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RESEARCH ARTICLE

THE INFLUENCE OF A COMBINATION OF ANTIOXIDANTS ON SUB FERTILE MALE RAT.

Fadhil k. Hassooni[1], Karam M. K.[1], Muayad S. Abod[2] and Mohammad Oda Selman[2].
1. College of Veterinary Medicine /University of Al-Qadisiyah.
2. High Institute of Infertility Diagnosis and ARTs / Ai-Nahrain University.

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Abstract

Background: Prescription of a combination of antioxidants might increase the quality of sperm parameters and decrease the serum level of cholesterol and serum level of triglycerine in men who suffer from infertility. Therefore, the present study investigated protective effects of alpha-Lipoic acid (ALA), L-Carnitine (CAR), N-Acytel cysteine (NAC) on sperm parameters and seminiferous tubules epithelium in high-fat diet model. **Objectives**: is to investigate the serum cholesterol and its effect on male rat's fertility and evaluate the effect of treating them with a certain combination of antioxidants mixed together they were (ALA), L-(CAR) and N-Acytel (NAC). **Material and Methods**: thirty male rats were administered cholesterol for 2 months to induce hypercholesterolemia to reduce fertility in male rats and consequently treated with a combination of antioxidants to repair the harmful effect of cholesterol. **Results**: cholesterol administered group were significantly different with those of control group ($P<0.05$) cholesterol levels were153.51±5.39 and 93.61±4.68 mg/dl while triglyceride was 176.71±12.49 and 83.14±7.08 mg/dl in treated and control group respectively. Also semen parameters showed significant difference between treated and control group ($P<0.05$) they were 28.46±3.56 and 74.4±6.36 ×106 sperm/ml, 34.23±2.12 and 89.34±2.41%, 32.45±5.23 and3.76±1.22%, 20.34±3.81 and 3.21±1.08% for sperm count, live sperms, sperm agglutination and abnormal sperm percentage in treated and control groups respectively. While after antioxidant administration showed significant difference ($P<0.05$) with the control group in sperm count, bigger live sperms percentage and lesser abnormal sperms was seen in combination of three antioxidants were ($P<0.05$) compared to positive control group (non-antioxidant group), sperm agglutination had a significant difference ($P<0.05$) between all antioxidant treated group compared with positive control group (non-treated group). **Conclusion**: As it is shown in our study, Oral administration of a combination of antioxidants with diet, decreasing the serum levels of cholesterol and serum level of TG, improving the sperm parameters by decreasing the ROS and protect the liver tissue from the harmful effect of cholesterol. Finally, it seems that the antioxidants in patients with subfertility or infertility is a new and efficient strategy with few side effects.

Corresponding Author:- Fadhil k. Hassooni.
Address:- College of Veterinary Medicine /University of Al-Qadisiyah.

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Introduction:-

Male reproductive function can be weakened by chronic diseases, advanced aging, medications, exposure to contaminants and the feeding with diet supplemented with fat which leads to the accumulation of free radicals in the body causing oxidative stress and functional disorder of hypothalamo-pituitary gonadal axis associated with damage of spermatogenesis [1,2].

Free radicals are unstable and highly reactive molecules or atoms with an unpaired number of valence electrons [3,4]. Reactive oxygen species (ROS) consist of free and non-free radical reactive molecules [5].

Antioxidants is a substance that prevents oxidation and have a widespread influence in male fertility, these scavenge ROS produced by leukocytes, reduce cryo-damage to spermatozoa, block premature sperm maturation, and stimulate spermatozoa, keep sperm cell from over production of ROS which is harmful and produce abnormal spermatozoa, improve semen quality in smokers, prevent DNA fragmentation, and improve assisted reproductive techniques (ART) outcome [6].

