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Histopathoogical, immunohistochemical and biochemical study of liver male mice and its relation to testosterone deficiency

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Abstract

The objective of the current research was to study the histological ,immunohistochemical and biochemical changes in the liver of castrated mice and possible treatment with testosterone, for this purpose 45 albino mice (3weeks) aged were divided into 3 groups control group , castrated group and castration with testosterone treated group for six weeks. From period January to April 2017. Histopathogical changes were detected in the liver of induced castrated mice is the form of disturbed hepatic architectures narrowing in sinusoids most of hepatocytes showed variable degrees of cytoplasmic vacuolation, accumulation of lipid in hepatocytes all these changes associated with inflammatory cellular infiltration in addition, decreased in number of hepatic stellate cells that was confirmed by immunohistochemical ,in addition, these structural changes were less pronounced in animals treated with testosterone more over there is significantly increased in (ALT,AST,ALP) level in castrated group as compared with control group , all so there is significant decreased in level of testosterone in castrated group as compared with control group, these findings suggested that castration has dangerous effect on liver

Key words: Castration, Testosterone, Mice, Non alcoholic fatty liver disease, hepatic stellate. cells.

1. INTRODUCTION

The Surgical castration method is a bilateral orchiectomy (removal of testis, or testicles) of male animals; farm animals (1, 2). It has the important effect of increased the progression steatosis to steatohepatitis through promoting activation of the endoplasmic reticulum (ER) stress pathway and increased of macrovesicular steatosis or droplet protein expression. Testosterone inhibits ER stress, suppresses the formation of macrovesicular lipid droplets, promotes lipid export, and ameliorates steatohepatitis induced by castration. Castration is a way of investigating the consequences of extreme testosterone deficiency in animal models (3).

Testosterone, a steroid hormone, that stimulates or controls the development and maintenance of male characteristics in vertebrates by binding to androgen receptors. This includes the activity of the accessory male sex organs and development of male secondary sex characteristics (4).Serum testosterone levels decline gradually and progressively with aging in men. Testosterone deficiency after castration caused atherosclerosis, abdominal obesity, impaired fasting glucose, excess accumulation of liver triglyceride(steatosis) (5).Common to all of these conditions, results in diminished glucose utilization and conversion of the excess glucose into fat. all of which contribute to the development of nonalcoholic fatty liver disease (NAFLD) (6) Higher circulating triglycerides then lead to an overspill of fat into ectopic storage in the liver and arteries as well as increasing the accumulation of visceral fat. (7) Low serum testosterone levels have a higher risk of developing hepatic steatosis (8,9) Testosterone deficiency promotes atherosclerosis Increased central adiposity, percentage body fat, fatty liver and insulin resistance, improving lipid profiles, insulin sensitivity and inflammatory profiles (10,11)

Hepatic stellate cells (HSCs) also called Ito cells, fat-storing cells, lipocytes , perisinusoidal cells and vitamin A-storing cells located in the space of Disse between hepatocytes and sinusoidal (12), it has important role in such a way modulating the liver sinusoidal blood flow. When the liver is damaged, the hepatic stellate cells change their shape and transform (via a process named activation) into the myofibroblast (13) Hepatocyte injury(steatosis) promoting hepatic inflammation, hepatic stellate cell activation, and the onset of fibrosis (14). Thus with disease progression, the cellular composition of the liver shifts toward increased proportions of inflammatory and fibrotic cells, and possibly cancerous cells. (15)

2. MATERIALS &METHODS

2.1. Chemicals

Testosterone obtained from LTD, Austuralian, ketamine and xylazine obtained from Gbhm, Germany, all other chemicals from Sigma, Italy

2.2.Animals

45 Male albino mice weighting between (14-20) g and aged (3weeks) were used in the present study, the mice were obtained from Animal House faculty of science/ university of Kufa, animals were kept in ventilated cages, with a temperature of (25±2C°) A 12:12 h light, dark cycle was also regulated for these animals balanced rodent food pellets and water was provided ad libitum (16) Animals were scarified at the end of the experiment (17).

