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Effect of Toxic Compounds Extracted from Microalgae Oscillatoria limosa (Roth) Agardh on the Fertility of White Male Mice Mus musculus L.

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Abstract
The effect of toxic compounds extracted from cyanobacteria *Oscillatoria limosa* isolated from Abu-Alkaseeb rivers in the southern Iraq was studied, water samples were collected from rivers, and cultured in chu-10 medium .Supernatant of toxic extract from biomass was extracted and test its effects on the fertility of male mice .The present study reveal that di(2-ethyl hexyl) phthalate and phytol compounds effected on the fertility of male mice based on the sperm count ,sperm abnormalities and testosterone hormone level. The current study concluded that the toxic compounds extracted of *Oscillatoria limosa* had a positive effect on fertility of *Mus musculus* these effects represented in increasing in abnormal sperms, decreasing of sperm count and testosterone hormone levels compared with control group.
Keywards: *oscillatoria limosa*, sperm count, testosterone hormone**.**

1.Introduction

Cyanobacteria are found in a variety of habitats, live in terrestrial, fresh, brackish, or marine water. They are usually too small to be seen individually but sometimes can form visible colonies (mankiewics et al. ,2003; Briand et al.,2003; Mazur *et al*.,2003) Cyanotoxins are toxic secondary metabolites produced by about 40 species of cyanobacteria (Westrick *et al*.,2010) It appears likely that cyanotoxins are produced and contained within the actively growing cyanobacterial cells. Release to the surrounding water during cell senescence death and lyses, rather than by continuous excretion (Lehtimaki *et al*.,1997)

Toxic blooms of cyanobacteria have been observed in different places of the world (Sivonen & Jones, 1999), and it was not effect only in water quality, but also produce highly toxic, Poisoning and grievous chronic effects in humans (Zanchett, & Oliveira-Filho,2013) the existence of these toxic microorganisms in water bodies used either for irrigating or drinking or for recreational activites may present serious health risks for the human inhabitants, several species are capable to produce Potent toxins that occur acute fatality in animals (Carmichael & Falconer, 1993), and disease in humans (Kuiper-Goodman *et al*., 1999). People could beexposre to the cyanotoxin effects by drinking the contaminated water or by in halation the vapors, as well as, by using the contaminated water in haemodialysis(Shoemaker,2007;Koreivienė *et al*, 2014).

The toxins of cyanobacteria were divided into five groups dependence on the function : neurotoxins, hepatotoxins, dermatotoxins, cytotoxins and endotoxins(Wiegand & plugmacher,2005). Among the most important cyanobacterial genera producing one or more of these toxins: *Oscillatoria*, *Lyngbya* ,*Microcystis*,*Nostoc* ,*Anabaena* ,*Nodularia* ,*Aphanizomenon Cylinderospermopsis* and *Raphidiopsis* (Sivonen & Jones,1999 ; Fastner *et al*.,2001).Genus of *Oscillatoria* is characterized by its ability to produce hepatic , dermato and neurological toxins which are toxic to the human and animals, (Carmichael,1997; Codd *et al*., 2005).Hepatotoxins and neurotoxins were isolated from in five species of *Oscillatoria* (*O.agardhii, O.brevis, O.limnetica,O. rubescens*, and *O.tenuis*) in Makka area (Mutawie,2012). Anatoxins can block the transmission of signals from neuron to neuron and from neuron to muscle, whereas microcystins cause bleeding in the liver (Madigan *et al*., 2000). Anatoxin-a, apotent neurotoxin, is one of several toxins produced by cyanobacteria, and its toxicity was evaluated on testes and sperm count of male mice (Yavasoglu *et al*.,2008).

A few studies have indicated that microcystin(MC-LR) are accumulative in the gonads of invertebrates and thus, gonads are considered as second target organ of microcystin after the liver (Chen & Xie,2005). The effects of chronic low-dose exposure to microcystins were preliminarily studied on sperm quality and testicular function in male mice, causing structural damage to the testis and Leydig cells exhibited apoptosis, therfor declines in sperm quality, the rate of sperm abnormality was higher, levels of LH and FSH increased and decreased levels of serum testosterone (Chen *et al*.,2011) . MC-LR affects hormones level of male mice by damaging hypothalamic–pituitary system(Wang *et al*.,2012).

