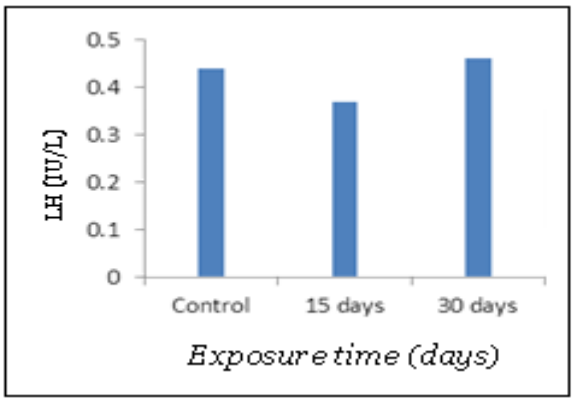

Fig. 4. The LH hormone in mice after exposure to sub-lethal dose of algal toxins (MC-LR).

In contrast, different degrees of degenerative changes such as necrosis in testis and reduce in diameter of seminiferous tubules, speramtogonium and diameter of germ cells were observed in two periods of exposure (15 and 30) days (Fig.9, 10).

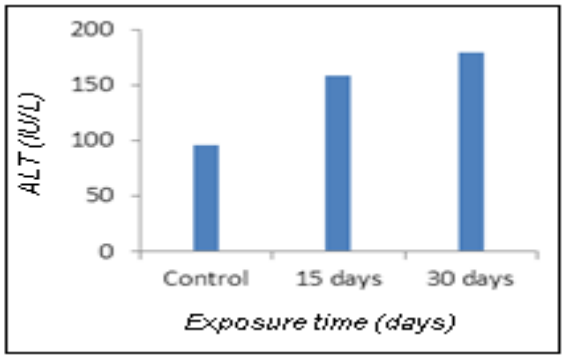

Fig. 5. The ALT enzyme in mice after exposure to sub-lethal dose of algal toxins (MC-LR).

Finding revealed a significant reduce in diameters of seminiferous tubules ($p \leq 0.05$): Control (54±1.21) µm, 15days (45.57±2.25) µm and30 days (37.50±1.88) µm. A significant decrease in the number of spermatogonium was also detected: control group (35±1.125) µm unit, 15 days (28.37±1.253) µm unit and 30 days (28±1.10) µm unit, while results showed that there was no considerable reduction in the diameters of germ cells for two periods of exposure 15 days (14.37±2.20) µm and 30 days (11.25±1.82) µm comparing with control group (16.75±1.34) µm (Table 2).

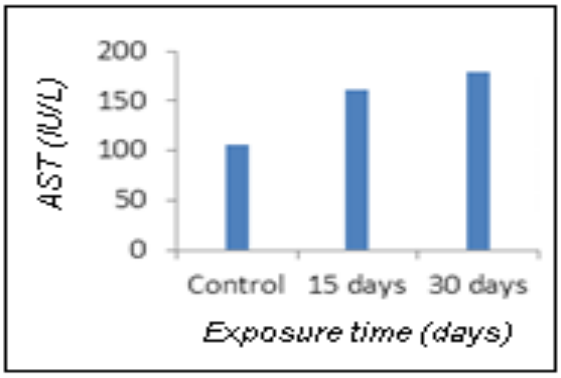

Fig. 6. The AST enzyme in mice after exposure to sub-lethal dose of algal toxins (MC-LR).

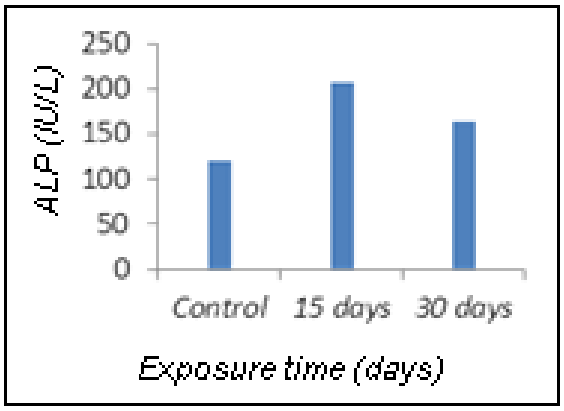

Fig. 7. The ALP enzyme in mice after exposure to sub-lethal dose of algal toxins (MC-LR).

