In vivo Study of Effects of Citric from *Aspergillus Niger* and Lemon Juice on the Hormonal Level and Histoarchitecture of the Testis

Worood Kamil Shalash and Aiden Mazen Hussain

***Abstract*—**To study the qualitative changes in testis tissue after carbon tetrachloride (CCl4) administration and to determine whether citric acid (CA) has a protective effect against testis damage induced by CCl4. This study compared two types of CA by measuring the histoarchitecture of the testis and serum levels of progesterone, estrogen and testosterone on mice. One of the most produced organic acid is citric acid. In this study, CA produced by microbial fermentation using Aspergillus Niger 5mg/kg and derived from citrus limon 400mg/kg (lemon). Mice were treated with daily intraperitoneal (i.p.) injection for seven successive days after randomly separated into six groups: (1) control, (2) CCl4 (0.02%), (3) limon citric acid (400 mg/kg), (4) CCl4 (0.02%) plus limon CA (400 mg/kg), (5) Aspergillus Niger citric acid (5 mg/kg), and (6) CCl4 (0.02%) plus Aspergillus Niger CA (5mg/kg). Mice were sacrificed, then the testes were histopathological examined under light microscopy, serum levels of progesterone, estrogen and testosterone tested.

Histopathological results indicated that CCl4 severely damage to mouse testis tissues, however, the protective effects on testes was observed when CCl4 combined with CA. Hormone estimation results showed that the treatment of CCl4 exhibited reduction in testosterone level of serum as compared to the control group of mice, in contrast exhibited high in the level of estrogen and progesterone in CCl4 treated group in correlation to that of the standard control group. Sex hormone serum levels were improved by lemon citric acid (CA) and Aspergillus Niger citric acid but did not reach the mean control level.

***Keywords*—** histopathology, lemon citric acid, Aspergillus Niger citric acid, carbon tetrachloride.

I. Introduction

Citric acid is a naturally occurring weak organic acid which known to be a non-toxic and pleasant with a sour taste of 0.2 pH level [1]. The corrosive it is found in all citrus natural products in its unadulterated structure that promptly solvent in water and colorless [2]. Citric acid, 2-hydroxy-propane-1,2,3 and tricarboxylic acid with a formula ($C_6H_8O_7$.H2O), has a molecular weight of 210.14 g/mol with three diverse pKa values, at pH 3.1, 4.7 and 6.4, attributable to the presence of three useful gatherings of carboxylic acids in its structure, it is strong at room temperature. Citrus extract has a dissolving point of 153°C and Boiling point of 310 °C [3].Citric acid can be derived from natural sources (lemon, lime, orange berries, tangerine and grape) as well as synthetic sources (chemical reaction and microbial fermentation). The strategy for separating citrus extract from lemon juice was spearheaded by a Swedish scientific expert, Karl Wilhelm Scheele (1742–1786). The flexibility and non-poisonousness of citrus extract are its primary positive attributes. It is acknowledged worldwide as sheltered (GRAS) and has been supported by the FAO/WHO advisory group on food added substances [4,5]. Citric acid is mostly used as a common ingredient in food products some use the acid as a preservative, acidulate, buffer in food industry, emulsifier, and antioxidant also used in pharmaceutical industry. Therefore, the compound is highly demanded across the globe. [6,7]. Several physical and chemical methods was used to produce of citric acid. In any case, such traditional strategies are discovered to be a mind boggling, costly and not eco-accommodating [8, 9, and 10]. It's produced mainly by solid state fermentation or submerged fermentation. Fermentation process using species of microorganisms species of Aspergillus such as A. wenti, A. foetidus, A. aculeatus, A. awamori, A. fonsecaeus, A. phoenicis and A. carbonaries, as well as Trichoderma viride and Mucor pyriformis, have been found to produce significant amounts of citric acid [11]. Aspergillus Niger a fungus is the world's leading source of commercial citric acid making this process the subject of many studies. Until 1920, all commercial citric acid was produced from lemon and lime juices [12].