Three different antioxidant protection systems play important roles in reducing ROS in males include:
Alpha Lipoic Acid (ALA) is a potent type of antioxidant that used as a cofactor for several important mitochondrial enzymes [7]. **N-acetyl cysteine (NAC)** commonly abbreviated to NAC, is a precursor of the amino acid L-cysteine and the antioxidant glutathione (GSH) [8]. **L-Carnitine** is an endogenous type of antioxidants that acts as a carrier for fatty acids across the inner membrane of mitochondria essential for subsequent beta-oxidations and ATP production [9].
Therefore, the **aims of our study** was to:

- **Induction of hypercholesterolemia in male rat which consider as a model of hyperlipidemia and its effect on decreasing the reproductive performance in male fertility.**
- **Detect the effect of a combination of a certain antioxidant on reproductive performance of hyperlipidemic male rat.**

Materials and Methods:-

The present study is performed on 45 apparently healthy male albino rats; their ages ranged between two months with a body weight ranging between 200-250 grams. Rats were obtained from the animal house of the college of veterinary medicine, Al-Qadisiyah University. The rats were housed in standard environments. Rat were fed with the standard balanced pellet that contains special dietary supplement to keep normal activity and growth, prepared in the animal house of the Institute and presented to the mice in addition to tap water *ad libitum* [10].

Rats were divided randomly in equal groups 15 male rats each. First of all, they were to 2 groups cholesterol treated group and non cholesterol group. Groups consists of (G1=cholesterol with combination of 3 antioxidants) and (G2 control=cholesterol with no antioxidant). Groups of G1 and G2 were administered orally with cholesterol supplement starting at 60 days of age as to induce hyperlipidemia (induction of partial subfertility) till day 90. Two days after ceasing the cholesterol supplement (day92), antioxidants are supplied orally to a group as follows for 30 days.
1- (G1) are given orally a combination of antioxidants Alpha lipoic acid (ALA) 100mg, L- (CAR) Carnitine150mg and N-Acetyl cysteine (NAC) 50mg/kg.B.W./daily.
2- (G2 control) no antioxidant, only fed and drenched normally without any antioxidants after cholesterol supplement.
3- (G3 control) no cholesterol supplement and no antioxidant is added for the entire process of investigation.
Blood samples were collected 5ml from the animal's left ventricle and aorta and stored at -8 °C until further analysis. Serum is used for biochemical assay. Total cholesterol concentration and triglyceride concentration were analyzed. Semen samples are collected from the tail of epididymis and used for sperm functions analysis [11,12]. The rats were sacrificed, Liver, testes are taken from the sacrificed rats of all groups and fixed in 10 % formalin and are used for the histological study [13].

Statistical Analysis:-
A computerized program, the statistical package for social sciences (SPSS) was used to calculated the statistics analysis. The statistical analysis of data for physiological and histological parameters had done [14].

Results:-

Results of cholesterol supplement showed that body weight and lipid profile of male rats before and after cholesterol administration. All male rats were weight checked in the first before any cholesterol administration they were 96.87± 7.33 and 84.25±6.72 grams for treated and control groups respectively, while after cholesterol administration they were 167.4±12.32 and 133.96±10.43 grams in treated and control group respectively, showing significant difference between the two groups after administration of cholesterol (P<0.05).

On the other hand, lipid profile showed highly significant differences in cholesterol level in treated rats 153.51±5.39 mg/dl and control group 93.61±4.68 mg/dl (P<0.01) also triglyceride levels were 176.71±12.49 mg/dl and 83.14±7.08 mg/dl in treated and control group respectively, showing highly significant difference between them (P<0.01) Table (4-1) and fig (4-1).

Results of sperm parameters after cholesterol administration Sperm count in cholesterol treated rats was 28.46±3.56 $\times 10^6$sperms/ml while untreated control group were 74.4±6.36× 10^6 sperms per ml with significant difference (P<0.01). Live sperm percentage were 34.23±2.12% in treated group while 74.4±6.36% in control group with marked significant difference between groups (P<0.01).

On the other hand, sperm agglutination was 32.45±5.23 and 3.76±1.22 in treated and control groups respectively, with highly significant difference between the two groups (P<0.01) while abnormal sperm percentage were 20.34±3.81 and 3.21±1.08 in treated and control group respectively with marked significant differences between groups (P<0.01).

Results of antioxidant treatment the body weight were taken two times after antioxidant administration one month apart, after one month of antioxidant administration they were 241.75±16.83, 201.93±12.45 and 178.32±12.32 grams for groups G1, G2 and G3 respectively, groups G1, G2 and G3 showed significant differences among them but highly significant difference compared between G1 and G3 (P<0.01). While after 2 month of antioxidant administration (end of experiment) body weight were 274.75±16.43, 234.33±15.32 and 239.51±13.47 grams for all groups G1, G2 and G3, groups respectively, showed no significant difference between G2 and G3 but both showed significant difference compared G1 (P<0.01). As shown in table (2).