Effects of sub lethal dose of algal toxins (MC-LR) on liver enzymes

Finding showed significant increase in the liver enzyme AST values ($p \leq 0.05$)reach to 161.66 IU/L after 15 days and these level showed an obvious increase to hit179IU/L after 30 days of microcystin exposure compared with control group 106.66 IU/L (Fig. 5). Similarly, the ALT enzyme appeared significant increasing after two exposure periods

reached to 158.33 and 180.33 IU/L respectively compared with control group 96 IU/L Fig.6. Whereas no significant increase in the ALP enzyme values reach to 208.33 and 164 IU/L for two period respectively compared with control group 120 IU/L (Fig. 7) .

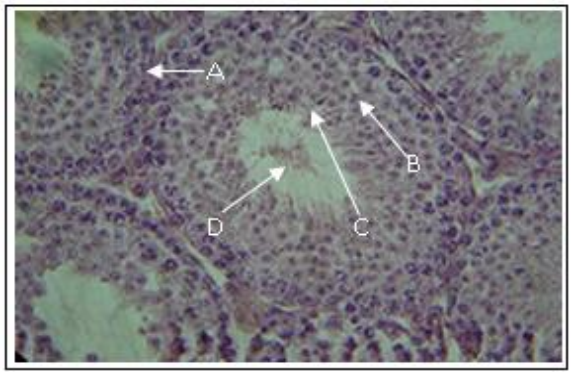

Fig. 8. Image shows the testis in mice (control group): A: (Spermatogonium), B: (Primary spermatocytes), C: (Spermatids) and D: (Spermatozoids). Staining with (H & E) 40×).

Histopathological changes

Finding showed many histopathological changes in testes of micecombined with increasing toxin exposure period from 15 to 30 days as compared with control group (Fig. 8, 9).These changes represented by congestion in blood vessels (Fig.10, 11), thickness of capsule wall with necrosis in seminiferous tubules (Fig.12, 13), excessive vacillation (Fig.14, 15), closured of some seminiferous tubules, narrowing in some seminiferous tubules with inhibition of spermatogenesis (Fig.16, 17).

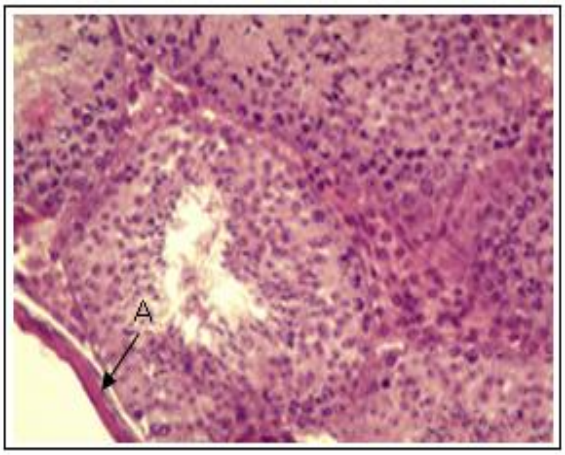

Fig. 9. Image shows the testis in mice (control group): A: (Normal thickening of capsule wall). 40x.

Discussion

Mice are considered a good model provides on alternate for humans to curry out the physiological study of toxic substances involve in male mice reproductive function.

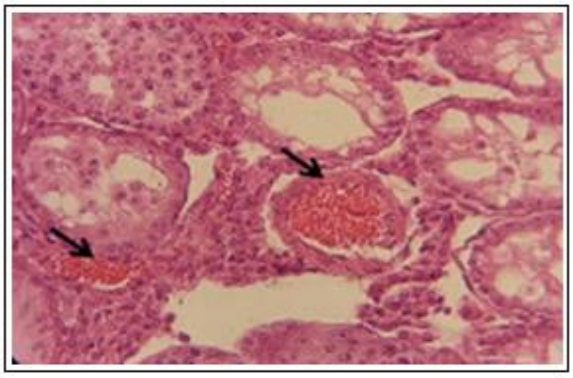

Fig. 10. Image shows the congestion of blood vessels after 15 days of MC-LR exposure (arrow) 40x.

The significant decrease in sperm account is most important signs indicator of male infertility (Kumar *et al.*, 2006).In the field and laboratory studies showed that the toxins (MCs) which produced from blue-green algae(Cyanobacteria)accumulate in highly concentration not only in liver or hepatopencreas but also in gonads (Testis and ovaries).