The aim of the present study is isolation and identification of toxic compounds and determine the LD50 of algal toxic extract of *Oscillatoria limosa* (Roth) Agardh and studing the physiological effects of toxic compound on fertility in male mice.

2. Materials and methods

2.1 *Isolation of micro algae***:** *Oscillatoria limosa* was isolated from Abu- Alkaseeb Rivers in city southern Iraq

from Sptemper to April 2015 .Primary culturing was done chu-10 medium. After incubation, pure culture of the living specimens were prepared by sub culturing with agar plate method in chu-10 medium (Stein,1975).Preserved specimens were prepared and the living specimens were inoculated in 100 ml- conical flasks. constant illumination was used at 60 ME m^{-2} sec^{-1} intensity using white fluorescent lamps.Incubated at 25±2ºC. Algal culture is identified based on their morphological characteristic following the taxonomy schemes of Prescott (1975) and Sant Anna et al.(2004)

2.2 *Preparation of extract*
Axenic culture for *O. limosa* reaped by centrifuge at 3000 rpm, the reaped algal cells were lyophilized by using freezing drier, preparation of toxic extract was made according to Yin *et al*.(1997) by weighting 1g of lyophilized mass alga then extracted for (12 to 24) hour by using the magnetic stirrer in acidified PH(3.5) of 80% ethanol extraction at 4ºc . The extract was dried by freezing drier and Filtered by a buchner funnel.

2.3 *Identification of the toxic compounds by GC-mass.*
Gas chromatography mass (GC-mass) (gilent Technologies GC- mass 7-890 A GC system) method was applied for the identification and determination of the molecular weights and chemical structure of the isolated toxic compound.

2.4 *Determination of Lethal Dose (LD_{50})*
Males of the *Mus musculus* Balb/C albino strain were injected in intrapretonal (I.P) by syringe 1ml volume to determine the LD_{50} of toxic extract from *O.limosa* for 72 hours,. Injection started with low dose then continued to high dosages according to the equation dependence on Litchfield and Wilcoxon,(1949).

$$\mathbf{LD_{50}} = \text{highest dosage} - \frac{\sum ab}{n}$$

Where LD_{50} the lethal dose 50, highest dosage the dose with 100% mortality of mice, is the value of difference between the previous and next dose, is the summation of dead animal for each dos (previous dose + next dose \ 2) ,$\sum$**ab**: summation of multiplied **a** with **b**, **n**: is the number of animals used for each dose.

2.5 *Preparation of Animals*
Male mice *Mus musculus* L.strain Balb /C were used for the present study. the animal house of Biology Department/ College of Education/ Basrah University. Animals were maintained at a temperature of (23±2cº) with controlled light-dark cycle through the period of experiment .The food and water given orally ,were fed with standard diet (Bell,1962)

2.6 *Experimental Design*
Adult male mice(8-10)weeks of age ranging (23-27)g in weight body were used in the present study . The male mice divided into three different treatment groups (n =8): a control group injected I.P with (0.1 ml of distal water), second group injected I.P. with low dose 0.1 ml of algal extract solution (50 mg/ kg) and third group injected I.P. with high dose 0.1 ml of algal extract solution (100 mg/ kg). The mice treated with algal extract of 15 doses daily , one dose of each 24 hr.

2.7 *Method of sperms count*
After the last injection on the 16^{th} day Male mice were torpid by using chloroform. and the right epididymis were uprooted and mashed in Petri dish by 1ml (0.9%) physiological saline. Eosin 1% was mixed with suspension aqueous Y (10:1) . and kept for half-hour for the staining of sperms. Then an suspension withe Eosin was taken in white blood cell pipette up to the 0.5 degree and dilutive further up to degree 11 with physiological saline . The mixture was shaken and Embattle into chamber in hemocytometer and sperm count was performed as per standard procedure. The sperm count in egiht squares of $1mm^2$ each area except the central erythrocyte counting area of chamber was performed and multiplied by 50000 factor to calculate the total number of sperms(vega *et al*.,1988)

2.8 *Determination the Percentage of normal and abnormal sperms.*
Determine Percentage of normal and abnormal sperms male mice was made according to Wyrobek, & Bruce.(1975) .