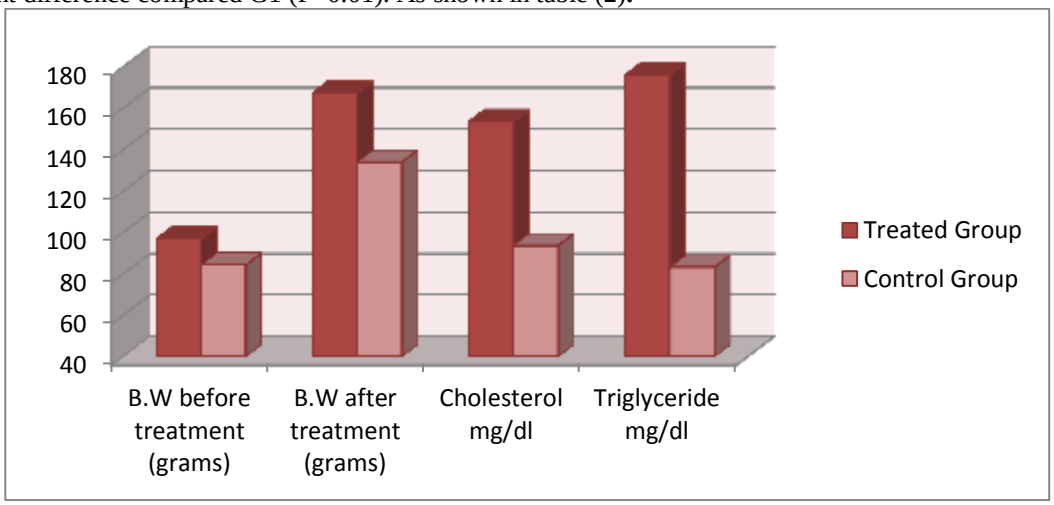

Fig. 1:- Body weight (grams) before and after cholesterol administration, cholesterol and triglyceride levels (mg/dl) in treated and control groups.

Table (1):- Body weight, lipid profile and sperm parameters for cholesterol treated and control group

Groups Parameters	Treated Group (M±SD)	Control Group (M±SD)
B.W before treatment (gram)	**96.87± 7.33**	**84.25±6.72**
B.W after treatment (gram)	**167.4±12.32** [a]	**133.96±10.43** [b]
Cholesterol mg/dl	**153.51±5.39** [a]	**93.61±4.68** [b]
Triglyceride mg/dl	**176.71±12.49** [a]	**83.14±7.08** [b]
Sperm count $\times 10^6$	**28.46±3.56** [b]	**74.4±6.36** [a]
Live sperm %	**34.23±2.12** [b]	**89.34±2.41** [a]
Sperm Agglutination	**32.45±5.23** [a]	**3.76±1.22** [b]
Abnormal sperms %	**20.34±3.81** [a]	**3.21±1.08** [b]

***Small letters refers to significantly different (p<0.05) in rows**

Table (2):- Body weight (grams) of all groups one month and two months after antioxidant administration(M±SD)

GROUPS	G1	G2	G3
Cholesterol	**97.12±4.23^{b}**	**133.15±4.91^{c}**	**95.32±3.85^{b}**
Triglyceride	**83.71±2.06^{b}**	**181.06±3.56^{c}**	**87.51±2.96^{a}**

***ab = significant difference in rows (P<0.05)**
***bc = significant difference in rows (P<0.05)**
***ac = significant difference in rows (P<0.01)**

On the other hand, lipid profile at the end of experiment were checked and cholesterol was 97.12±4.23, 133.15±4.91 and 95.32±3.85 mg/dl in groups G1, G2, G3 respectively, G1 and G3 showed significant difference from G2 (P<0.05). While triglyceride was 83.71±2.06, 181.06±3.56 and 87.51±2.96 mg/dl for groups G1, G2 and G3 respectively, groups G2 and G1 s showed significant difference (P<0.05). Among them but showed highly statistical difference compared with G3 (P<0.01) and G3 when compared with G1(P<0.05). As shown in table (3).

Table (3):- showed highly statistical difference compared with G3 (P<0.01) and G3 when compared with G1 (P<0.05).

