The immune and metabolic treatment approach of testosterone on mice model of liver injury

Johnny Amer[1*], Ahmad Salhab[1], Hadeel Snobar[1]

[1]An-Najah National University, Palestine

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Author contribution statement

JA contributed to the study design, manuscript writing, and revision. AS contributed to practical work, and revision. HS contributed to implementation of the experiments and data analysis. All authors contributed to the article and approved the submitted version.

Keywords

liver injury, Testosterone, NK cells, IL-6, IL-6 receptor

Abstract

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Background: Natural killer (NK) cells showed an anti-fibrotic effect; however, their function is thought to be impaired in advanced liver injury. In the current study, we aimed to assess the immune and metabolic impact of testosterone on mice model of liver injury. Methods: Carbon-tetrachloride (i.p injected) of acute (2 weeks) and chronic (4 weeks) models of male mice (n=108) of liver injury was performed. Testosterone (4 mg/kg mouse body weight) was injected i.p following the first week of acute model of CCl4 and following the second week of the chronic model of CCl4. At the end of experiments, mice were sacrificed, and serum were collected for assessing liver enzymes of ALT, AST, inflammatory marker of IL-6, metabolic makers of C-peptide levels as well as for lipid and glucose profiles. Livers were harvested and used for histological assessments for inflammation and for fibrosis. Fibrosis profile from liver extracts; aSMA and Collagen III, were assessed by RT-PCR. Moreover, liver tissue-resident NK cells were isolated and evaluated for their activity through assessing INF-g and IL-6 receptor by the ELISA and flow-cytometry respectively. Results: Serum ALT, AST, IL-6, and metabolic assessments of cholesterol, triglyceride, C-peptide, fasting blood sugar, and fibrotic profiles were linearly correlated with disease progressions. Histological characterization of the liver was worsened in the chronic model of liver injury. Testosterone-treated mice exhibit a significant reduction in collagen depositions with less dense fibrosis tissue associated with reduced liver injury enzymes and metabolic markers in both the acute and the chronic CCl4 mice model in favor of the later one ($P<0.05$). Moreover, testosterone treatments displayed significant decrease in serum IL-6 of 2.4-fold ($p=0.0001$) and 2.3-fold ($p=0.0003$) in the acute and chronic models, respectively ($p=0.002$) and data were associated with increase in INF-g release from NK associated with a reduction in their IL-6 receptor expressions ($P<0.05$). Conclusion: Our results showed effects of testosterone on mediating a decreased expressions of NK IL-6 receptors and consequently induced their activation, results that in part could explain the amelioration in liver injury findings. Our results suggest an anti-inflammatory and anti-fibrotic treatment approach of testosterone for delaying disease progressions.

Contribution to the field

Testosterone therapy is a relatively common treatment for men with documented testosterone deficiency. Several studies suggest that long-term testosterone therapy in hypogonadal men improves liver function, however, the immune and metabolic influence of testosterone therapeutic approach was not addressed previously. The increase in the interests of the role of immune cells such as NK cells become of a clinical significance. These cells exhibit an anti-fibrotic effects and in advanced liver injury they become impaired and this could lead to propagation of the disease. Manipulation of NK cell activation has become a potential liver injury target and moreover the use of testosterone could be a novel therapy in particularly in men and athletics. Our results showed amelioration in liver injury findings following testosterone treatments. These data were mediated through a decreased expressions of NK IL-6 receptors and consequently induced their activation. Our results suggest an anti-inflammatory and anti-fibrotic treatment approach of testosterone for delaying disease progressions.

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Data availability statement

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The immune and metabolic treatment approach of testosterone on mice model of liver injury

Johnny Amer[1]†, Ahmad Salhab[2], Hadeel Snobar[3], Yazan AlHabil[4]

[1] Department of Allied Sciences, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, Palestine. Johnny Amer: j.amer@najah.edu

[2] Department of Biomedical Sciences, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, Palestine. Ahmad Salhab: ahmad.salhab@najah.edu

[3] Department of higher education, Faculty of Medicine and Health Sciences, An-Najah National University, P.O. Box 7, Nablus, Palestine. Hadeel Snobar: hadeelsnober04@gmail.com

[4] Department of Medicine, Faculty of Medicine and Health Sciences, An-Najah National University, Nablus, Palestine. Yazan AlHabil: yazanalhabil@gmail.com

† **Correspondence:**

Dr. Johnny Amer

Department of Allied Sciences, Faculty of Medicine and Health Sciences, An-Najah National University, P.O. Box 7, Palestine. Mobile: +972-523718231, j.amer@najah.edu