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II. Materials and Methods

A. Citric acid

Citric acid produced by microbial fermentation using Aspergillus Niger and derived from natural sources (lemon).

2.1.1. Collection, cultivation and production of Aspergillus Niger citric acid

-Isolation sources: A group of damaged onion.

-Culture media and stains: The medium Potato Dextrose Agar (PDA) was used and prepared according to manufacture instructions (OXOID Company) using 39 g of the medium which dissolved in the distilled water. Sterilized for 15 minutes and pressure 121 pound/inch2. The purification of isolates has done by this culture medium [13].

-The screening medium: The Czapek-Dox Agar prepared by dissolving the components listed below in one liter of the distilled water with starch with 30 g/L of distilled water .The components are: MgSO4 0.5 g, KCl 0.5 g, NaNO3 3 g ,K2HPO4 1.0 g, Fe2 (SO4)3 0.01 g, and agar 13 g. pH of the medium was adjusted to 6 (14).

-Isolation and screening: A number of local isolates that their characteristics in the culture media are similar to fungus of Aspergillus are used in this study; all isolated were collected from damaged onion sieving medium used of the screening process and then methyl red was added with a few drops for each 10 ml of the medium. The medium was inoculated with active isolates of fungi and incubate at 30 ° C for 5 days, and the diameter of growth zone (cm) of citric acid was recorded after 4, 5 and 6 days of incubation. The diameters of growth zone were as initial indication of the ability of the isolates in the citric acid production [15].

-Production of citric acid from Aspergillus Niger:

Citric acid production method by Aspergillus Niger is solid state fermentation was used included moisten 10 g of raw material, which is sunflower wastes, with ratio of 1: 1 and pH=4, then all the suspension sterilized and inoculated with 1 ml of 5 × 106 spore in a 250 ml conical flask . The flasks were incubated at 30° C for 3 days. Citric acid extracted from the fermentation medium with 25 ml of distilled water, and filtered with Whatman No.1, and then filtered solution was centrifuged at 6000 rpm for 20 minutes and then pH estimated by pH-meter and determination of total acidity it estimated according to [16] by adding 10 ml with sodium hydroxide (NaOH 0.1 M) using phenolphthalein reagent. The concentration of acidity was calculated according to the following equation:

The percentage of citric acid (%) = [volume of alkaloid consumed x normality of alkaloid × equivalent weight of citric acid) / (volume of acid x 1000)] x 100

2.1.2 Recovery processes of citric acid from citrus limon

Citric acid was produced from lemon using solutions 1000 ml lemon juice, NaoH solution,(CaCl 28.5g in 100 ml water), H_2So_4(15 ml in 85 ml water).Lemon acidity was measured it was 3 then drops of NaoH added until the solution from acidic to basic and became dark orang color .filter the solution and add cacl2 and heated for 10-15 minutes with boiling then filtered and add H_2So_4 to the precipitate and heated until the volume reached 60 ml and filtered and heated and observed citric crystals [17].

B. Dose of citric acid

In albino male mice, a single dose of (400 mg/kg) of limon extract was used, while it decreased on citric acid production by A. Niger to (5mg/kg).

C. Dose of drug (CCL4)

The daily only dose of CCl4 was (0.02% (in albino male mice.

D. Animal acclimatization

Healthy mice of Albino Swiss male (Mus musculus) were acclimatized to the conditions of laboratory. They procured from Al-Nahrain University, Biotechnology Research Centre. The animals ranged between 8-10 weeks, and weight from 23-27g. They divided into 6 groups, and each group was kept in a separate plastic cage (4mice/cage). The creatures kept up at a temperature of 23 – 25°C, relative stickiness run between 30-half with standard pellets and water.