2.3. Preparation of Testosterone solution

The hormone was administrated as oily injection, testosterone prepared by using the oil vehicle for this injection was olive oil .50 mg of testosterone (one tablet) was taken and dissolving in 100 ml of olive oil to give concentration 50μ g/ml testosterone, and injected as 0.1 ml daily (18).

2.4. Surgical castration

mice were anesthetized (given intrapritonial) prior to surgery in 3 weeks aged with (50mg ketamine + 20 mg 4/1 xylazine) by using sterile instruments and gloves a transversal scrotal incision was made testicular exposed and removed the scrotal incision was then closed using simple stitch, Bilateral castration was the basic operation performed in these experiments. Mice were transformed upon recovery to clean cages.

2.5. Injections:-

Hormonal solutions were usually injected daily for 42 days into the thigh Muscle (alternating legs on consecutive days).

2.6. Experimental Design

A total number of 45 albino male mice were used in this study, animals were divided in to 3 groups, (N=15) Grouped-housed animals of comparable age (3 weeks) were allocated as below into categories:

Group I: Intact male mice received tap-water as a control (N=15).

Group II: Castrated male mice (N=15

Group III: Castrated male mice treated with 50µg/kg/day of Testosterone. (Treated daily for 6 weeks with 0.1 ml) (N=15)

After 42 days, fasting animals were euthanized under (ketone and xylin). Blood was collected from a puncture to heart by

using a syringe in to clean dried centrifuge tubes .the tubes were then centrifuged at 3000rpm for 15 minutes , serum samples were carefully separated using pasture pipette and frozen at -20 ℃ until biochemical and level of testosterone analysis, alanin aminotransferase ,aspartate aminotransferase and alkaline phosphates, were measured with seamaty kits that were obtained from(biodiagnostic ,china). Determination of total testosterone was done by Rat T (Testosterone) ELISA Kit NOE-EL-R0033

2.7. Preparation for paraffin sections for light microscopic study

The abdominal lumen was opened and the liver was removed and immediately fixed in10% formaldehyde solution for 24 hours .dehydration was then carried out in ascending of alcohole followed by with xylol followed by embedding with hard paraffin. Sections of 2-3 microns in thickness from each block were cut by the microtome, then stained with H&E to study the general histological structures (19). Some sections of paraffin were stained with avidin-biotin Immunohistochemical technique for detection viminten protein within hepatic stellate cells. sections were dewaxing and endogenous peroxidase blocking by using H2O2, 10% for 10 minutes, sections were pre-treated with microwave treatment (twice for 5 minutes at 450 W in a citrate buffer pH 6, with a 20 minute .the interval between the two treatments) to induce antigen retrieval. Sections were then incubated overnight at room temperature in a humid chamber with primary antibody (viminten antibody) code674M from Dakocytomation with dilution 1:10, and the reaction products were visualized with a freshly prepared solution of 3.3-diamino- benzidine tetrahydrochloride (DAB, Sigma, Italy), 10 mg in 15 ml of a 0.5 M Tris buffer at pH 7.6, containing 1.5 ml of 0.03% H_2O_2. To ascertain structural details, sections were slightly counterstained with Mayer's haematoxylin.(20)

2.8.Statistical analysis:

Data were expressed as Mean ±S.E. statisticall analysis was performed using one way Anova followed by Duncan test for multiple comparison by using SSPS version 16 computer program the $p \leq 0.05$ were considered significant for all data