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Abstract

Background: Natural killer (NK) cells showed an anti-fibrotic effect; however, their function is thought to be impaired in advanced liver injury. In the current study, we aimed to assess the immune and metabolic impact of testosterone on mice model of liver injury. **Methods:** Carbon-tetrachloride (i.p injected) of acute (2 weeks) and chronic (4 weeks) models of male mice (n=108) of liver injury was performed. Testosterone (4 mg/kg mouse body weight) was injected i.p following the first week of acute model of CCl_4 and following the second week of the chronic model of CCl_4. At the end of experiments, mice were sacrificed, and serum were collected for assessing liver enzymes of ALT, AST, inflammatory marker of IL-6, metabolic makers of C-peptide levels as well as for lipid and glucose profiles. Livers were harvested and used for histological assessments for inflammation and for fibrosis. Fibrosis profile from liver extracts; αSMA and Collagen III, were assessed by RT-PCR. Moreover, liver tissue-resident NK cells were isolated and evaluated for their activity through assessing INF-γ and IL-6 receptor by the ELISA and flow-cytometry respectively. **Results:** Serum ALT, AST, IL-6, and metabolic assessments of cholesterol, triglyceride, C-peptide, fasting blood sugar, and fibrotic profiles were linearly correlated with disease progressions. Histological characterization of the liver was worsened in the chronic model of liver injury. Testosterone-treated mice exhibit a significant reduction in collagen depositions with less dense fibrosis tissue associated with reduced liver injury enzymes and metabolic markers in both the acute and the chronic CCl_4 mice model in favor of the later one ($P<0.05$). Moreover, testosterone treatments displayed significant decrease in serum IL-6 of 2.4-fold ($p=0.0001$) and 2.3-fold ($p=0.0003$) in the acute and chronic models, respectively ($p=0.002$) and data were associated with increase in INF-γ release from NK associated with a reduction in their IL-6 receptor expressions ($P<0.05$). **Conclusion:** Our results showed effects of testosterone on mediating a decreased expressions of NK IL-6 receptors and consequently induced their activation, results that in part could explain the amelioration in liver injury findings. Our results suggest an anti-inflammatory and anti-fibrotic treatment approach of testosterone for delaying disease progressions.

Introduction

Testosterone has been shown to adjust the carbohydrates, fats and proteins metabolism and effect muscle growing and adipogenesis (Yassin A *et al.*, 2019). As the major males circulating androgen, Testosterone provides for a variety of biological processes in many tissues and organs such as muscle and bones (Kelly DM *et al*., 2013). Testosterone therapy has become a moderately common treatment for men suffering from testosterone deficiency (Morgentaler A *et al.*, 2018). However, testosterone therapy is the standard practice in otherwise healthy hypogonadal men, with prostate cancer history (Natale C et al, 2021). Up to 90% of men with liver cirrhosis have decreased serum testosterone levels, which continue to decline as the liver condition worsens (Sinclair M *et al*, 2015). Advanced liver illness shares many characteristics with hypogonadal males, such as sarcopenia, osteoporosis, gynecomastia, and reduced libido. (Yurci A *et al*., 2011). Al-Qudimat A *et al.* suggest that long-term testosterone therapy in hypogonadal men improve liver function. (Al-Qudimat A *et al*., 2021). However, it is not fully proven how much testosterone deprivation contributes to the symptoms of severe liver disease. Natural killer (NK) cells play critical roles in innate immune defense against bacterial, viral, and parasitic pathogens, as well as tumor suppression through the natural cytotoxicity and cytokine secretion (Wei Y *et al*., 2022), (Fasbender F *et al.*, 2016). Manipulation of NK cell activation has become a potential liver fibrosis immunotherapy, such as adoptive transfer of allogeneic NK cells, genetic engineered NK cells, NK cell-targeted chemotherapy and others (Zhang Y *et al.* 2022). In our current study, we aimed to assess the molecular and metabolic aspects of Testosterone and their modulatory effects on liver tissue resident NK cells phenotype activations in mice model of liver injury.

Methods

Experimental design

C57BL/6 male mice at week 12 of age and weighted 22.5±1.5 g received care according to the An-Najah National University ethical guidelines. All animal protocols were approved by institutional animal care ethical committee (Ref: Med. Oct/2018/59).

Testosterone effects on liver injury

Liver injury mice model was induced using carbon tetrachloride (CCl_4; Sigma, C-5331) introduced by i.p injections of 0.5 µl pure CCl_4/g body weight (one to nine dilution in corn oil) twice a week for two and four weeks as an acute and advanced chronic liver injury. In the middle of the liver injury duration (two week) in the chronic model and one week in the acute model, mice were i.p injected with

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testosterone (Merck; T1500; purity ≥ 98%) in the concentration of 100 µg/mouse [4 mg/kg mouse body weight] twice a week for the rest weeks. In all experiments, mice were sacrificed two days after the final CCl_4 injection. To this end, the animals were weighed and anesthetized with inhaled 5% isoflurane for 10 seconds before cervical dislocation.

