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REPRODUCTIVE IMPAIRMENT INDUCED BY THE BLUE-GREEN ALGA *LYNGBYA AESTUARII*

F. S. Kata[1], E. Y. A. Al-Sultan[1] and A. M. Eassa[2]*

[1]Department of Biology, College of Education for Pure Science, University of Basra, Iraq.
[2]Department of Marine Chemistry, Marine Science Center, University of Basra, Iraq.
*e-mail: amal_0770@yahoo.com

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ABSTRACT : A severe problem has emerged in the aquatic environment by endocrine disrupting cyanotoxins that may adversely affect the reproduction function of human beings and wildlife. So, the aim of present study is to investigate the reproductive toxicity of the blue-green alga *Lyngbya aestuarii* on laboratory male mice. The current results showed a significant decrease in testosterone concentrations in both high and low dose treatment respectively comparable with control treatment. While, concentration of LH and FSH hormones showed significantly decrease in high dose treatment comparable with control treatment. Reproductive ability showed 100% of fertility for control treatment, 37.50% of fertility for high dose treatment and 100% of infertility in male treated with low dose of alga extract. Also, histological sections of testes showed several histological defects in both low and high dose comparable with control. These histological defects suggest that toxic algal extract were either capable of permeating the blood-testis barrier or it induced these defects via its impacting on hypothalamus-pituitary-testes axis. The number of newborn pups was significantly decreased in high dose treatment only comparable with control one.study concluded that cyanobacterial bloom in Basra rivers is a real environmental problem which has a detrimental effects on fertility of citizens in the future.

Key words : Lyngbya aestuarii, fertility, spermatogenesis, reproductive hormones.

INTRODUCTION

Cyanobacterial blooms in aquatic systems has been regarded as a serious global public health problem and a major environmental issue. According to WHO (2003) approximately 50-75% of the cyanobacterial blooms are toxic. The first documentation of animal poisoning correlated with cyanobacteria was recorded by Francis (1878), who registered deaths of several animals such as sheep, cattle, dogs, horses and pigs near lake Alexandrina, South Australia. Since this initial poisoning, mortality of animals linked to blooming of toxic cyanobacteria have been numerous. Human illnesses caused by cyanotoxins fall into three categories; gastroenteritis, allergic and irritation reaction and liver diseases (Madkour *et al*, 2015). The first case of human disease due to cyanobacteria was in 1844 where the cyanobacterium *Oscillatoria* identified in fecal samples of people lived in London. A high incidence of primary liver cancer in people living in some areas of China has been linked to chronic exposure to low levels of microcystins through drinking water (Yu *et al*, 1995). Human deaths caused by acute exposure to cyanotoxins are limited to few cases so far. In (1996) many cases of liver failure and human death occurred in Brazilian dialysis clinicwhich named Caruaru syndrome, being caused by the exposure of the patients to microcystins-contaminated water when used for dialysis (Pouria *et al*, 1998; Carmichael *et al*, 2001). Recently, a severe problem has emerged in the aquatic environment by endocrine disrupting cyanotoxins that may adversely affect the reproduction function of human beings and wildlife (Essa *et al*, 2013; Al-Sultan *et al*, 2017). In Iraq, the study of Kataa *et al* (2017) is the only study on the impact of toxic blue-green alga "*Oscillatoria limosa*" on reproductive aspects on mice. So, the aim of present study is to investigate the reproductive toxicity of the methanolic extract of the blue-green alga *Lyngbya aestuarii* on laboratory male mice.

MATERIALS AND METHODS

Algal sampling and extraction

The algal bloom were collected from Al-Dawoodi river, a branch of Shatt Al-Arab river at Basrah governorate southern Iraq during the desiccation period in Winter 2018. The identification of the collected bloom was with Leica microscope according to Desikachary

(1959) and Prescott (1975). It has been found the algal bloom was comprised of only one alga and the alga was identified as *Lyngbya aestuarii*. Twenty grams of lyophilized alga were weighed and extracted twice with 75% methanol (1 liter) then it re-extracted twice with 1% acetic acid (40 mililiter), then both extractions were mixed together before desiccation with Freeze dryer (Rangel *et al*, 2013).