E. Experimental Scheme

All mice were separated into six groups, four mice per group. Group 1 was normal control and received normal saline only. Group 2 was toxic, given a dose of CCl4 i.p. injections for a week. Group 3 and group 4 were only given 400 mg/kg lemon citric acid and 5 mg/kg fungal citric acid i.p. for 7 days, respectively. Group 5 and group 6 on the first day, CCl4 was given, posttreatment with 400 mg/kg lemon citric acid and 5 mg/kg fungal citric acid i.p. form (2-7) days. All groups were administered i.p. with (0.1 ml) of the dose. Then, the mice were scarified at day 8. Before sacrificing the mouse, blood was collected by heart, and then serum was obtained.

The serum was used for hormone estimation. After blood assortment, the mouse was relinquished and analyzed to assessment of histological segments.

F. Hormone Estimation

Serum levels of progesterone, estrogen and testosterone were estimated by the kit which supplied from the local market that was synthesized by (Monobind Inc. Lake forest CA 92630, USA. Accu Bind). All reagents, serum references, and control were prepared in room temperature (20-27°C) before starting with the assay. Then pipette 10 µL of serum reference, control or specimen into the microplates well, followed by adding (50 ml) of the working enzyme reagent and swirled the microplate gently for 20-30 seconds to mix, then 0.050 ml of biotin reagent was added to all wells followed by swirling the microplate gently for 20-30 seconds to max. Then incubate the microplate for 60 minutes at room temperature and discard the contents of the microplate and added 350 ul decant (tap and blot) or aspirate, the process repeated two additional times to have three washes. To all wells, 0.100 ml of the working substrate solution was added then incubated at room temperature for (15) minutes. The absorbance was checked at 450 nm. After 0.050 ml of stop solution was added to each well.

G. Preparations of Histological Specimens

Mice were sacrificed by cervical dislocation and the testis as well as epididymis (caput and cauda) of the animal was prepared for examination as explained in [19]. Specimens were treated in a formalin 10% fixative solution for 24-hour, serial of dilution with a gradual (30-100%) of alcohol used for

dehydration for (5) min each. Before inserted in paraffin wax for sectioning, the specimens cleared in two xylene changes. Each cross section was (5) μm in thickness. Finally, the staining process for sections with hematoxylin (Harrison) and eosin prior to histological analysis according to the standard procedure [18].

H. Statistical Analysis

The comparison between groups were conducted utilizing Minitab 16 (Minitab Ltd, Coventry, UK). Variance among bunches are controlled by Student t-test. Information are communicated as mean and standard error.

III. RESULTS

A. citric acid production

Nine isolates were selected from samples of Aspergillus Niger that isolated from spoiled onion on PDA medium. the PDA medium also use to purified then the Czapek-Dox Agar media prepare to screened to detect their ability to form citric acid, by culturing it on that containing starch , a source of carbon, and methylene red reagent; all isolates were incubated at 30° C for 5 days. The transformations of culture medium color from yellow to red is the indicator to production of citric acid due to decreases of pH. The comparison was made of isolates in citric acid production by calculating the ratio between the diameter of the colored zone to the diameter of the growth zone during 4, 5 and 6 days of culturing (Figure1). The best isolation was selected after re-screening. This isolated known O5, which characterized by the high efficiency to produce the citric acid with concentration 5mg/kg; the citric acid also measured using pH meter, it was 3. While the concentration of lemon citric acid was 400 mg/kg.

B. Hormone Estimation

Level of testosterone, estrogen and progesterone in serum of various gatherings is displayed in (Table 1). Treatment of CCl4 showed decrease in testosterone level of serum (1.2ng/ml) as contrasted to the control group (2.4ng/ml) of mice. Administration of lemon and fungal citric acid in this trial demonstrated a height in the degree of testosterone (3.13 ng/ml, and 3.63 ng/ml) in comparison to that of the CCl4 treated group and to the negative control group. Treatment of lemon and fungal citric acid in combination with CCl4, increased the level of testosterone (2.68ng/ml, and1.98 ng/ml) in comparison to that of the positive group.