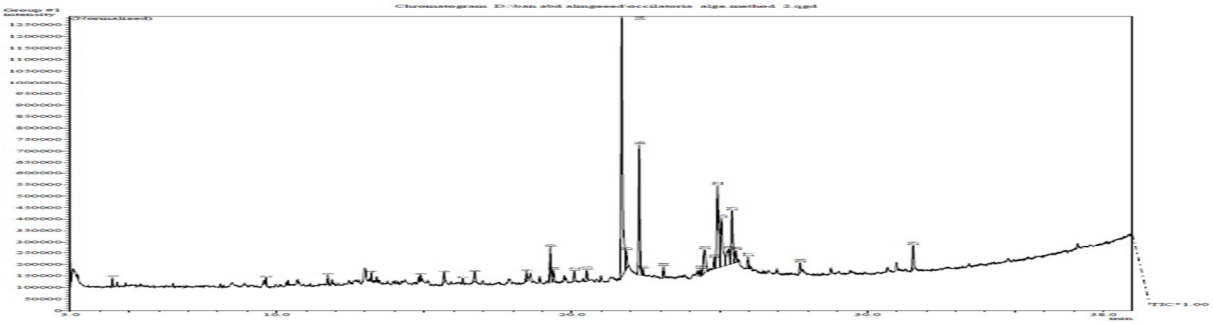
2.9. *Histological testes*
Preparation of histological sections of male mice testes from each groups were taken for both matrices according to Humason.(1972) using the fixed in Bouin's solution, dehydrated in ethanol, and embedded in paraffin wax. The sections of Tissue (6-7 µm-thick) were cut on a microtome Machine , mounted the section on glass slats. and staining with eosin and hematoxylin.

2.10 *Assays of testosterone hormone*.
Serum concentrations of testosterone hormone were measured by enzyme-linked immune sorbent assay (ELISA) as described in the instructions provided by manufacturer's kits (Biolabo, French).

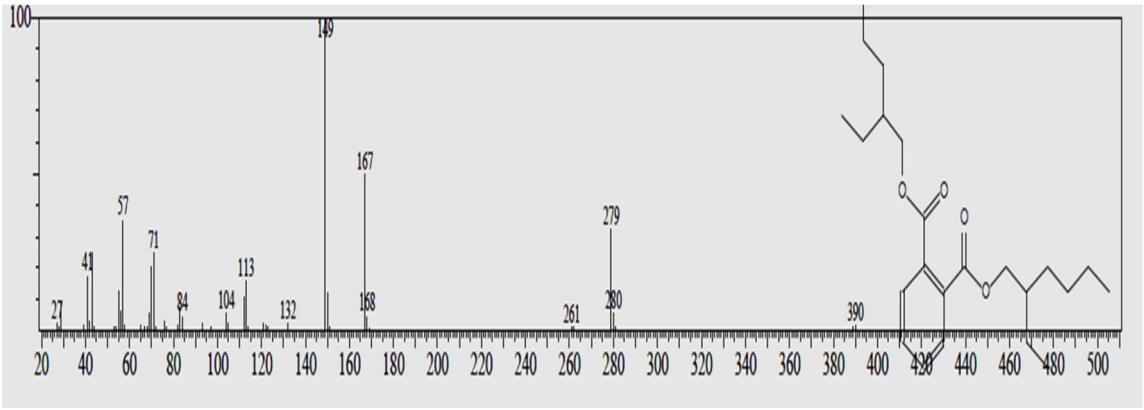
2.11 *Statistical analysis.*
The statistical analysis was conducted in SPSS version- 19. One-way ANOVA were done to evaluated the significant difference between treatments under propability $p \leq 0.05$ and calculate the revised least significant difference(R.L.S.D).