Median lethal dose test (LD_{50})

The median lethal dose (LD_{50}) for methanolic extract of *Lyngbya aestuarii* was 560 mg/kg according to Al-Sultan *et al* (2019).

Experimental design

Twenty four adult male mice weighing about 25 gram were divided into three groups, each group comprised of eight mice. Two sub-lethal doses were prepared from the toxic algal extract based on LD_{50} values, they are: 40 mg/kg (i.e. 1/14 LD_{50}) and 80 mg/kg (1/7 LD_{50}) and the mice groups were injected with 0.1 ml of distilled water (control group), 40 mg/kg (low dose) and 80 mg/kg (high dose) for fifteen days. At the end of injection period, mice were anesthetized by chloroform and sacrificed for blood collection by heart puncture. Blood samples were left to clot and then centrifuged at 3500 r.p.m for 10 minutes at room temperature to separate the serum and finally the serum is stored at -20°C until assayed (Sood, 1995).

Hormonal aspect

Three hormones were determined they are: follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone using enzyme-linked immunosorbent assay (ELISA) kit manufactured by Monobind company at 450 nm according to instructions of kits.

Reproductive ability

Male mice groups were intraperitoneally injected for a period of fifteen days. Then, the three groups of male mice were transferred to individual cages and mated with fertile females. Male and female mice were mated for eight days then they were separated. Pregnant mice were allowed to complete their pregnancy. After birth the following reproductive parameters were analyzed: 1) number of pregnant females and fertility percentage. 2) number of newborns. 3) newborns weight. 4) mortality percentage and 5) congenital malformation.

Spermatogenesis

Histological section were made according to the procedure of Humason (1972). Testes of three groups of treatment were removed and fixed in bouin's fluid for twenty four hours. Then they were washed with 50% ethanol for six times and stored in 70% ethanol. Preparation of tissue sections includes dehydration, clearing, infiltration and embedding in wax.

Statistical analysis

Results are expressed as mean ± standard deviation. The difference between the means was determined by one-way analysis of variance (ANOVA) using SPSS for Windows, Version 21.

RESULTS AND DISCUSSION

Effect of *Lyngbya aestuarri* extract on hormonal aspects

The present results of *Lyngbya aestuarri* crude extract on reproductive hormones are shown in Table 1. The endocrine hypothalamus, the pituitary and the testes are responsible for secretion of male reproductive hormones. The testes require stimulation by the pituitary gonadotropins, *i.e.*, LH and FSH, which are secreted in response to hypothalamic gonadotropin releasing hormone (GnRH) (Manjithaya and Dighe, 2006). The effect of LH and FSH on germ cell development is mediated by the testosterone and FSH receptors that are present on leydig and sertoli cells, respectively. FSH acts directly on the germinal epithelium. Whereas LH stimulates the secretion of testosterone by leydig cells (Wang *et al*, 2012). So, we infer that may be the alternation of testosterone concentrations was caused by the alternation of LH concentrations. Low testosterone and low pituitary LH and FSH hormones in serum confirm the hypogonadotropic hypogonadism disorder (HH) as a consequence of hypothalamus and/or pituitary gland dysfunction. In the hypogonadotropic hypogonadism (HH), secretion of GnRH is absent or scarce. Also, lack of/or scarce production of pituitary gonadotropins may also result in HH. HH can be caused by some diseases such as hemosiderosis (Felitti and Baer, 1999; Fraietta *et al*, 2013).

Table 1 : Effect of *L. aestuarii* on reproductive hormones for male mice (n = 8) after 15 days of i.p. injection. The parameters results are expressed as mean ± SD.

Parameters / Treatments	Testosterone (ng/ml)	LH (ng/ml)	FSH (ng/ml)
Control	a3.33±0.75	a18.20±14.93	a9.02±0.04
Low dose (40 mg/kg)	b2.34±0.62	ab10.36±6.63	ab9.06±0.08
High dose (80 mg/kg)	b1.94±0.28	b3.02±1.00	b8.97±0.03
R.L.S.D.	0.59	11.65	0.06

a,b significant difference ($p \leq 0.05$) among treatment groups.