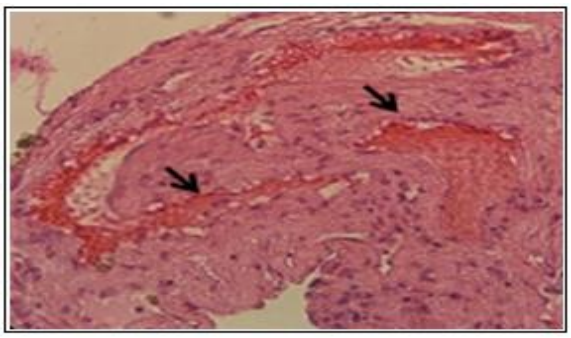

Fig. 11. Image shows the congestion of blood vessels after 30 days of MC-LR exposure (arrow).40 x.

In this study the exposure of sub-lethal dose of MC-LR was led to a significant decrease in sperm account, spermatogenesis and testosterone levels in particular the long term exposure after 30 days (chronic exposure).So this indicates to the cytotoxicity for sub-lethal dose of toxin (MC-LR)on all types of germ cells and spermatogenesis by causing the death of the developing germ cells in the seminiferous tubules as is the case when exposed to high concentrations of the toxin.

Because, spermatozoa are extremely susceptible to cytotoxic agents because of their rapid proliferation (Wyrobek *et al.*, 1983; Reddy and Reddy, 2009).In addition the reproductive system is considered the second important target of microcystins (Chen and Zie, 2005).

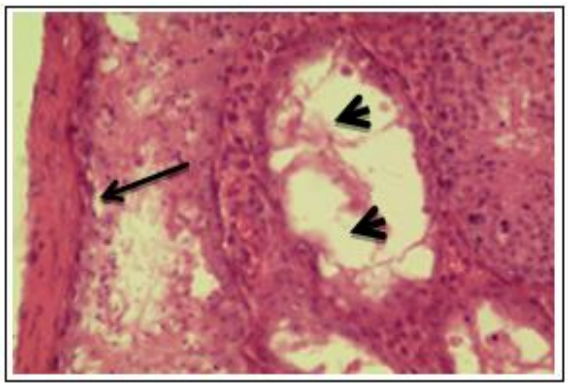

Fig. 12. Image shows the thickening of capsule wall(arrow) and necrosis in tubular cells (head arrow) after 15 days of MC-LR. exposure. 40 x.

A few studies agree with these results which shown that MCs where toxic to the male reproductive system and in particular the testes were more sensitive than the liver or other organ (Li *et al.*, 2008) and the study of Li *et al.*, (2011) have confirmed that MCs induce germ cells apoptosis associated with the mitochondrial dependent apoptotic pathway in rat.

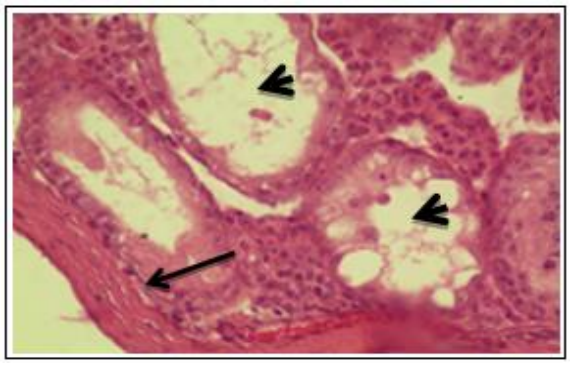

Fig. 13. Image shows the thickening of capsule wall (arrow) and necrosis in tubular cells (head arrow) after 30 days of MC-LR exposure.40x.

Testosterone is a type of steroid hormone which play an important role in reproductive tract development such as spermatogenesis and erected functions, however it might be the target of many environmental toxicant (Li *et al.,* 2008).

Microcystins can indirectly affect male mice serum hormones and mRNA expressions by damaging the hypothalamic pituitary gonad (HPG) axis (Wang *et al.*, 2012), Therefore the sub-lethal dose of MC-LR is cause decreasing in the levels of testosterone, so this matter was led to decrease in sperm account in male mice with increasing of exposure period, because testosterone is required for differentiation of sex organs and production of sperms and maintenance of testosterone levels very critically for spermatogenesis (Watanabe *et al.*, 1986; Pidoux *et al.*, 2007). Therefore, the decline of this hormone led to lower numbers of sperm with increasing duration of exposure periods.

