Testosterone Replacement Protects Against Non-Alcoholic Fatty Liver Disease in Castrated Male Mice

Liqaa Oday Ali¹, Merza Hemza Homady²

Department of Basic Sciences Dentistry College Babylon University, Iraq.
Department of Biology College of Sciences University of Kufa Iraq.

*Corresponding Author: Liqaa Oday Ali

Abstract

The present work aimed to study histopathological, histochemical and biochemical changes in the liver of castrated male mice and effect of possible treatment with testosterone. For this purpose Forty five (45) male albino mice (3 weeks) aged were divided into 3 groups (n = 15). The Group I was used as a control, Group II was castrated group, Group III was castrated treated with testosterone group. For 6 weeks from period February to June 2017, lipid profile was measured. There are significant p ≥ 0.05 increased in (cholesterol, triglycerides, and low density lipoprotein LDL) levels, but there is no change in the level of HDL in the castrated group as compared with the control group. In addition, histopathological changes of the liver were examined. Castration enhanced lipid accumulation liver (macrovesicular steatosis), ballooning degeneration in hepatocytes all these changes associated with inflammatory cellular infiltration. Histochemically accumulation of lipid in hepatocytes were strongly positive to Sudan Black B reaction in liver of castrated mice as compared with the control group and these structural changes were less pronounced in animals treated with testosterone. From the present results, it can be concluded that testosterone replacement ameliorates role in non alcoholic fatty liver disease.

Keywords: Non-alcoholic fatty liver disease, Testosterone, mice, steatohepatitis, hepatic lipid accumulation.

Introduction

Non-alcoholic fatty liver disease (NAFLD) affects nearly one third of the General population [1]. NAFLD may progress from simple steatosis to steatosis with inflammation and Liver cell injury steatohepatitis (NASH) or further progress to cirrhosis and hepatocellular carcinoma [2]. NAFLD is strongly associated with testosterone deficiency, type 2 diabetes, obesity and metabolic syndrome [3; 4].

Higher body mass index in men is correlated with low total serum testosterone [5]. Approximately 20% to 50% men with steatosis and metabolic syndrome have hypogonadism. T treatment reverses the visceral fat accumulation that is associated with androgen deficiency [6]. Hypogonadal men are at higher risk to develop NASH, which may be attenuated by Treatment [7]. Clinically, low serum total testosterone level is independently associated with NAFLD [8]. Generally, the findings are supported by demonstration of hepatic steatosis in castrated male chickens and androgen receptor knock out (ARKO) male mice [9]. Hepatic lipid accumulation results from an imbalance of lipid production and turnover.

Sources of fat in the liver include fatty acids made by de novo synthesis, uptake of free fatty acids released by lipolysis, and fatty acids from exogenous (dietary) sources.) [10].

Lipid droplet formation from the ER (endoplasmic reticulum) mediated by specific lipid droplet proteins may also be implicated in formation of macro-vesicular droplet in NAFLD/NASH [11]. Hepatocyte damage, apoptosis, and inflammation also play key roles during NAFLD development and progression. It is clear that T reduces hepatic fat, apoptosis, and inflammation, the objective of this study was to determine the protective effect of testosterone against steatosis and steatohepatitis in castrated...
mice. We hypothesized that the development of steatohepatitis in castrated male mice may result from a combination of increased lipid production, increased macro-vesicular lipid droplet formation, but the testosterone-replacement would ameliorate the severity of fat accumulation, cell apoptosis, and inflammation through reversal of these mechanisms.

**Materials and Methods**

**Chemicals**

(Testosterone was obtained from LTD, Austutalian, other reagents (Sudan black b, obtained from Sigma, USA). Hematoxylin &Eosin obtained from Fulka, Switzerland and solutions obtained from Sigma, USA)

**Animals**

Swiss albino Male mice weighting between (14-20) g and aged (3weeks) were used in present study, the mice were obtained from Animal House Faculty of Science/ University of Kufa, animals were kept in ventilated cages, with temperature of (25±2Cº) A 12:12 h light dark cycle was also regulated for these animals balanced rodent food pellet and water was provide ad libitum [12]

Animals were scarified at the end of experiment, [13]

**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.[14]

**Sudan Black B Solution**

This stain was prepared by dissolving 0.7 g in 100 ml propylene glycol with stirring at 100 °C for a few minutes, and then the solution was filtered through Whitman# 2 filter paper. This solution is stable for one year

**Surgical Castration**

mice were anesthetized(given intra pritonal) 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 testicales 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. [15]

**Experimental Design**

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

Group 1: Intact male mice received tap-water as a control (N=15).