3. Results & Discussion

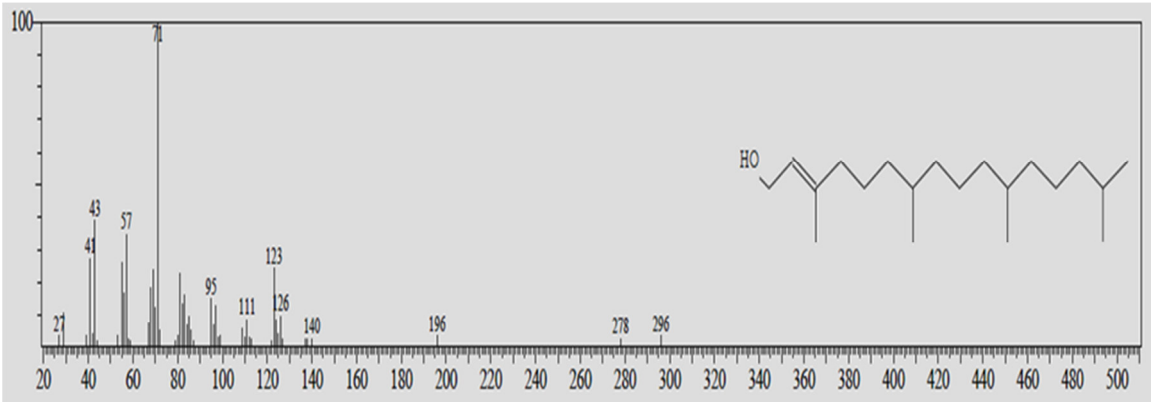
3.1 *Toxic extract*
The present study, (29) peaks were detected by the GC- mass as results of algal extract componens analysis (Fig.1), one of the compound is Di(2-ethylhexyl) phthalate with Retintial time 31.55 min and molecular weight (390) Killo Dalton(K.D).(Fig.2) . And the other compound was phytol, with R.T. 24.48 min and molecular weight of (296) K.D.(Fig.3)



(Fig.1) Chromatogram of GC-mass analysis



(Fig.2) mass spectrum of di(2-ethylhexyl) phthalate with chemical structure



(Fig.3) mass spectrum of phytol with chemical structure

3.2 *Values of lethal dose (LD_{50}) of toxic extract*

The results of LD_{50} of the toxic extract of *O.limosa* records from the following values (Table 1).

Table(1) Determination of LD50 for toxic extract of *O.limosa* in male of mice

Dose Mg/kg	Number of animals	Number of dead animals	a:Differences between doses	b:numberof dead animal (previous+next\2)	a×b
10 mg	6	0	0	0	0
15 mg	6	2	5	1	5
20 mg	6	3	5	2.5	12.5
25 mg	6	4	5	3.5	17.5
30 mg	6	6	5	5	25
					∑Ab=60

$LD_{50} = 30 - \frac{60}{6}$

$= 20$ mg

$= 20 \times 40 = 800$ mg/Kg

The value of LD_{50} equal 20mg was multiplied with 40 and divided on 8 to be high dose 100 mg/kg and low dose equal 50 mg/kg of the body weight.

3.3. *Effect of algal extract on sperm count and sperm abnormalities* .

The effect of toxic extract on sperm count and sperm abnormalities are illustrated in (Tab.2). The results showed a significant difference ($p \leq 0.05$) decrease in sperm count of male mice treated with two doses (50 and 100) mg/kg compared with the control group . As well as , the a significant differences ($p \leq 0.05$) increase in abnormal sperms of the male mice treated with high and low dose compared with the control group. The abnormalities included the changes of tail and head shape (Fig.4).

3.4 *Effect of algal extract on spermatogenesis.*

The injection of mice with high and low doses for toxic extract showed a significant alteration in histological sections of testis represented by a dissolution of spermatogenic cells in the seminiferous tubules and destruction in the interstitial tissue. (Fig. 5)

Table 2. Effect of algal extract on the abnormalities and count of the mice sperms
(N=8) (Mean ± standard error)

Treatments	Sperm count ($mm^3 \times 106$)	Normal sperm (%)	Abnormal sperms	
			Abnormal head (%)	Abnormal tail (%)
Control group Distal water	^{a} 21.28 ± 4.66	^{a} 89.28 ± 6.23	^{b} 1.87 ± 0.73	^{b} 8.84 ± 1.38
algal extract 50 mg/kg	^{b} 13.15 ± 3.24	^{b} 19.16 ± 3.06	^{a} 11.06 ± 3.10	^{a} 69.70 ± 4.66
algal extract 100 mg/kg	^{b} 9.35 ± 1.48	^{b} 27.19 ± 4.44	^{a} 12.57 ± 2.45	^{a} 60.22 ± 5.21
R.L.S.D	3.136	7.255	2.101	3.726

(a,b) There is a significant difference($p \leq 0.05$) compared with the control group .