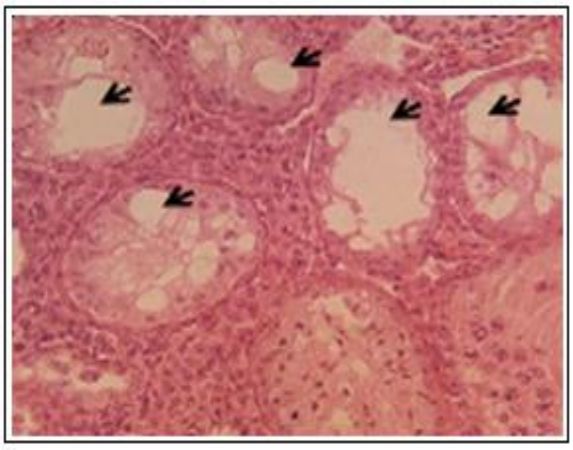

Fig. 14. Image shows the excessive vacillation after 15 days of MC-LR exposure(arrow).40x.

The study of Chen *et al.,* (2016) showed when male mice exposing to high dose of MC-LR (i.p) reach to 5µg/kg/day led to decrease sperm motility, testis weight, sperm concentration and the level of serum testosterone. Findings did not show any significant increasing or decreasing of FSH and LH hormones levels when exposed to sub-lethal dose of algal toxins MC-LR as compared with control group for two exposure periods.

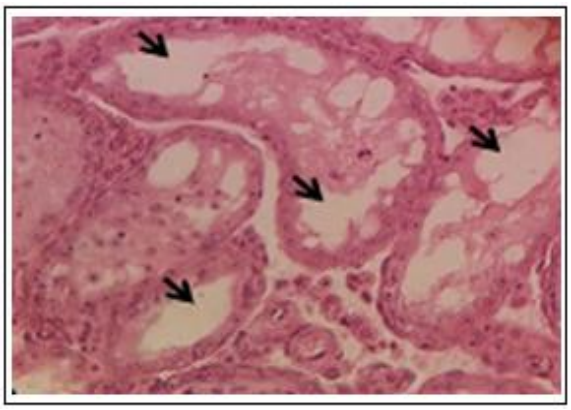

Fig. 15. Image shows the excessive vacillation after 30 days of MC-LR exposure(arrow).40x.

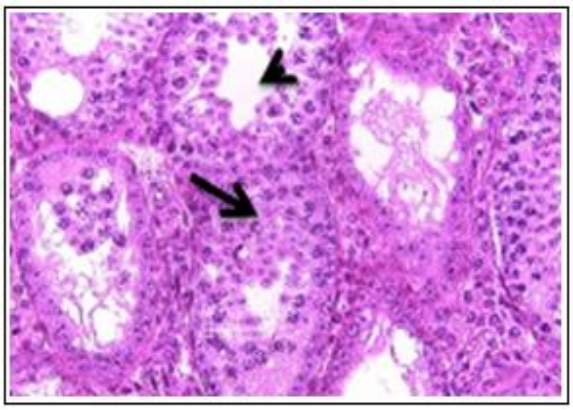

Fig. 16. Image shows some of somniferous tubules are closured (arrow) and narrowing seminiferous tubules (head arrow) inhibition of spermatogenesis after 15 days of MC-LR exposure.40x.

These results did not agree with several studies which showed significant decreasing of these hormones in rats and mice when expose to high dose of microcystins ranged between (5-15) µg/kg/day (Lankoff *et al.,* 2003; Zhou *et al.*, 2012; Li and Han, 2012) and also study of Li *et al.* (2008) who showed that microcystincause decline of follicular stimulating hormones (FSH) Luteinizing hormone (LH) in addition to testosterone levels. The maintaining LH serum levels is very important for initiating and supporting spermatogenesis , hence degradation of sertoli cells and germinal cells may be due to high concentrations of circulating LH hormone(Shan *et al.*, 1995 ; Sharkar *et al.*, 2000). In addition the spermatogenesis disruption might be due to increase in the serum LH concentration which is determined to the germinal cells (having a very important role in the first steps of spermatogenesis process (Boekelheide and Schoenfeld, 2001 and Izumi *et al.,* 2005). Liang *et al.*, 2015 assumed that MC-LR exposure can significantly affect cytoskeleton organization in rate testis which lead to the altered expressions of MFs, MTs and IFs, thus leading to the morphological changes and excrete prominent toxicity to the reproductive system.