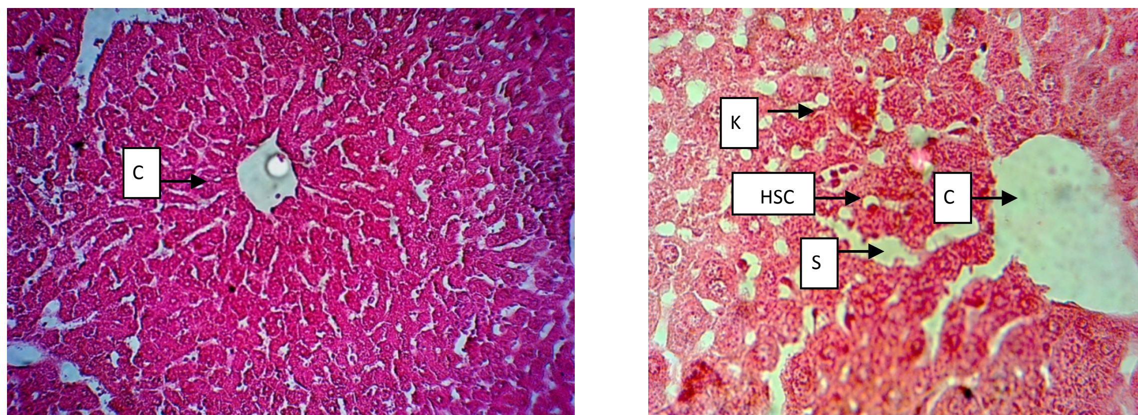
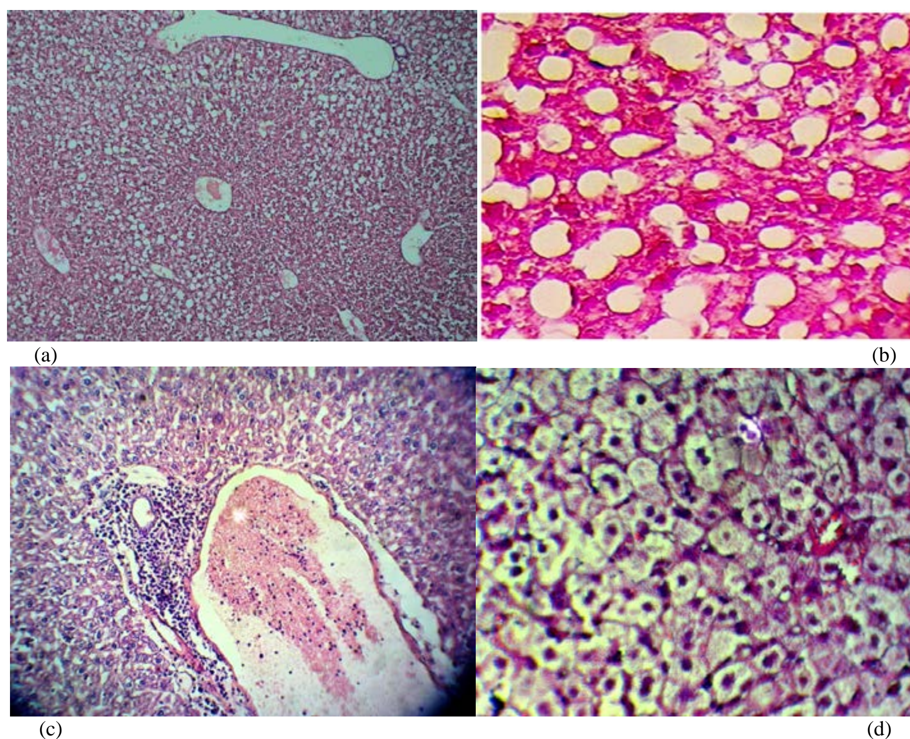
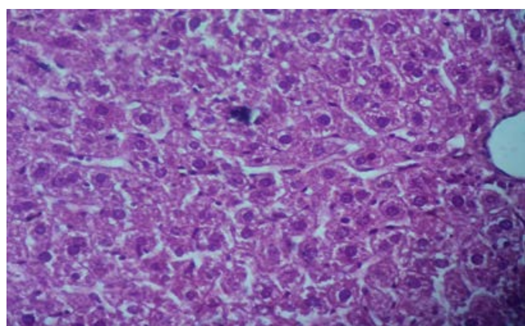


Figure1. Liver sections for control group showed a) normal liver architectures containing central vein (C)b) classic hepatic lobules containing central vein and radiation cord of hepatocytes with blood sinusoids (S) lined with kupffer cells (K) and there is some HSC cells also found between hepatocytes (H&E 200X ,400X)