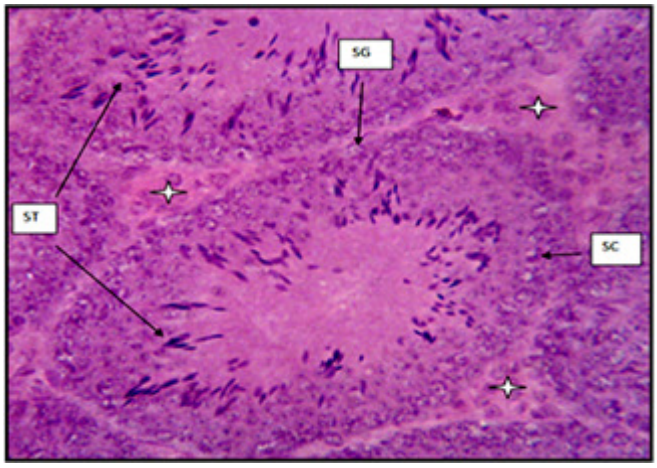

Fig. 1 : Transverse section of seminiferous tubules for control mice showing various stages of spermatogenesis. Spermatogonia (SG), primary spermatocytes (PS) and spermatides (ST) (black arrows) and leydig cells (stars) (H and E staining, 400x magnification).

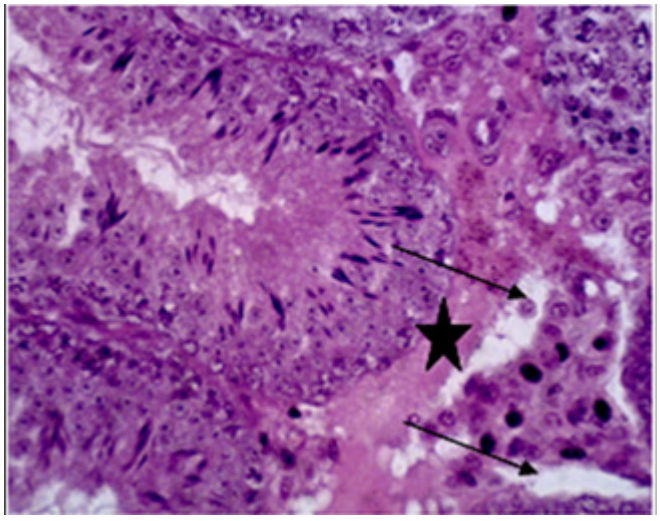

Fig. 2 : Transverse section of seminiferous tubules for low dose group (40 mg/kg) showing necrotic area in interstitial tissue between seminiferous tubules (black arrows) and accumulation of pinkish-stained liquids (oedema) between seminiferous tubules (black star) (H and E staining, 400x).

Effect of *L. aestuarii* extract on reproductive capacity of male mice

Following delivery, the newborn pups were separated from female, counted and weighted in the first day then they were returned to the breeder cage with their mothers. Also, pups were monitored for registered their mortalities. The present results as shown in table 2 below showed 100% of fertility for control treatment, 37.50% of fertility for high dose treatment and 100% of infertility in male treated with low dose of algal extract. The number of newborn pups was significantly decreased in high dose treatment comparable with control one. Also, there are high percentages of mortality in newborn pups for high dose treatment. There are several reasons for infertility.