The study of Chen *et al.*, 2013 also showed that male rats when exposure to MC-LR, the testis index significantly decreased under highly dose 10 µg/kg/day body weight group compared with group treated with 1µg/kg/day. However another study demonstrated that MC-LR exposure led to an initial increase and subsequent decrease in secretion of FSH, LH and testosterone (Wang *et al.*, 2012). Similar results was found in study of Katá (2013) who showed that anticancer drug (Cisplatin) cause many physiological effects on hormones and testicular tissues and histopathological effects on male mice testis when expose to 1 and 2 mg/kg of drug for long term exposure reach to 16 days.

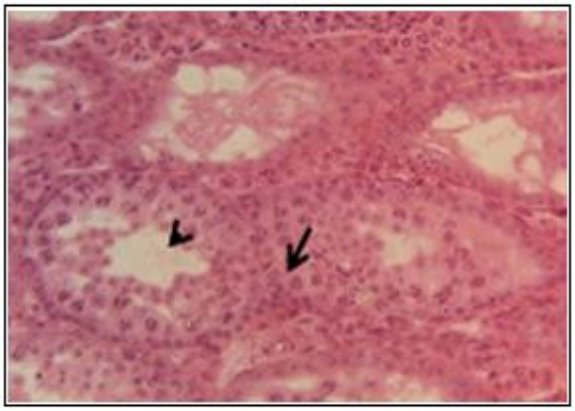

Fig. 17. Image shows some of somniferous tubules are closured (arrow) and narrowing seminiferous tubules (head arrow) inhibition of spermatogenesis after 30 days of MC-LR exposure.40x.

Results showed significant increasing in in blood serum enzymes especially AST,ALT and non-significant increasing in ALT enzyme. , these results agree with the findings of Gupta and Guha (2006) when fresh water fish *Heteropneuster fossilis* (Bloch) after injection with microcystin after 24h of treatment, because that microcystin toxicity cause damage in the liver and Kidney.

Robergh *et al.*, 1991 reported that blood serum enzymes ALT, ALP and LDH increase two hours after intrarperitonial injection of toxin as a consequence of hepatocyte necrosis. Also Knopp and Hetesa (2000) reported that there were increase in ALT and AST in the juvenile carp (*Cyprinus carpio* L.) when exposure to different natural populations of cyanobacterial water blooms. In addition our result was agreed with study of AL-Sultan *et al.* (2011).

The principles mechanism of MCs toxicity is the inhibition of protein phosphatases 1 and 2a which then leads to an increase in protein phosphorylation's (Yoshizawa *et al.*, 1990). This study was appeared the sub-lethal dose of MC-LR was led to several histopathological effects on testis tissue with increasing period of exposure represented by congestion, thickening of capsule wall, necrosis in seminiferous tubules, excessive vacillation, closured of some seminiferous tubules, narrowing in some seminiferous tubules with inhibition of spermatogenesis. Chen *et al.* (2013) showed microcystins can pass through the blood–testis barrier (BIB) can cause morphological damage at testis and some ultra-structural observation indicated some typical apoptosis features, including cell membrane blabbing ,cytoplasmic shrinkage ,swollen mitochondria and denervation of nucleus. Another studies showed that MC-LR induced reproductive functional impairment in medaka fish by detecting exacerbated physiological stress and tissue damage in gonads (Trencher *et al.*, 2011).Noticeable damage to testicular ultrastructure and oxidative stress also found in zebra fish testes (Zhao *et al.*, 2012). Quad *et al.* (2013) observed marked histological lesions and cell apoptosis in zebra fish gonads after MC-LR exposure, and also showed that reproductive toxicity in female zebra fish were more vulnerable than in males upon to MC exposure. A few studies showed that treatment with highly concentration of MC-LR on mice and rats reach to 5-10 µg/kg B.W., which led to causes slight testicular atrophy associated with changes and blockage in seminiferous tubules , slight deformation of and rogonial and spermatogonic cells , thinning of spermatogenic epithelium as well as depopulations of lyding cells , reduce numbers of interstitial cells, stertolic cells and mature sperm (Ding *et al.,* 2006; Li *et al.*, 2008 ; Chen *et al.*, 2011; Wang *et al.*, 2013). Therefore, the present study showed that low concentrations of hepatotoxins (MCs) have very detrimental effects on the long-term level of chronic exposure, especially on the histological structure of testicular mice and on fertility and effect on male hormones, which may cause infertility.

Acknowledgement

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Conclusion

The daily exposure of sub-lethal dose of microcystin-LR led to many physiological and histopathological effects as the same as the high dose exposure. Also major changes in the testicular functions and structure as well as hormonal changes could lead to a loss of reproductive ability or infertility.

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