The estrogen concentration in the current study, was quantified and was high in CCl4 treated group (43.8ng/ml) in correlation to that of the standard group (36.7 ng/ml). However, the administration of lemon and fungal citric acid alone to mice reduced the level of testosterone in serum (34.99 ng/ml, 30.98ng/ml) as compared to the control group. Treatment of animals with CCl4 in mix with lemon and fungal citric acid reduced the level of testosterone in serum (33.82ng/ml ,25.66 ng/ml) as compared to the control group.

The level of female hormone progesterone directly effects by CCl4 drug. The high concentration of drug increased the progesterone level to (23.5 ng/ml) in compared with control group (13.9 ng/ml). Treatment of animals with CCl4 in combination with lemon and fungal citric acid reduced the level of progesterone in serum (17.65ng/ml,10.96 ng/ml) as compared to the control group. However, the administration of lemon and fungal citric acid alone to mice increased the level of progesterone in serum (14.57 ng/ml, 13.75ng/ml) as compared to the control group.

C. Histopathological Changes of Testes

In normal testes, histological examinations of negative control mice demonstrated a normal arrangement of spermatogenic, spermatids and seminiferous at different development stages (Figure2). This response was the same in Aspergillus citric acid treatment that showing normal development of seminiferous tubules with spermatogonia cells development and presence of sperms in good quantity inside the lumen and Leydig cells are normal looking in appearance (Figure 3). On the other hand, there were no differences with lemon treatment in testicular tissue that showing the seminiferous tubules well mature of spermatogonia cells development and presence of sperms inside the lumen of tubules in comparison of negative control (Figure 4), but showing Leydig cells hyperplasia (Figure 5). The histological section in the testes of mice treated with CCl4 on the first day, posttreatment with lemon citric acid form (2-7) days showed seminiferous tubules with few sperms inside the lumen (Figure 6), while the treated mice with CCl4 on the first day, followed with fungal citric acid treatment form (2-7) days showed normal maturation of spermatogonia cells but there was diminution of sperms inside the lumen with normal structure of Leydig cells (Figure 7).

IV. DISCUSSION

Citric acid is found in numerous microorganisms , tissues ,fluids and in almost all plants. Because of citric acid extremely less in toxicity, it predominantly utilized as a flavoring, food additive, drinks, soft drinks, chemicals, pharmaceuticals, and in cosmetic preparations. It can be argued that citric acid play a major role in treating or slow down oxidative stress regarded disorders in fertility, and can improve fertility, because it acts as an antioxidant and bacterial inhabitant [19].

industrially by fermentation, citric acid is produced and the Aspergillus Niger filamentous is exclusively used due to its high citric acid productivity at low pH without the toxic byproducts secretion [20].

synthetic concoctions including ecological poisons, eco-microorganisms, and even clinically valuable medications can cause cell harms in different organs of the body through metabolic actuation to profoundly responsive substances, for example, free revolutionaries [21].

The cycle of development of spermatozoa from spermatogonia undeveloped cells is controlled by different hormones and this entire cycle in controlled by the hypothalamic-pituitary testicular axis. Any minor internally or externally environmental disturbance such as chemicals, cause problems in males fertility. Aggregation of reactive oxygen species (ROS) in testis prompts hypogonadism [22]. The

account of CCl4 treatment prompted higher creation of H2O2 and nitrite in testicles tests which suppresses the antioxidant defense while upgrades the cellular injuries [23].

The occurrence of butcher germinal cells and their event in the epididymis and seminiferous tubule luminal are conceivable because of the contact loss as a result of cellular junctions disruption [24]. Spermatogonia are influenced by the treatment by which some seminiferous tubules contained spermatogonia however do not finish and at a low level. The spermatogonia present as being the most resistant spermatogenic cells to toxicity, however, chronic exposure to the chemotherapeutic medication according to the reported studies [25].

The endocrine balanced of hypothalamus, pituitary and the testis is the basic for fruitful and complete male germ cell development. A gonadotropin-delivering hormone delivered by the nerve center initiate the discharge of gonadotropins hormones from the pituitary organ [26]. FSH ties with receptors in the Sertoli cells and animates spermatogenesis. The creation of testosterone in the Leydig cells stimulates by LH hormone, which thus may follow up on Sertoli cells and animates spermatogenesis [27].