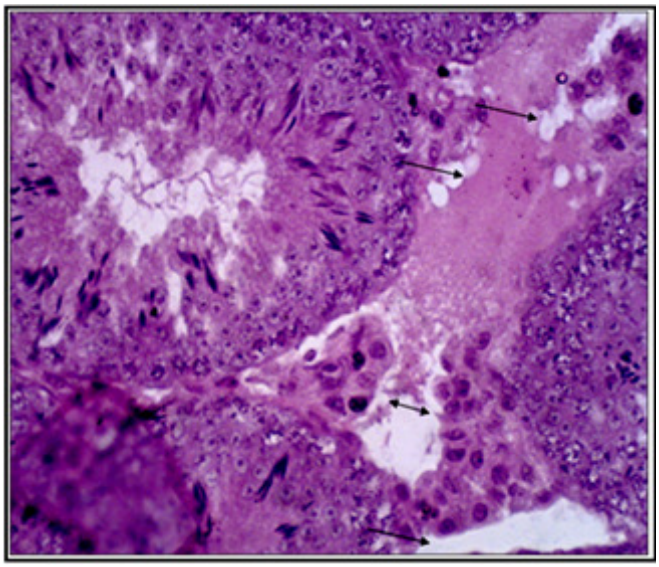

Fig. 3 : Transverse section of seminiferous tubules for low dose group (40 mg/kg) showing necrotic area in interstitial tissue between seminiferous tubules (long arrows) and necrosis in leydig cells (bi-headed arrow) (H and E staining, 400x).

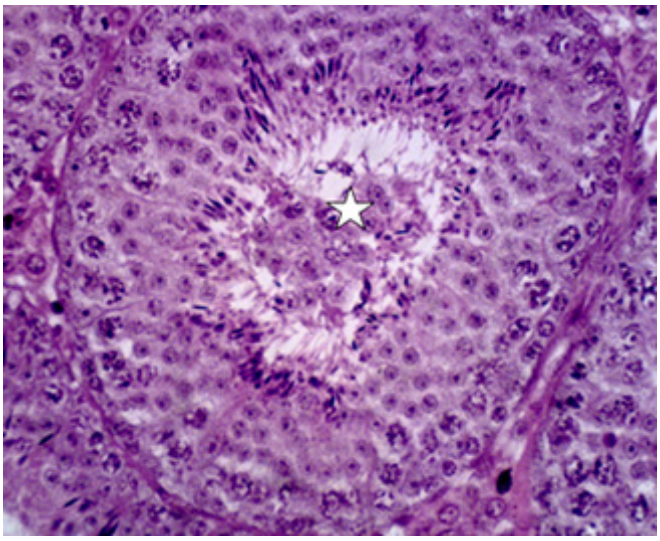

Fig. 4 : Transverse section of seminiferous tubules for low dose group (40 mg/kg) showing detachment of germinal mass into tubular lumen (star) (H and E staining, 400x).

The most common reason of male infertility is spermatogenesis defects. Spermatogenesis is the long process of differentiation begins from diploid spermatogonia to haploid spermatozoa. The complexity of this process implies that mutations in many several genes can interfere with spermatogenesis and thereby result in infertility (Oberheide *et al*, 2017). Also, oxidative stress in testes and epididymis are considered as a potential contributor to male infertility (Gautam *et al*, 2006).

The hypoxia caused by algal extract stimulated production of ROS in plasma membrane of sperms because their plasma membranes contain considerable

Table 2 : Reproductive capacity for male mice groups (n = 8) after eight days of mating after treated with methanolic extract of *L. aestuarii* for 15 days (mean ± SD).

Parameters / Treatments	Pregnant females	Fertility (%)	Number of pups	Deformities (%)	Mortalities (%)	Weight of pups (g)
Control	8	100	a8.37±1.06	0	0	a1.48±0.19
Low dose (40 mg/kg)	0	0	c0	0	0	c0
High dose (80 mg/kg)	3	37.50	b3.66±2.62	0	40.17	b0.89±0.64
R.L.S.D.	-	-	1.99	-	-	0.50

a,b,c significant difference ($p \leq 0.05$) among treatment groups.