GROUPS	Sperm count ×10^{6}	Live %	Abnormal sperms %	Sperm Agglutination %
G1	**127.39±7.61^{a}**	**86.75±5.92^{a}**	**4.86±1.58^{c}**	**4.32±1.32^{c}**
G2	**53.33±4.65^{c}**	**67.33±3.28^{c}**	**13.24±3.28^{a}**	**20.21±2.87^{a}**
G3	**116.27±6.92^{b}**	**82.33±5.51^{b}**	**4.33±1.77^{c}**	**4.22±1.02^{c}**

***ab = significant difference in rows** (P<0.05)
***bc = significant difference in rows** (P<0.05)
***ac= significant difference in rows** (P<0.01)

Results of sperm parameters after antioxidant administration the sperm count in antioxidant treated rats were 125.39±10.61, 53.33±4.65 and 116.27±6.92 (×10^{6}sperm/ml) in all groups G1, G2 and G3 respectively, showing significant differences between G1 and G2 (P<0.01) while both showed significant difference with G3 (P<0.05) as shown in table (4).

Table (4):- Sperm parameters of all groups

GROUPS	G1	G2	G3
B.W after 1 month Antioxidant (grams)	**241.75±16.83^{a}**	**201.93±12.45^{b}**	**178.32±12.32^{c}**
B.W after 2months antioxidant (grams)	**274.75±16.43^{b}**	**234.33±15.32^{c}**	**239.51±13.47^{c}**

***ab = significant difference in columns (P<0.05)**
***bc = significant difference in columns (P<0.05)**
***ac = significant difference in columns (P<0.01)**

While live sperm percentage were 86.75±5.92, 67.33±3.28 and 82.33±5.51 % for all groups G1, G2 and G3 respectively, showing significant differences between G1 and G2 (P<0.01) while both showed significant difference with G3 (P<0.05) as shown in table (4). Abnormal sperms percentages were taken also and were 4.86±1.58, 13.24±3.28 and 4.33±1.77 % for all groups G1, G2 and G3 respectively, G2 showed highly significant difference to both groups G1 and G3 (P<0.01). While sperm agglutination percentage were 4.32±1.32, 20.21±2.87 and 4.22±1.02 for G1, G2 and G3 respectively, G2 showed highly significant difference to both groups G1 and G3 (P<0.01) as shown in table (4).

Histological findings in liver after two-month cholesterol administration showed congestion of central vein and lose of hepatic architecture, the bile duct showed congestion with hyperplasia, marked vacuolation of the hepatocyte, present of fatty change within hepatocyte, Hepatocyte showed as binucleated, also there is infiltration (aggregation) of the inflammatory cells (mainly macrophage) within hepatic tissue, hepatocyte show as signet - like shape as shown in fig (2, 3).

While in G3 showed normal hepatic tissue, which characterized by presence of radially arranged hepatocytes around normal central vein, in higher magnification, the hepatocytes showed hexagonal and normal shape. As shown in fig (4, 5).

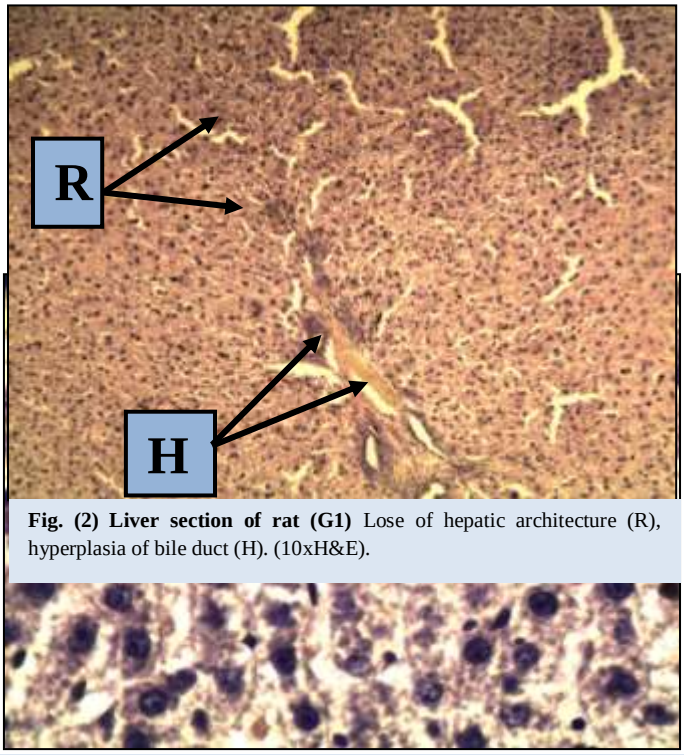

Fig. (2) Liver section of rat (G1) Lose of hepatic architecture (R), hyperplasia of bile duct (H). (10xH&E).