3.5. *Effect of algal extract on testosterone hormone.*

Treated of male mice with 50 and 100 mg/kg of toxic extract of *O. limosa* caused a significant differences($P \leq 0.05$) decrease in testosterone hormone levels compared with control group shown in (Tab. 3).

Table 3. Effect of algal extract on testosterone hormones of male mice (N=8) (Mean ± stander error)

Treatments	Testosterone Hormone ng/ml
Control group Distal water	[a] 4.001 ± 0.701
algal extract 50 mg/kg	[b] 1.538 ± 0.714
algal extract 100 mg/kg	[b] 0.292 ± 0.145
R.L.S.D	0.527

(a,b) There is a significant difference($p \leq 0.05$) compared with the control group .

Over the last ten years, numerous global lethal animal poisonings and a number of situations of human illness caused by toxic cyanobacteria blooms, and the level of risk to the human health depends on the cyanotoxin levels and exposure pathways have drawn the heedfulness of WHO, the scientific community and the public(WHO,1998; Dziga *et al*., 2007). In the present study, there was a reduction of sperm count and testosterone level as well as increasing sperm abnormalities in the both dose compared with control group.

In general the reproductive system is particularly susceptible to the endocrine-disrupting activity of phthalates, and the compound of (DEHP) di(2- ethylhexyl)phthalate had an effect on reproductivesystem represented in reducation of fertility (Agarwal *et al*., 1989).Some phthalates and their metabolic products are Known to cause reproductive and developmental toxicity in laboratory animals some of the reproductive effects observed in animals are: alteration hormone levels ,reduction survival of offspring and fetal defects. In developing males , some compound of phthalates cause sertoli and leydig cell damage , prostate injury, testicular atrophy,and reduced sperm production and motility (Digangi *et al*.,2002; Kavlock *et al*., 2002). The exposure of male laboratory mice to (DEHP) led to atrophy of testis and effect in spermatogenesis as well as decreasing in sperm count , this is due to the role (DEHP) in generation of reactive oxygen species(ROS) that cusses oxidative stress to the germ cell and apoptosis to the spermatocytes, with concomitant decrease in the concentration of glutathione and ascorbic acid in the testis(Kasahara et al.,2002). Dietary exposure of adult male to (DEHP) resulted in testis reduction and epididymis weights, degenerative changes in testis, decreasing testicular zinc , reduction sperm density and motility increasing occurrence of abnormal sperm, and reduction testosterone with increasing luteinizing hormone and follicle stimulating hormone in serum(Agarwal *et al*.,1986). The physiological role of zinc is sperms maturation and fending of germinal epithelium, therefore decreasing of zinc causing depression of spermatogenesis (Underwood,1971). Also, Foster *et al* .(1982) Fount thateexposure of (DEHP) caused the degradation of seminiferous germinal epithelium a spermicidal or a spermicidal . The activation of apoptosis in testes by DEHP could be due to its induction of mitochondrial damage and increasing ROS production, and this that leads to decreasing testicular ATP levels which causes problem in male fertility because of spermatozoa motility depends on sperm ATP (Perchec et al.,1995; Huang *et al*., 2013).

The other compound is phytol, a saturated fatty acid with a branch chain . The toxic activity of thiscompound was represented by histopathological effects . phytol enters the mice body, it turns into phytanic acid and pristanic acid which are oxidized in mitochondria by special enzymes such as thiolase. So the oxidation of these fattyacid resulted in production of hydrogen peroxide H_2O_2 and reactive oxygen species ROS (Seedorf *et al*., 1998 ; Komen *et al.*, 2007; Mackie *et al*., 2009).

The hypothalamic–pituitary–gonadal axis is an instrumental pathway for endocrine regulation and proper function, of reproduction Hypothalamic gonadotrophin releasing hormone (GnRH) stimulates from the interior of pituitary gland to releasing of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) also (GnRH) plays a key role in the neuro-hormonal control of reproduction(Wang *et al*.,2012).Effect of MC-LR represented modulating the hypothalamic–pituitary–gonadal axis by disrupting of spermatogenesis and causing changes in serum testosterone hormone level(Li *et al*., 2008; Schipani & Kronenberg, 2009).