Group 2: Castrated male mice (N=15)

Group 3: Castrated male mice treated with 50µg/kg/day of Testosterone. (Treated daily for 6week s with 0.1ml) (N=15)

After 42 days, overnight fasting animals were euthanized under mild ether anesthesia. Blood was collected from a puncture to the heart using syringes into clean dried centrifuge tubes. The tubes were then centrifuged at 3000rpm for 15 minutes, the serum samples were carefully separated using pasture pipette and frozen at -20 until biochemical analysis,(cholesterol, triglycerides, HDL, and LDL) were measured with seamaty kits that were obtained from(bio diagnostic ,china)

**Preparation of Tissues**

**Preparation for Paraffin Sections for Light Microscopic Study**

After scarified the animals the liver was removed and immediately fixed in10% formaldehyde solution for 24 hours dehydration was then carried out in ascending of alcohol followed by with xylool 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 [16].

**Frozen Section Preparation**

Fresh tissues were washed in PBS before mounting in OCT (Optimum Cutting Temperature). Tissues were mounted on metal chucks. The tissues were sectioned at 4 µm in -24°C, and then collected on charged microscopic slides. Slides were allowed to air dry for 30min prior to stain with be dried,The frozen sections were fixed with 10% formalin, then they were washed well in distill water to drain off excess water, propylene glycol was
added for 5 minutes, and Sudan Black was added for 7 minutes. At last Propylene glycol 85% was added for 3 minutes with washing in distill water. The sections were mounted with aqueous mounting media, Glycerin Jelly. [17]

Statistical Analysis
Data were expressed as Mean ±S.E. statistically analysis were performed using one way Anova followed by Duncan test for multiple comparison by using SSPS version 16 computer program the p ≤ 0.05 were considered significant for all data.

Results
H&E stain
Group I (control group)
A histological sections of male mouse liver (control) for 6 weeks period show normal histological structure as in Figure1 (a&b) and can identify the boundaries of the liver lobule by locating the central vein at its centre. The hepatocytes appear with regular arrangement in section and are arranged in plates one cell thick, usually, they separated by sinusoids. The hepatic sinusoids usually contain some blood cells and kupffer cells.

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 degrees of cytoplasmic vacillation some contain multiple small vacuoles and other have large vacuoles (macro &micro vesicular steatosis) the other appeared ballooned with peripheral nuclei as in Figure2 (a, b, c and d) Group III (castrated treated with testosterone) showed similar to the control group and showed the normal histological structure of the liver. Figure 3

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) (H&E 200X ,400X)

Figure 2: Liver sections for castrated group showed a) disturbed hepatic architecture with ballooning hepatocytes .b)lipid accumulation (macrovesicular steatosis) .c) cellular infiltration between hepatocytes with narrowing blood sinusoids .d) ballooned hepatocytes with micro vesicular steatosis 200 xs
Frozen Sections Stained with Sudan Black B

Most of lipid droplets stained with Sudan black B was distributed in the hepatocytes within liver. In control group as in Figure 4 (a & b) shows (moderate intensity to lipid droplets). Castrated group have (strong intensity to stain lipid droplets) as in Figure (5). Castrated treated with testosterone group in male mice (moderate intensity to lipid droplets) as in Figure (6).
Biochemical test (lipid profile)

Results of the biochemical analysis (M±SD) showed that the castration group has a significant increase in levels (cholesterol, triglycerides and LDL), with no changes in HDL Level as compared with control group more over there is no significant changes in castrated treated with testosterone as compared with control group histogram (1, 2, 3 and 4)

Histogram 1: showing the level of total cholesterol in different experimental groups similar letters indicate no significant with different letters indicate significant compared treated vs. control group (six weeks) n=15 for each group
Discussion

Our data investigated that the histological section of male mice obtained from castrated liver mice (a and b) show histologically lipid accumulation (micro vesicular to macro vesicular steatosis) hepatocytes degeneration, cellular chronic inflammation, severe degeneration with ballooning degeneration. The accumulation of hepatic triglycerides is closely related to the development of liver injury.