Fig. (4) Liver section of rat G3: Radially arrangement of hepatocytes (R) around normal central vein (CV), hepatocytes showed with hexagonal and normal shape (HE)(40×H&E).

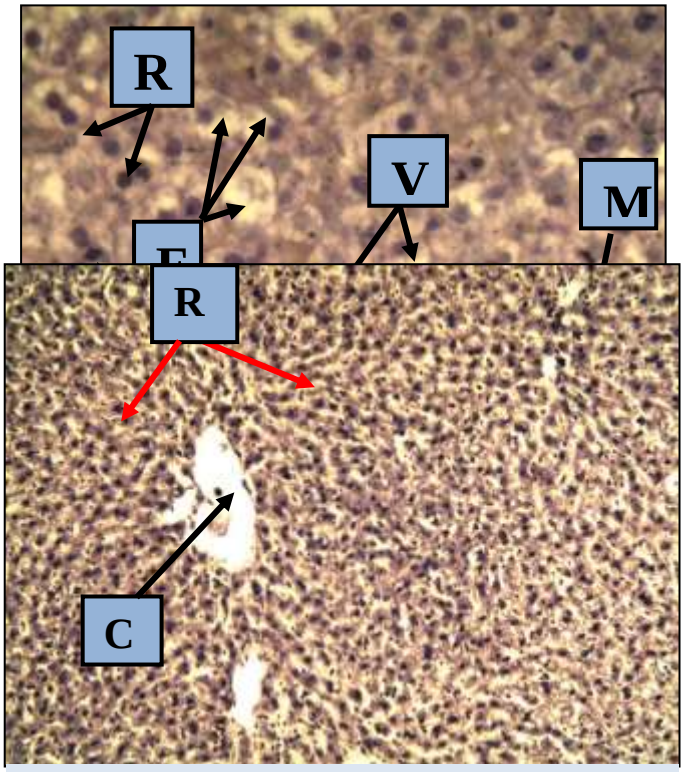

Fig. (5) Liver section of rat G3: Radially arrangement of hepatocyte (R) around normal central vein (CV). (10×H&E).

Testicular tissue which characterized by presence of complete spermatogenesis, the seminiferous tubules showed compact, circular and normal tubules, there are high numbers of spermatogonia, primary and secondary spermatocyte and spermatids in the lumen of seminiferous tubules as seen in G3 in fig (6,7).

On the other hand, histological finding of testes in cholesterol administered group showed suppression of spermatogenesis characterized by vacuolation of spermatogonia**,** with few numbers of primary and secondary

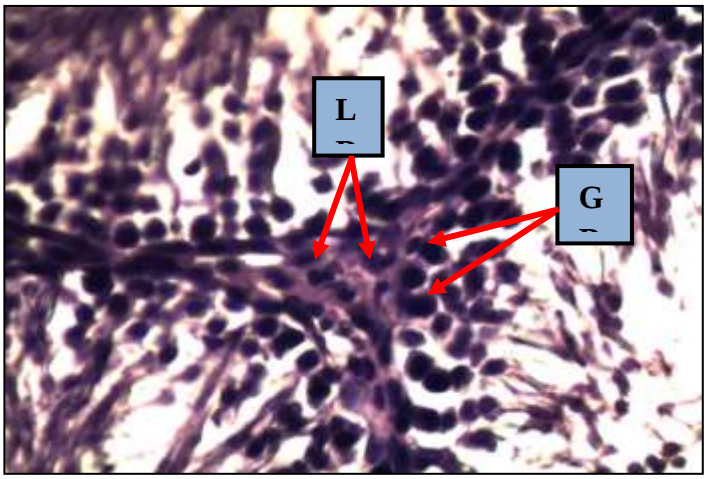

Fig. (6) Testis of rat (G3): spermatogonia(G), leydig cells (L). (40xH&E).