V. Conclusions

The aftereffects of the current investigation supposed the antioxidant activity of the citric acid against the oxidative stress induced by CCl4 in testicles of male mice. The adjusted histopathological changes initiated with CCl4 were likewise decreased with co-treatment of citric acid. These outcomes proposed the assessment of citrus acid for sexual behavior. CCl4 like any others synthetic drugs which have a side effect, and the consequences of this examination found that treatment of mice with it negatively effect on the level of male sex hormones.

References

[1] Makut, M. and Ekeleme, I. (2018). Citric Acid Production by *Aspergillus Niger* and Trichodermaviride Using Hydrolysed Potato Peels Substrate. Asian Journal of Advances in Agricultural Research, 5(1): 1-7.

[2] Angumeenal, A.; Kamalakannan, P.; Prabhu, H. and Venkappayya, D. (2003). Effect of transition metal cations on the production of citric acid using mixed cultures of *Aspergillus Niger* &Candida guilliermondii. Journal of the Indian Chemical Society, 80:903-906.

[3] Papagianni, M. (2007). Advances in citric acid fermentation by *Aspergillus Niger*: Biochemical aspects, membrane transport and modeling. Biotechnology Advances, 25:244–263.

[4] Soccol, C.R.; Vandenberghe, LPS.; Rodrigues, C.and Pandey, A. (2006)N ew perspectives for citric acid production and application. Food Technology & Biotechnology, 44:141–149.

[5] Grewal, H.and Kalra, K. (1995). Fungal production of citric acid. BiotechnolAdv.,13:209–234.doi: 10.1016/07349750(95)00002-8.

[6] Radwan, H. H.; Alanazi, F. K.; Taha, E. I.; Dardir, H. A.; Moussa, I. M. and Alsarra, A. I. (2010). Development of a new medium containing date syrup for production of bleomycin by *Streptomyces mobaraensis* ATCC 15003 using response surface methodology. Afr. Journal Biotechnol, 9(33), 5450-5459.

[7] Vasanthabharathi,V. N.; Sajitha, N. and Jayalakshmi, S. (2013). Citric acid production from U-V Mutated Estuarine.

[8] Gupta, G. K.; De, S.; Franco, A.; Balu, A. M. and Luque, R. (2015). Sustainable biomaterials: current trends, challenges and applications. Molecules, 21(48), 1-11*Aspergillus Niger*. Advances in Biological Research, 7(3):89-94.

[9] Yin, X.; Hyun-dong Shin, H.; Li, J.; Du, G.; Liu, L. and Chem, J. (2017). Comparative genomics and transcriptome analysis of *Aspergillus Niger* and metabolic engineering for citrate production. Sci Rep, 7, 41040.

[10] Yu, B.; Zhang, X.; Sun, W.; Xi, X.; Zhao, N.; Huang, Z. and Ying, H. (2018). Continuous citric acid production in repeated-fed batch fermentation by *Aspergillus Niger* immobilized on a new porous foam. Journal Biotechnol, 276–277, 1–9.

[11] Berovic, M.and Legisa, M. (2007). Citric acid production. Biotechnology Annual Review. 13:303–343. doi: 10.1016/S1387-2656(07)13011-8.

[12] Rohr, M. and Kubicek, C. P. (1981) Regulatory aspects of citric acid fermentation by *Aspergillus Niger*. Journal Process biochem., June-July,pp. 34-37.

[13] Selvankumar, T., Sudakar, C., Selvam, K., Govarthanan, M., Senthil Kumar, B., Anbarasan, P. M., … & Kamala-Kannan, S. (2014). Statistical optimization of citric acid production from alkali-treated coffee husk with lemon peel pomace by using *Aspergillus Niger*. Int J adv Sci Engineer, 1(2):14-19

[14] Al-Samarrai, S. M. A. (1995). Production Citric Acid from starch by Aspergillus Niger. MSc Thesis, College of Agriculture -University of Baghdad.