quantities of unsaturated fatty acids and their cytoplasm contains low concentrations of scavenging enzymes. Oxidative stress attacks not only plasma membrane of sperms but also the DNA in the nuclei and mitochondria (Hallak *et al*, 2001). If DNA damage occurs during spermatogenesis before the final nuclear compaction, the damage cannot be repaired in the mature sperm because the chromatin increases six-folds in condensation (Swanson *et al*, 2012). Samplaski *et al* (2010) have shown that the sperms of infertile men possess more DNA damage than their fertile ones. DNA damage in the male germ line has been associated with bad semen quality, injured pre-implantation development, raised abortion and raised illnesses in progeny. In addition to hypoxia, generation of ROS may be as a result to accumulation of hemosiderin in testes as emphases by tissue sections (Fig. 7), which may lead to testicular dysfunction. The seminal plasma contains antioxidant enzymes that arecapable of overcoming these ROS as well as protecting the sperms against any possible damage. But excessive ROS production leads to surpass the antioxidant capacity within the semen and the fatty acids of sperms plasma membrane undergo lipid peroxidation by ROS and then multiple DNA defects can occur (Vargas *et al*, 2011; Kohgo *et al*, 2008; Adewoyin *et al*, 2017).

The reproductive system is a potential target for endocrine disruption. Several scientists investigated the correlation between exposure to cyanotoxins and disruption of reproductive hormones (Ding *et al*, 2006; Damkova *et al*, 2009; Coster *et al*, 2012). The present study showed that reproduction failure was in low dose group. Because some EDCs haveunique U-shaped dose–response curves whose maximum detrimental effects are either at low doses or at high doses only but not at median ones (Clotfelter *et al*, 2004). A number of other factors can trigger infertility such as destruction of leydig cells in testes as shown in transverse section of testes in low dose treatment, where there are necrosis in leydig cells (Figs. 2 and 3) led to decreased testosterone concentration in serum. Necrosis in leydig cells may result from oxidative stress mediated by toxic algal extract (Gautam *et al*, 2006; Kataa *et al*, 2017). Also, the complete infertility in low dose treatment may due to failure in maturation of sperms in epididymis and their capacitationin female tract. Capacitation of sperms enables them both to undergo the acrosomal reaction and to develop a vigorous flagella movement termed hyperactivation. Defects in both acrosomal reaction and hyperactivation might be responsible for affect the sperm ability to penetrate the zona pellucida (ZP) for fertilization oocyte. The decline in fertility of high dose mice may be as a consequence to accumulation of hemosiderosis in interstitial tissue of testes in addition to decline in testosterone levels. In the present study, the hemosiderosis in testes as emphases by tissue sections of high dose treatment (Fig. 7) may generate ROS, which led to reduced fertility. Also, decreased testosterone concentrations as emphases by hormonal analyses (Table 1) caused reduction in libido of male mice which led to reduced percentage of fertility in the present study (Felitti and Baer, 1999; Hotchkiss *et al*, 2008).

Effect of *Lyngbya aestuarri* crude extract on spermatogenesis

Transverse sections of testes of control group animals showed normal architecture (Fig. 1). While, the transverse sections of testesfor both low and high dose groups (Figs. 2-4) showed several defects in spermatogenesis. Defects in low dose group comprised dysplasia in seminiferous tubules, disappearance of testicular lumen, detachment of germinal mass into tubular lumen, necrotic in interstitial tissues and leydig cells while defects in high dose group (Figs. 5-7) comprised dysplasia in seminiferous tubules, degeneration of the basement membrane of some seminiferous tubules and accumulation of hemosiderin among seminiferous tubules. These histological defects suggest that toxic algal extract were either capable of permeating the blood-testis barrier or it induced these defects via its impacting on hypothalamus-pituitary-testes axis. Thus, necrosis in leydig cells may be resulted from oxidative stress mediated by toxic algal extract (Gautam