The secretion of FSH and LH is reguiated by testosterone in a negative feedback method. Li *et al*. (2008) observed that implication exposure to the lower concentration of MCLR in rats caused increasing LH and FSH levels where as the high concentration of MC-LR Leading to impaiar FSH and LH production and destroy the negative feedback or otherwise impair FSH and LH.

Exposure of Leydig cells to MC-LR in culture led to plasma membrane and DNA damage because of excessive production of ROS that considered as a causal factor in lipid peroxidation. MC-LR cytotoxicity to Leydig cells resulted from oxidative stress of ROS on the that promoted the cell to apoptosis (Guzman& Solter, 1999; Botha *et al*., 2004; Fu *et al*., 2005).

Treatment male mice with algal toxin leading to decrease in sperm count and decline epididymis weight because of seminiferous tubules damage (Yavasoglu *et al*., 2008). Also, treatment male mice by algal toxins treatment of mice with MC-LR causes slight testicular atrophy and occlusion in seminiferous tubules, slight deformation of spermatogenic cells, expansion of the cavity of the seminiferous tubules, thinning of the spermatogenic epithelium degeneration of Leydig cells, and reduction numbers of interstitial cells, Sertoli cells and mature sperm. All these changes cause reduction in sperm count and testosterone level in male mice(Chen *et al*.,2011;Wang *et al*., 2013).maight be due to the passage of MC-LR through the testicular blood barrier to the testis(Li et al., 2008).

MC-LR was observed to mediate its toxic effect on testis mainly by oxidative stress and induction DNA damage and also via affecting the motility and morphology of sperm (Lone et al., 2015). Abnormalities indicate that occure on sperms had been arise in spermatogonial cells. They might be caused damage to the pre-meiotic stages of spermatogenesis during DNA synthesis. Therefore, any abnormalities observed in the sperm heads presumably occurred in spermatogenesis during shape develop of the sperm head (Monesi,1962; Beatty,1970).

As well as any distortion in sperms is due to the exposure of nuclear material DNA to toxic substances that causing mutation during the spermatogenesis and sperm abnormalities such as detached tail because of abnormalitiy chromosomes(chemes *et al*.,1999; Bakare *et al*., 2005)

One of the forms of sperm abnormalities in the is the loss of acrosome from the sperm head ,as a result of exposure of male mice to mutagenic sub stances causing the loss of the responsible gene for the formation of the acrosome, and the transformation of the hade shape into aspherical causing loss its ability to fertilize the egg and disrupt fertilization process(Kang-Decker *et al*., 2001).

The decreasing in testosterone levels found in present study might be responsible for the reduction sperm count and spermatogenesis in mice injected with toxic extract of *O.limosa* depression . In addition, the results indicate that hormonal perturbation caused by algal toxins are mediated by its effects on the hypothalamic-pituitary-gonadal axis, such as microcystin lead to decrease of testosterone level (Ciccone& Kaiser,2009).Due to passage of toxins through Blood-brain barrier Causing functional and structural changes of the hypothalamus cells and affecting the secretion of hormones and decrease in the level of FSH and LH from pituitary gland and cause a decrease in the level of testosterone (Feurstein *et al*.,2009) .As well as effect of DEHP in impairing testosterone biosynthesis in leydig cell is due to changes in steroidogenic enzyme activity, and the creasing of LH secretion and production for pituitary gland has adverse consequences on testosterone levels and male fertility (Akingbemi *et al*., 2001)

4- Conclusion

It was indicated the toxic activity on system male reproductive induced by toxic compounds of algal extract for *O. limosa* after isolation and identification by GC-MS technique then determined the lethal dose of 50 the effective dose to use in experiment, would be augmented by decreased serum testosterone levels as well as an inducing the damage in seminiferous tubules, in addition to the direct Cytotoxic effect on germ cells lead to decrease sperm count and increase of sperm abnormalities during 15 day-treated .