[15] Hussain, A. M. (2018). Citric Acid Production Using wheat bran by Aspergillus *Niger*. Indian Journal of Public Health Research & Development, June 2019, Vol.10, No.6

[16] Pearson, D. (1973). The chemical analyses of food. 6 the Ed. Chemical publishing Co., Inc., London.

[17] https://youtu.be/eWSzpjUUOWs.

[18] Alwachi, S.N. and Husain, D.K. (2014). Research articleTamsulosin hydrochloride (flomax) effects on fertility of albino male mice. International Journal of Recent Scientific Research,5(2):326-331.

[19] Goldberg, I. and Rokem, J.S. (2009). Organic and Fatty Acid Production, Microbial in Encyclopedia of Microbiology ,3d Edition.

[20] Kirimura, K. and Hattori,T. (2011). Industrial Biotechnology and Commodity Products in Comprehensive Biotechnology 2nd Edition .

[21] Lemike, T.L.; Williams, D.H. and Foye, W.O. (2002). Principles of medicinal chemistry. 6th Edition.

[22] Khan, M.R. and Ahmed, D. (2009). Protective effects of Digera muricata (L.) Mart. On testis against oxidative stress of carbon tetrachloride in rat. Food Chem Toxicol.,47:1393–9.

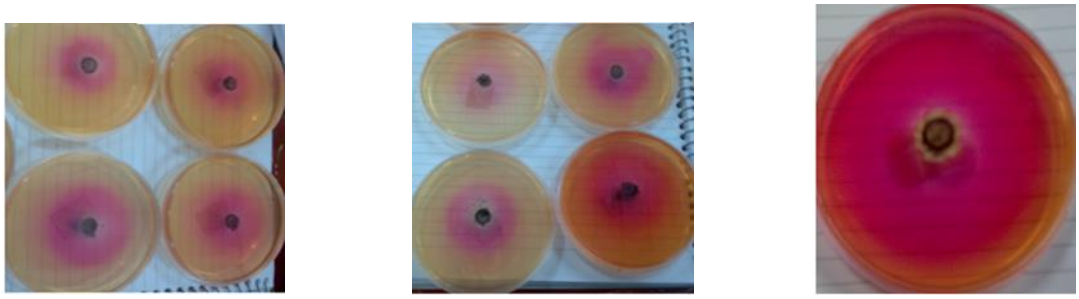
[23] Ojo, O.A.; Ojo, A.B.; Ajiboye, B.; Fadaka, A.; Imiere, O.B.; Adeyonu, O. and Olayide, I. (2016). Protective influence of Ficus asperifolia Miq leaf extract on carbon tetrachloride (CCl4)-induced testicular toxicity in rats. Journal of Applied Pharmaceutical Science,6(06):037–41.

[24] Niran, A. Ibrahim; Hanady, S. Al-Shmgani and Ruqaya, M.I. Al-ezzy. (2017). Cytarabine induced reproductive histopathological changes in albino male mice. Journal of Biotechnology Research Center, 11(1):6-12.

[25] Howell, S.J. and Shalet, S.M. (2001). Testicular function following chemotherapy. European society of human reproduction and embryology. Human Reproduction Update,7(4): 363–369.

[26] Lucas, B.K.; Ormandy, C.J.; Binart, N.; Bridges, R.S. and Kelly, P.A. (2019). Null mutation of the prolactin 36 431150 20 40 60 80 100 120 140 control 1000mg\kg 2000mg\kgserum prolactin concentration (ng\ml) treatmentsProlactin Oraibi AJB2T, 5(1): 1-7; Article no. AJB2T.48254 7 receptor gene produce a defect in maternal behavior. Endocrinology; 139(10):4102-2107.