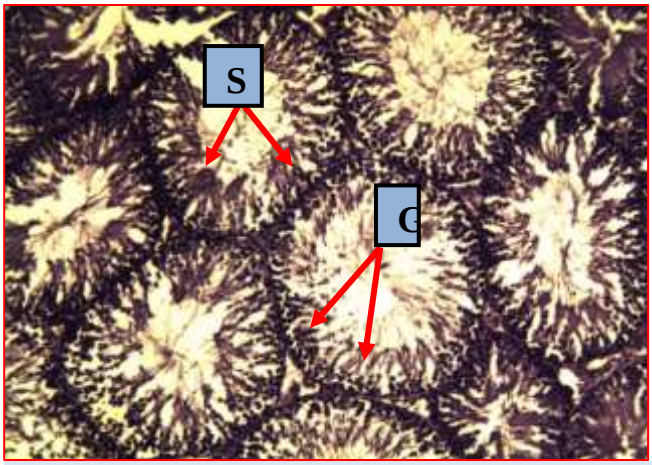

Fig. (7) Testis section of rat (G3): complete spermatogenesis (ST), spermatogonia (G). (10xH&E).

spermatocytes, absence of sperms in the lumen of the seminiferous tubules which showed very wide, few numbers of Leydig cells in the interstitial tissue with presence of adipose tissue as shown in fig (8,9).

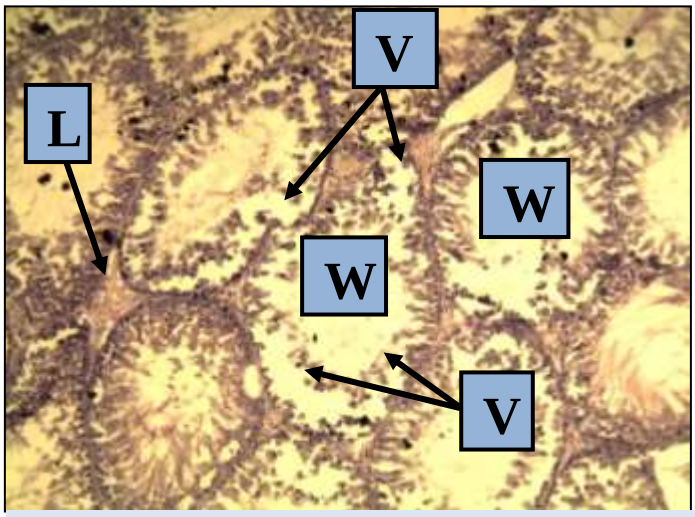

Fig. (8) Testis section of rat (G1): Vacuolation of spermatogonia (V), lumen of the seminiferous tubules showed very wide (W), Leydig cells in the interstitial tissue (L). (10×H&E).

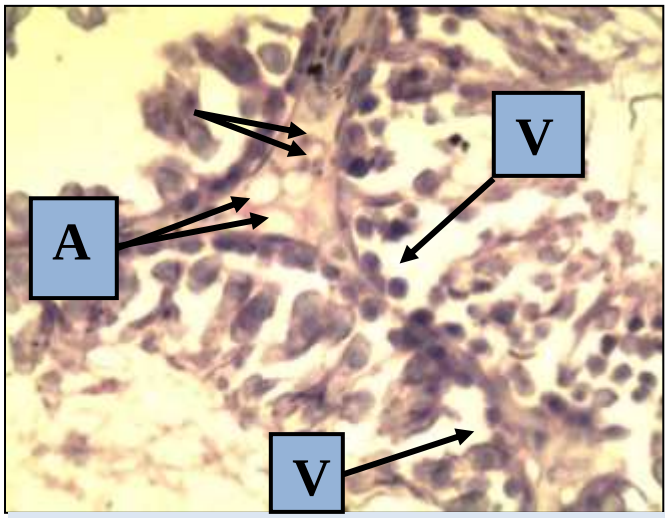

Fig. (9) Testis section of rat (G1): vacuolation of spermatogonia (V), Leydig cells in the interstitial tissue (L), adipose tissue (A). (40×H&E).