Figuer 2. Liver section for castrated group showed a) disturbed hepatic architecture with ballooning hepatocytes. b) lipid accumulation (macrovesicular steatosis). c) cellular infiltration between hepatocytes with narrowing and congested blood sinusoids. d) ballooned hepatocytes with peripheral nuclei (200x)



Figuer 3 Liver section for castrated treated with testosterone group showed return to normal histological structures

3. RESULTS

3.1. Light microscopic results

3.1.1. H&E stain

Group I (control group):

Examination of sections obtained from liver of the control group showed normal histological structure. The liver was formed of classic hepatic lobules with central veins forming their central axis the hepatocytes were polygonal in shapes containing rounded nuclei and other binucleated ,blood sinusoids were found as a network between the plate of hepatocytes converging towered the central vein , they were lined by 2 types of cells (endothelial and kupffer cells) most probably HSC arranged in rows between hepatocytes figure 1(a&b)

Group II (castrated group)

Examination of liver sections obtained from this group revealed several histological changes in the form of disturbed hepatic architecture narrowing and congestion of portal veins in addition, cellular infiltration that can see between hepatocytes most hepatocytes showed variable degree of cytoplasmic vacuolation. Some contain multiple small vacuoles and other have large vacuoles the other appeared ballooned with peripheral nuclei figure 2(a, b, c &d)

Group III (castrated treated with testosterone) showed similar to the control group and showed the normal histological structure of the liver. Figure 3

3.2..immunohistochecal results

Number of vimentin +ve cells

As assessed by image analyzer the staining reactivity had been represented by calculating the mean of the positivity percentage in the paraffin sections for both the control and treated groups. the castration induced group figure (5) showed statically significant increased in number of viminten +ve cells as compared with control group figure (4) there was no significant changes in number of viminten +ve cells in testosterone group figure (6) as compared with control group histogram (1)

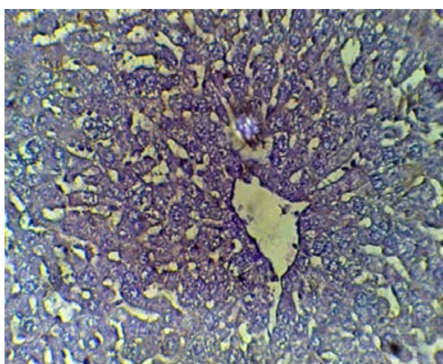


Figure (4)immunohistochemical stain liver section(control group) showed viminten +ve between hepatocytes

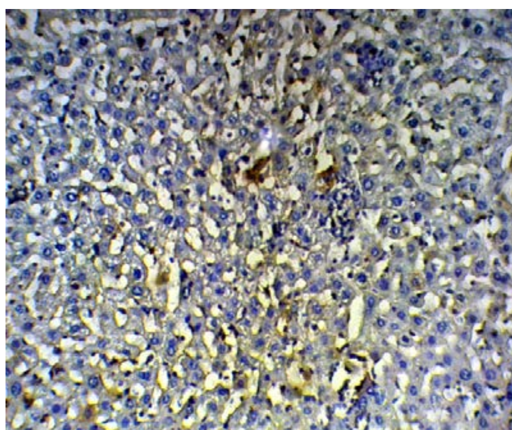


Figure (5)immunohistochemical stain liver section(castration group) showed increased viminten +ve between hepatocytes