The morphological sections confirmed the accumulation of intrahepatic lipids, which were mainly triglycerides. Accumulation of lipid inside the hepatocytes with peripheral nucleus this occurs because there is an imbalance between the inflow of fatty acids, lipid synthesis in the liver and β-oxidation can intensify fat deposition in hepatocytes [18] Hepatic fat accumulation can result from increased fat supply to the liver, increased hepatic lipogenesis (including fatty acid de novo synthesis), in the form of very low density lipo protein (VLDL) assembly and secretion. Macro vesicular steatosis this abnormality explained as inside delivery, metabolism, synthesis and export lipid [19].
In Figure 2(c) show cellular infiltrations with lymphocytes between hepatocytes this result as one of 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 severe fatty liver disease simple steatosis may progress to fibrous steatohepatitis with initiation of capillarization in sinusoids, sinusoids are characterized structurally by progressive loss of fenestrate in the sinusoidal endothelial cells concomitant with the development of basal lamina and deposition of collagen in the space of dissection this may be accompanying adhesion of leucocytes to sinusoidal endothelium, followed by leucocytes infiltration into the hepatic parenchyma to form inflammatory foci [20,21].

In Figure 2(d) showed vacuolation of hepatocytes was described as micro vesicular however micro vesicular steatosis associated with defective beta oxidation of fatty acids including mitochondrial cytopathies also reduced hepatic fatty acid beta-oxidation, and/or suppressed 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 [22,23].

The Histochemical and morphological (stained with Sudan black B) method confirmed the accumulation of intrahepatic lipids, which were mainly triglycerides. Histochemically current results showed that the lipid accumulation in hepatocytes were strongly reaction to Sudan black b as in Figure 5 but in figure 4 and 6 show there is moderated reaction to Sudan black b this results confirmed steatosis in castrated group. [24, 25, 26].

Previous study revealed that castration-induced testosterone deficiency results in elevated serum cholesterol levels in pigs and rats fed a normal diet [27, 28, and 29]. In addition to its effects on serum cholesterol levels, testosterone deficiency also caused a significant increase in serum (triglycerides) TG levels in castrated group. TGs in the liver are packaged into very low-density lipoproteins (VLDLs) and exported to the circulation. Excessive production of TG-rich VLDL (VLDL-TG) contributes to hypertriglyceridemia [30].

Many genes are involved in the assembly and secretion of VLDL-TG, such as the microsomal triglyceride transfer protein (MTP) and Apo lipoprotein A-V (Apo A-V). Previous studies showed that testosterone regulates hepatic MTP expression in rats and mice Apo A-V was recently identified as an important modulator of TG metabolism and shown to lower plasma TG levels in both humans and animals [31, 32].

The results of recent studies have shown decreased Apo A-V expression in obese subjects. Moreover, the treatment of hepatocytes cells with insulin decreased Apo-V expression Testosterone deficiency is associated with obesity and insulin resistance.

Thus, testosterone deficiency may affect serum TG levels by altering the expression of genes involved in lipoprotein assembly and secretion. [33, 34]

It is well known that LDLR (low density of lipoprotein receptors) is a crucially important modulator of plasma LDL-cholesterol levels in humans and animals. Increased LDLR expression was shown to result in reduced serum LDL-cholesterol levels [35].

Previous study found that LDLR mRNA expression and protein levels were significantly decreased in the livers of castrated pigs, compared to intact pigs.

This agreed with our findings that the castrated mice showed a significant increase in both serum LDL-cholesterol and TC levels. Moreover, these findings suggest that one of the mechanisms through which testosterone deficiency can increase serum cholesterol levels in castrated group is by decreasing LDLR expression, thus inhibiting the removal of LDL-cholesterol from the circulation [36].
References