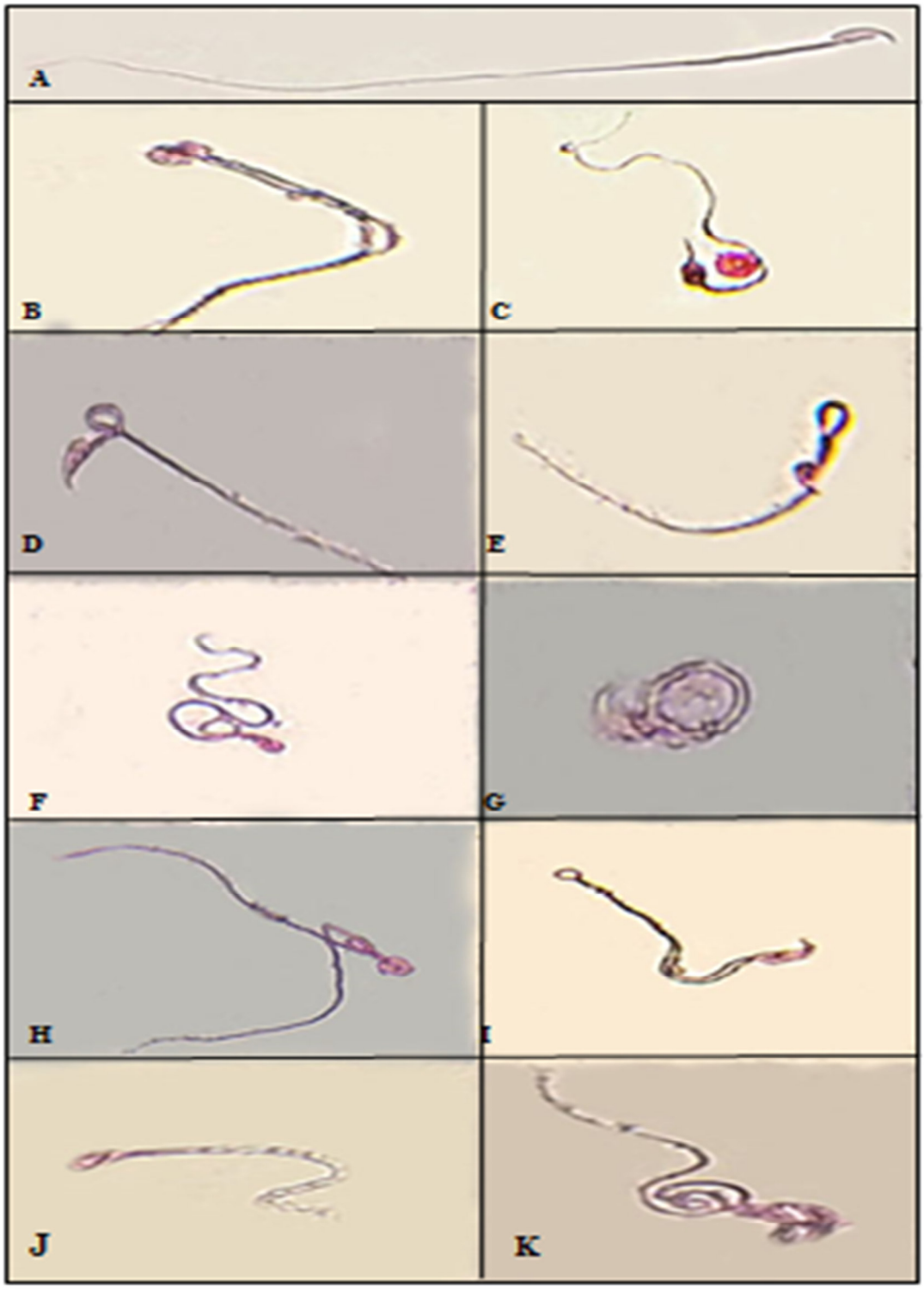


Fig.4. toxic extract induced sperm shape abnormalities in mice. (A) normal sperm with characteristic hook, (B) two-tail &head sperms,(C) irreguiar-head sperms (D) coiled- tailed sperm, (E) folded-tailed sperms, (F) a wand-head sperms, (G) a ring-shaped sperm,(H) a schizoid-tail sperm, (I) a ring tail- sperm (J) a pygmean sperms,(K)a snail shape-tail sperms, . 1% eosin Y; 400×.