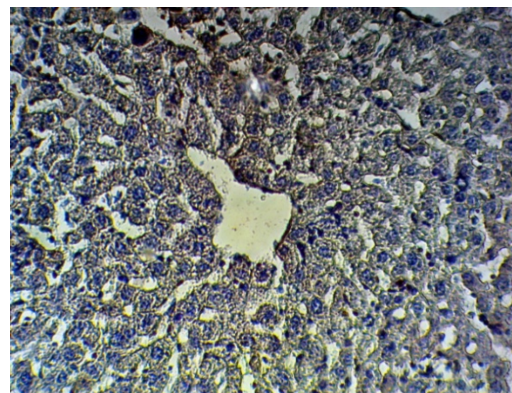
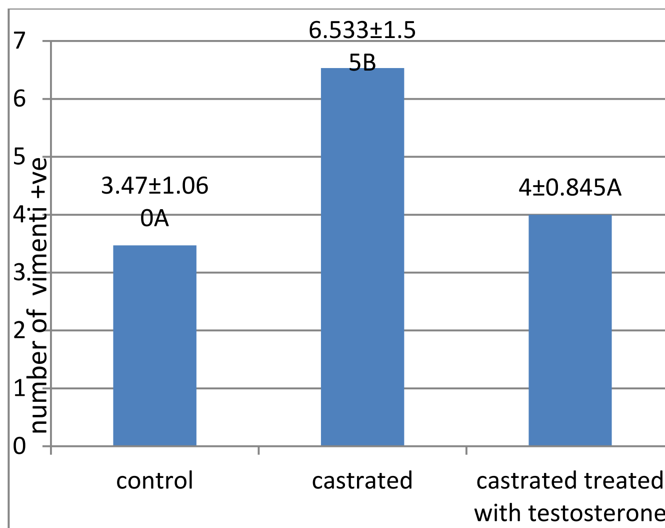


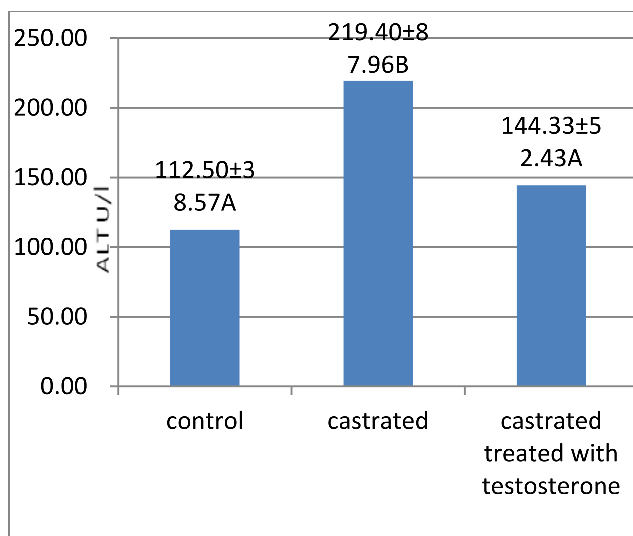
Figure (6)immunohistochemical stain liver section(castrated treated with testosterone group)



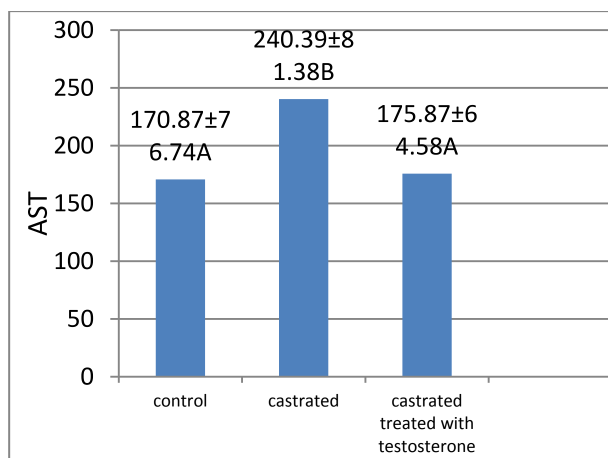
Histogram (1) comparison between different groups as regarded (mean±SD) of the number of viminten +ve cells