[27] Al-Hadidyai, A.A.A. (2003). Estimation of testosterone and some biochemical parameters in infertile women's serum. Thesis supplement to Microbiology Department, Medicinal College, Mosul.



A **B (O5)** **C**

Fig. 1 The ability of isolates (O1 to O9) on the production of *Aspergillus Niger* citric acid by changing the color of ethylene red reagent of the region surrounding the growth of fungi on Czapek-Dox Agar media after 6 days (figure B refers to best isolate known O5).

Table I: Effect of *Aspergillus Niger* citric acid, lemon citric acid and CCl4 on hormonal level in albino male mice.

Groups	Testosterone Mean±S.E.	Estrogen Mean±S.E.	Progesterone Mean±S.E.
Negative control	2.4 ± 0.04	36.7 ± 4.3	13.9± 2.2
Positive control CCL4 (0.02%)	1.2 ± 0.01	43.8± 4.2	23.5 ± 3.8
Lemon citric acid (400 mg/kg)	3.13 ± 0.12	34.99 ± 2.3	14.57 ± 0.19
Aspergillus citric acid (5 mg/kg)	2.68 ± 0.09	30.98±2.10	13.75± 0.20
CCL4+Lemon citric acid	3.63 ± 0.17	33.82± 1.7	17.65± 0.22
CCL4+*Aspergillus* citric acid	1.98 ± 0.03	25,66± 2.8	10.96 ± 0.18

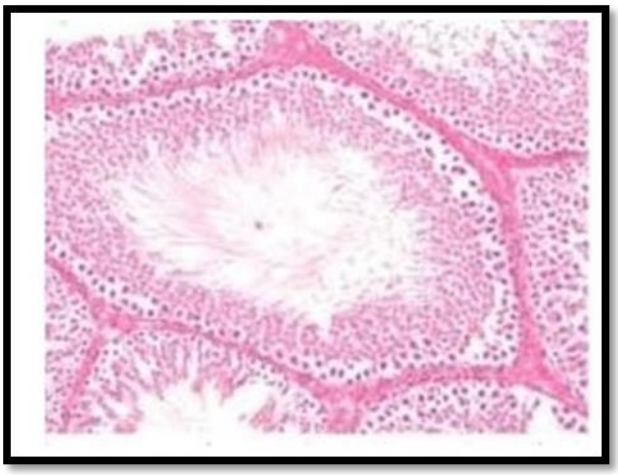


Fig.2: Section of testicular tissue in negative control mice showing normal seminiferous tubules, spermatogonia and spermatids cells (400x; H & E).

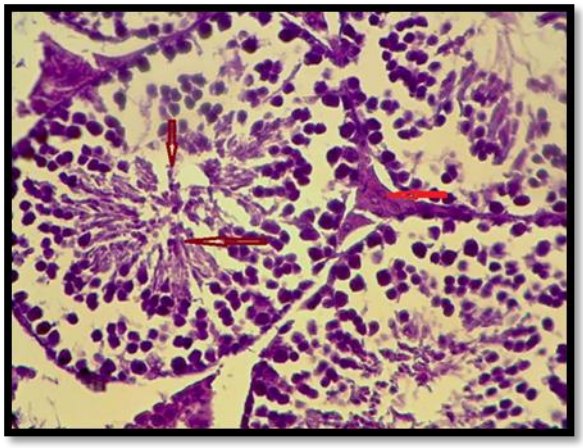


Fig.3: Section showing normal development of seminiferous tubules with spermatogonia cells development and presence of sperms in good quantity inside the lumen. Leydig cells are normal looking in appearance (400x; H & E).