Conclusions:-

According to the presented results and discussions of our study, indicated that oral administration of cholesterol powder with diet (15g/kg.diet) for two months for adult albino male rats will have a significant harmful effect on male reproductive performance through destruction of seminiferous tubules and spermatogenesis causing reduction in sperm parameters including sperm concentration, sperm motility, and normal sperm morphology, a marked abnormal histopathological changes in liver, testes, and epididymis, a significant hyperlipidemic agent by increasing the serum level of cholesterol, and oral administration of a combination of antioxidants with diet (ALA 100mg/kg.B.W, CAR 150mg/kg. B.W, NAC 50mg/kg.B. W) for two months will have a significant beneficial effect on male reproductive performance in improving the sperm parameters by decreasing the ROS, protect the liver, testis tissue from the harmful effect of cholesterol, decreasing the serum levels of cholesterol and increasing the serum level of TG, but its protective effects on sub fertile men needs to be clarify via future studies on human.

Discussion:-

In this study it was designed to administer cholesterol to induce infertility in male rats to to be treated subsequently by different antioxidants. In this study administration of two-month cholesterol to treated group showed marked increase in body weight other than untreated group [(15)] this due to highly lipid intake which played a role in increasing fatty change in liver showing marked vocualation of hepatocyte**.** [(16)] Reported that Hypercholesterolemia in rat model, which can be recognized by nurturing with 0.5%-1.0% cholesterol-supplement diet for several weeks. Dietary 0.5%-1.0% cholesterol can increase serum VLDL and LDL levels radically in rats. In our study, dietary cholesterol extremely distressed triglyceride (TG) metabolism, the hepatic TG content increased in doubles until hepatic steatosis forms this implicates what was seen in treated groups in histological finding in liver. These results are nearly similar to those observed by [(17)] who reported that, a significant increase in plasma total cholesterol, VLDL (very low density lipoprotein) and LDL (low density lipoprotein) cholesterol was observed in mice fed on an excessive cholesterol diet for 3 months. Also, [(15)] demonstrated that, feeding the cholesterol- enriched diet caused a significant increase in total cholesterol.

This indicates that administration of cholesterol made significant variation between treated and non-treated groups as seen in our study this declaration matches with [(18,19)] who reported that, rat receiving cholesterol- enriched diet showed sever hypercholesterolemia, elevated plasma LDL and VLDL- cholesterol compared to the control group of

rats fed on a normal diet. Furthermore, [20,21] showed that, in rats fed a rich lipid diet there was a significant increase in plasma cholesterol, triglycerides and lipid concentrations.

When semen checked and analyzed for sperm count, live and dead sperm percentage, agglutination and abnormal sperms [22] were highly different in cholesterol treated male rats, which is main trace of this study. It was obvious that cholesterol effect on the testicular tissue for the treated group showed excessive production of ROS as mentioned by [23,24] when exposed to chronic diseases or environmental pollution, while [25,26] confirmed that excessive amounts of lipids lead to harmful effect to cells and to oxidative stress.

The purpose of given cholesterol to male rats is to induce partial infertility has experienced this method in our study, its obvious that hyperlipidemia had a negative effect concerning sperm quality due to ROS reactive oxygen species which lead to hyper oxidative stress since both hypercholesterolemia and hypertriglyceridemia caused an increase of oxygen radicals production and lipid peroxidation level associated with decreased antioxidative effect of glutathione as few studies matched with ours [27].

Its seem to be a highly difference when male rats were exposed to antioxidant after two month which showed highly difference with un administered antioxidant groups (both control groups) and in the second month showed variance between antioxidant groups other than the control groups this may be due to the effect of dissimilar mode of action to the contributed antioxidants especially ALA and CAR where Lipoic acid acts as a coenzyme in mitochondrial multienzyme complexes in the oxidative decarboxylation of keto-acids such as pyruvate and ketoglutarate [28,29]. In addition, ALA is involved in the regulation of carbohydrate and lipid metabolism [30]. Being easily absorbed from the gastrointestinal tract and able to cross the blood brain barrier without exhibiting any serious side effects. While the gain weight of male rats does not agree with what [31] reported its affect to decreases weight gain and obesity. For the sperm count and live sperms percentage ALA and COMB were found significantly different than other groups due to the effect of ALA in strengthens other antioxidant effect which was obvious on the combination of few antioxidants [32] in producing more live sperms and this effect was seen more and clear on agglutinations of sperm in ALA and COMB group while less in abnormal sperm percentage was seen in CAR group which is may due to [33] in scavenging free radicals Maintaining mitochondria integrity in stress conditions and preventing ROS formation.

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