3.3. Liver enzymes

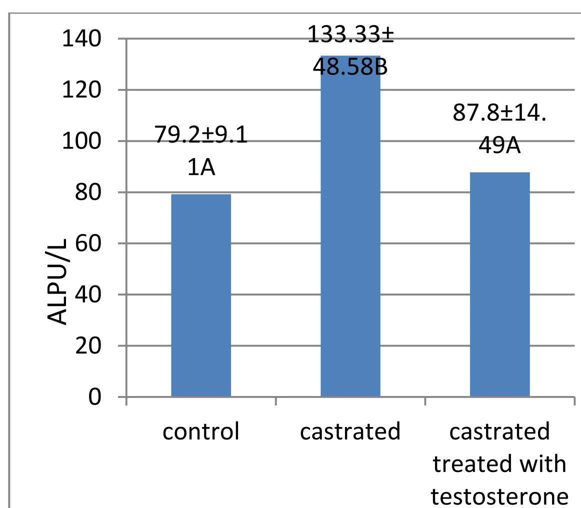
Results of the biochemical analysis showed that the castration group has a significant increase in levels (ALT, AST and ALP) as compared with the control group , concerning the testosterone group it was found there is no significant changes in levels of (ALT,AST and ALP) as compared with control group histogram (2,3 and 4)



Histogram (2) showing the level of ALT in different experimental groups Similar letters indicate no significant while different letters indicate significant compared treated vs. control group (six weeks) n=15 for each group



histogram (3) showing the level of AST in different experimental groups Similar letters indicate no significant while different letters indicate significant compared treated vs. control group (six weeks) n=15 for each group

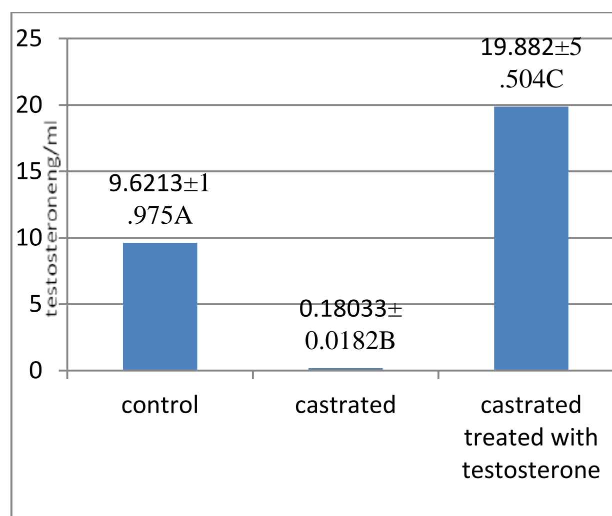


Histogram (4) showing the level of ALP in different experimental groups

Similar letters indicate no significant while different letters indicate significant compared treated vs. control group (six weeks) n=15 for each group

Testosterone hormone

Results of ELISA analysis showed that the castration group has significant decreased in level of (testosterone) as compared with the control group , concerning the castrated treated with testosterone group it was found there is significant increased in level of (testosterone) as compared with control group histogram (5)



Histogram (5) showing the level of testosterone in different experimental groups

Similar letters indicate non significant while different letters indicate significant compared treated vs. control group (six weeks) n=15 for each group

4.Discussion

Previous studies have detected the relationship between testosterone and fatty liver, and even less have tended to the potential connection between testosterone and NAFLD (21, 22)

Non alcoholic fatty liver disease (NAFLD) is a public health problem disease , and considered as the major common worldwide liver disease, the pathogenesis of (NAFLD) and nonalcoholic steatohepatitis (NASH) identified as accumulation of lipids and triglycerides within the hepatocytes in liver(steatosis) and the activation of (NAFLD) to(NASH) that is associated with other factors such as oxidant stress , mitochondrial injury , fatty acids lipotoxicity , innate immunity and inflammatory cytokines . steatosis is a characteristic histological features of (NAFLD) result from increased fatty acids accumulation or impaired fatty acids utilization in the hepatocytes (23)

Liver obtained from castration group in this research showed distributed of hepatic architectures was explained as a result of oxidative damage in hepatocellular proteins or necrotic changes in hepatocytes that lead to irregular in the orientation of the hepatocytes plate and distributing of hepatic architecturesas as in figure2(a) . In figure 2(b)showed accumulation of lipid inside the hepatocytes with peripheral nucleus this occur because there is An irregularity between the inflow of un saturated fats, lipid synthesis in the liver and β-oxidation can lead to fat deposition in hepatocytes (24) Hepatic fat accumulation can result from increased fat inflow to the liver, increased hepatic lipogenesis (including fatty acid de novo synthesis), (in the form of VLDL assembly and secretion)

macrovesicular steatosis this abnormalities explained as in delivery, metabolism, synthesis and export lipid (25).