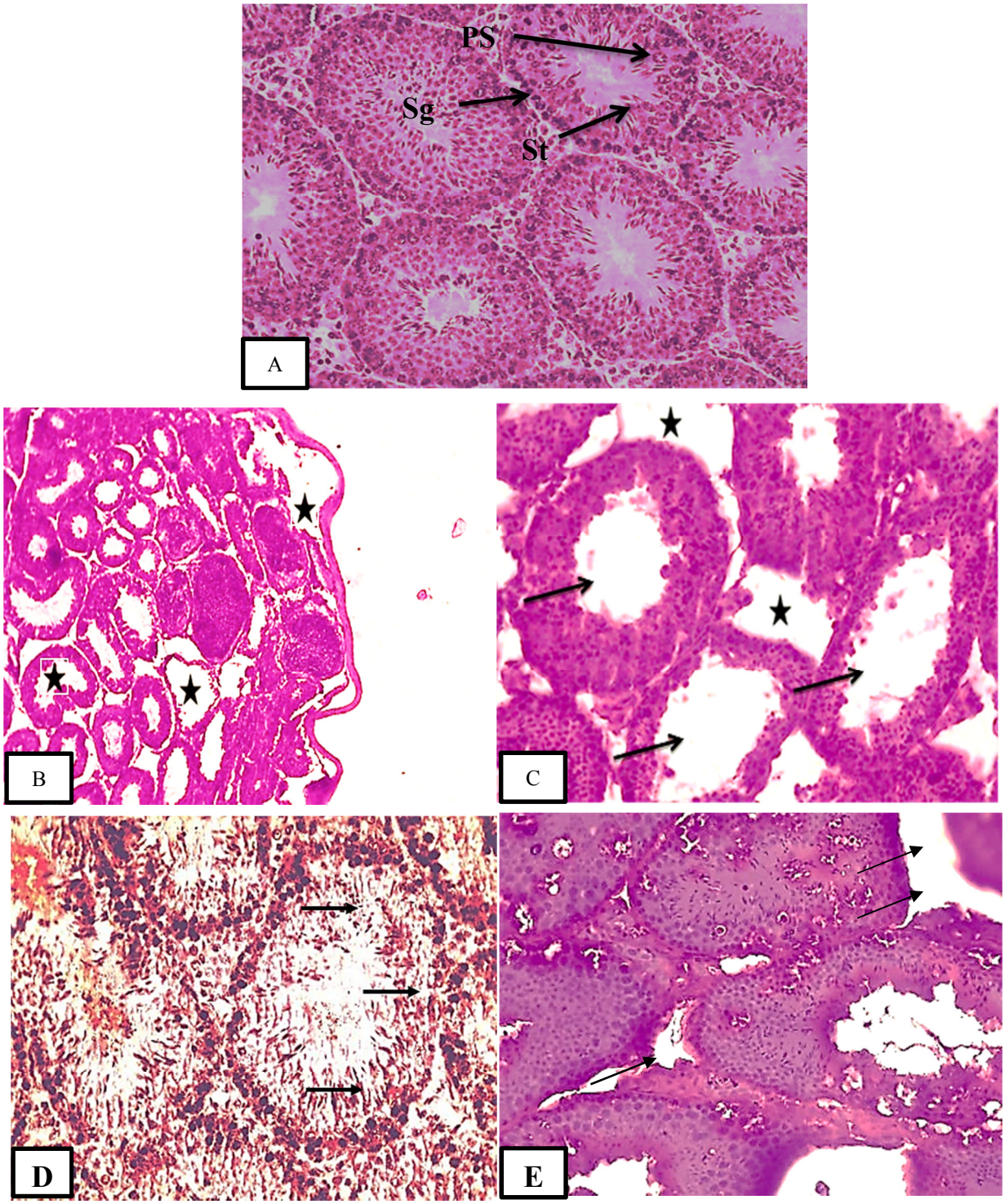


Fig.5. Testicular structure in control and toxic extract treated mice. A) A photomicrograph of testicular section from a control mouse showing normal germinal mass thickness, Spermatogonia (Sg), Primary spermatocyte (Ps), Elongated spermatide(St). B) Testicular section showing damge in seminiferous tubule in low dose (Stars).C) Dissolution of difference testicular stages for the spermatogenesis (arrow) with destruction in the interstitial tissue(stars). D) Testicular section showing loss and shorthand in number of Spermatogonia , Primary spermatocyte and Elongated spermatide in high dose (arrow). E) testicular section showing absent of tissue between seminiferous tubule in high dose (arrow) .

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