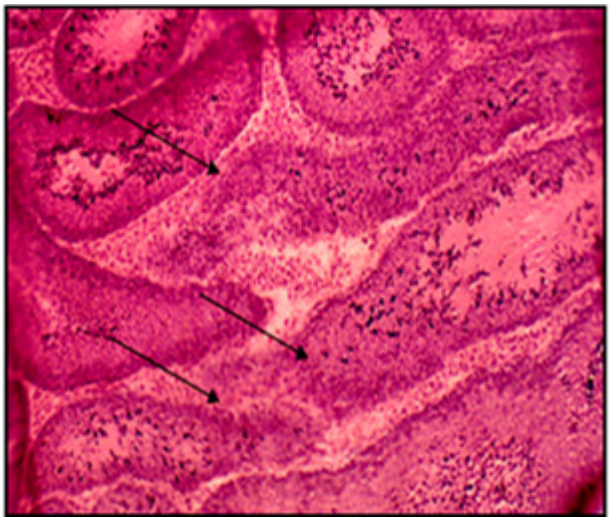

Fig. 5 : Transverse cross section of seminiferous tubules for high dose group showing severe degeneration of some seminiferous tubules (black arrows) (H and E staining, 100x).

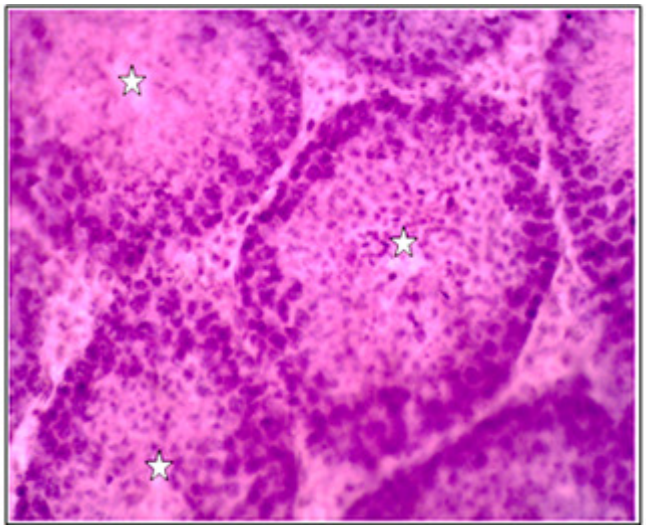

Fig. 6 : Transverse cross section of seminiferous tubules for high dose group showing dysplasia in seminiferous tubules, overlap in stages of spermatogenesis and disappearance of tubular lumen (stars) (H and E staining, 400x magnification).

et al, 2006; Kata *et al*, 2017). Oxidative damage can also affect spermatogenesis through the decline in serum testosterone and viability of sperms (El-Seweidy *et al*, 2010). While, degeneration of some seminiferous tubules for high dose treatment resulted from hypogonadism of pituitary and/or hypothalamus which causes atrophy of the seminiferous epithelium and degeneration in germ cells (Gosden and Spears, 1997). The present research give an obvious picture on impact of sub-lethal doses of toxic cyanobacterial extract on fertility rate in rodents. So, we concluded that cyanobacterial bloom in Basra rivers is a real environmental problem, which has a detrimental effects on fertility of citizens in the future.

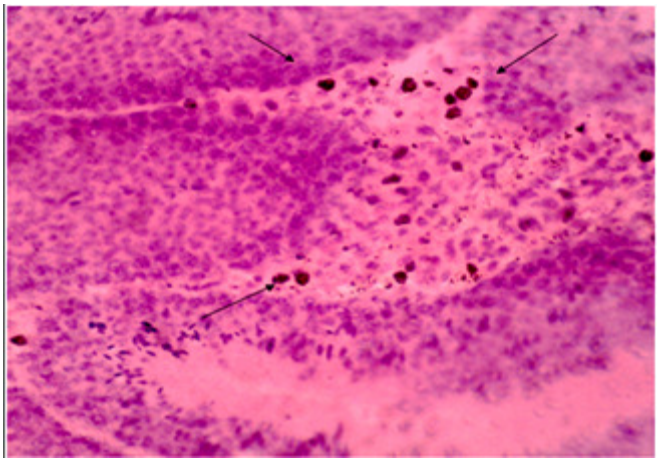

Fig. 7 : Transverse section of seminiferous tubules for high dose group (80 mg/kg) showing accumulation of brown particles in the interstitial tissue and in leydig cell between seminiferous tubules (black arrows) (H and E staining, 400x).

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