Figure 2(c) showed cellular infiltrations with lymphocytes between hepatocytes this result as one of the features of steatohepatitis. it was reported that the adipocyte in fatty liver are considered as active cells that secrete multiple immune modulators factors in the form of pro-inflammatory cytokines, interleukin -6 and tumor necrotic factors (TNF) with reactive oxygen species (ROS)all these factors contribute to the chronic inflammatory condition and hepatocytes injury in addition , inflammation can be explained as in sever fatty liver change. simple steatosis may lead to fibrosis, steatohepatities with initiation of capillarization in the sinusoids, steatosis is characterized structurally by loss of fenestrate in the endothelial cells (sinusoids) association with development of basal lamina and deposition of collagen in the space of disse , this may be adhesion of leucocytes to sinusoidal endothelium , followed by infiltration of leukocytes into the hepatic parenchyma to form inflammatory foci (26,27)

In figure 2(d) showed vacuolation of hepatocytes was described as microvesicular however microvesicular steatosis associated with defective beta oxidation of fatty acids including mitochondrial cytopathies also decreased hepatic fatty acid, beta-oxidation, and/or reduced lipid export from the liver further more cytoplasmic vacuolation was attributed to lipid per-oxidation because of oxidative stress that damage cell membrane as well as cell organelles and disturbance of the ion concentrations in the cytoplasm of cell organelles, ballooned hepatocytes can be attributed to micro tubular disruption and several cell injury(28,29)

Hepatic stellate cells (HSCs) also known as perisinusoidal cells, lipocytes, fat-storing cells or Ito cells.They are investigated by accumulation intracellular lipid droplets and by filamentous cytoplasmic material. Newly vimentin has been considered a predominant IF (intemediat filamentous) protein in the cytoplasm of mesenchymal cells; thus, this protein has been considered as a mesenchymal marker of epithelial-mesenchymal transition. Detection by immunohistochemistry. In compared with the control group, castration group with fatty liver showed strong positive immune expression of HSC markers “vimentin.” This result could be explained by the findings of some researchers (30) who stated that vimentin expression in the liver is linked to liver fibrosis and inflammatory infiltration, as activated HSCs secreted cytokines that attracted inflammatory cells. Further, many researchers had been reported the progression of HSC may be associated with the role of ROS and oxidative stress in stimulating the expression of pro inflammatory and pro fibrotic molecules (31).

Previous studies (32) reported that testosterone deficiency increased serum hepatic enzyme levels, alanine aminotransferase (ALT) and aspartate aminotransferase (AST). And alkalin phosphastase, this increase was due to an increase in the production of free radicals that initiate lipid peroxidation. The cellular damage resulted from induction of cytochrome P-450 in the liver producing reactive trichloromethyl free radical. This in turn in the presence of oxygen generated by metabolic leakage from mitochondria cause lipid peroxidation of membrane leading to loss of integrity of cell membranes and damage of hepatic cells (33)

In our research testosterone therapy in castrated treated with testosterone group modulator the previous changes. These results agree with the findings of other researchers (34) the addition of ERα blockade Significantly diminished the beneficial effects of testosterone treatment on hepatic lipid accumulation suggesting actions via aromatization of testosterone to estrogen and subsequent ER activation. ERα blockade, however, did not completely abrogate these effects indicating that testosterone may also act, at least in part, via alternate non-classical AR-independent mechanisms. Indeed, further testosterone replacement therapy in men has been observed to have an anti-inflammatory effect, with a reduction in TNF-α, IL-1b, and sIL-6r levels and an increase in IL-10 (35).

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