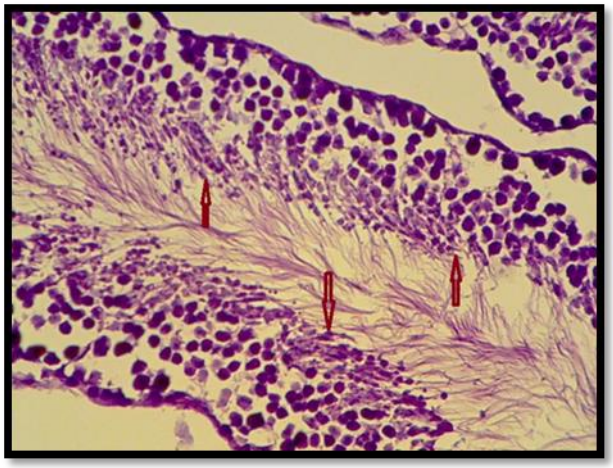


Fig.4: Section of testicular tissue showing that the seminiferous tubules well mature of spermatogonia cells development and presence of sperms inside the lumen of tubules (400x; H & E).

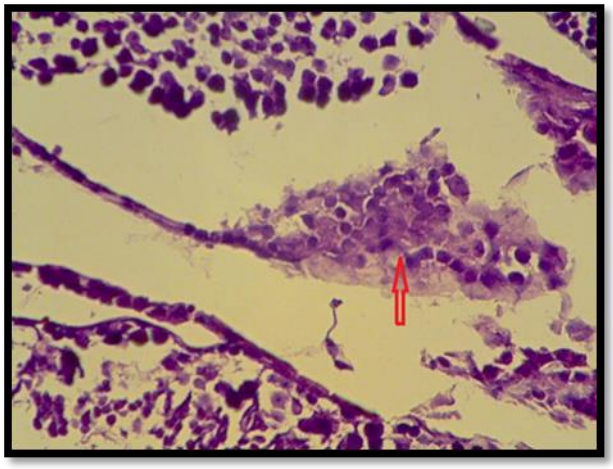


Fig.5: Showing Leydig cells hyperplasia (400x; H & E).

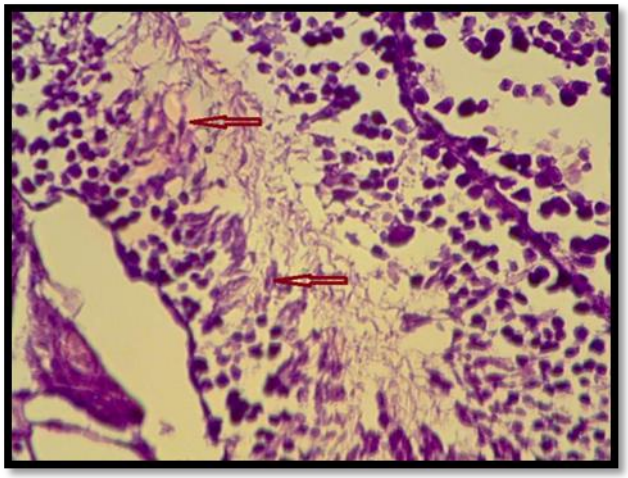


Fig.6: Section showing seminiferous tubules with few sperms inside the lumen (400x; H & E).

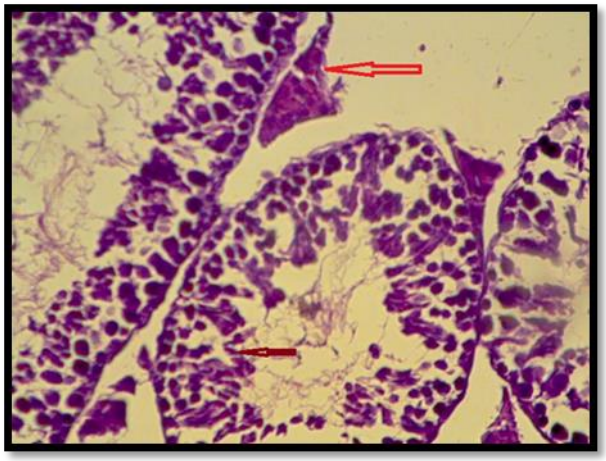


Fig.7: Normal maturation of spermatogonia cells but there was diminution of sperms inside the lumen with normal structure of Leydig cells. (400x; H & E).