Mice groups

The following mice groups were included: (A) Naive mice (mice untreated neither CCl_4 nor testosterone), (B) Mice group treated with testosterone only, (C) CCl_4-treated mice-acute liver injury mode (two-week injections), (D) CCl_4-treated mice-acute liver injury and treated with testosterone, (E) CCl_4-treated mice-chronic liver injury mode (4-week injections), (F) CCl_4-treated mice-chronic liver injury and treated with testosterone. Each experimental group included 6 mice and was repeated 3-times (a total of 108 mice).

Histological assessment

The posterior third of prostate and liver tissues were fixed in 3% formalin overnight and then embedded in paraffin in an automated tissue processor. Sections (7 µm) were stained with H&E to assess steatosis, areas regions of necroinflammation, and apoptotic bodies, and with 0.1% Sirius red F3B in a saturated picric acid stain (Abcam, ab150681) to visualize connective tissue. A veterinary pathologist assessed all histopathological findings and reported assessments and the grade of the assessment.

Serum biochemical assessments

Peripheral blood from the heart were collected on the sacrifice day was centrifuged at 5000 rpm for 15 minutes at 4°C to obtain the serum. Serum ALT (Abcam; ab285263), AST (Biocompare; MBS2019147), Fasting blood sugar (Biocompare; MBS7200879), C-peptide (Biocompare; MBS007738), cholesterol (Abcam; ab285242), and triglycerides (Biocompare; MBS726589) were determined using ELISA kits according to the manufacture protocols.

RNA isolation, cDNA preparation, and real-time PCR

RNA was obtained from liver tissue using trizol buffer (Bio-Lab; Cat# 90102331). Liver tissues were homogenized at RT, and 0.2 ml chloroform (Bio Lab; Cat# 03080521) was added. The samples were then incubated for 15 minutes at room temperature and centrifuged (1,400 rpm) for 15 minutes at 4°C. For RNA precipitation, the supernatant in each sample was transferred to a new micro-centrifuge tube,

and 0.5 ml of isopropanol (Bio Lab; Cat# 16260521) was added, followed by 10 minutes incubation at 25°C. The tubes were then centrifuged (12,000 rpm) for 10 minutes at 4°C, the supernatants were removed, and one ml of 75% ethanol was added to the pellet, followed by centrifugation (7,500 rpm) for 5 minutes. The pellets were air-dried at room temperature for 15 minutes, 50 µl of DEPC was added, and the samples were heated for ten minutes at 55°C. RNA purification from NK cells were assessed using RNeasy plus mini kit (CAT# 74034) according to manufacturer's guidelines. cDNA was obtained using High-Capacity cDNA Isolation Kit (R&D; Cat# 1406197). RT-PCR reactions were performed using TaqMan Master Mix (Applied Biosystems; Cat# 4371130) to quantify *αSMA*, *collagen III* mRNA levels, Results were normalized to *gapdh* as a housekeeping gene and analyzed using QuantStudio™ 5 Real-Time PCR System.

ELISA

Serum levels of testosterone and estradiol were assessed using abcam; ab285350 and Creative diagnostics; DEIA04927, respectively. Moreover, intracellular IL-6 and IFN-γ concentrations were assessed using Human IL-6 Quantikine ELISA Kit (R&D; D6050), Human IFN-γ Quantikine ELISA Kit (R&D; 285-IF), according to the manufacture protocols.

Liver tissue-resident NK (trNK) cells isolation

The livers were extracted and placed in Petri dishes containing 10 ml of DMEM medium (Biological industries; Cat# 01–055-1A). The liver tissue was thoroughly dispersed using a stainless-steel mesh, and the cells were collected along with the medium and transferred to 50 ml tubes containing 10 ml of DMEM. Subsequently, the cells were cautiously moved to new tubes containing Ficoll (Abcam; Cat# AB18115269) and subjected to centrifugation at 1600 rpm for 20 minutes at 20°C. The resulting supernatant from each tube was transferred to fresh tubes and centrifuged again at 1600 rpm for 10 minutes at 4°C. Following the second centrifugation, the cell pellet in each tube was resuspended in 1 ml of DMEM to isolate and purify NK cells using the Stem Cells kit (Cat# 19665).

Flow cytometry

The trNK cells isolated from harvested mice liver were diluted to a concentration of 1 million cells per milliliter in a saline buffer supplemented with 1% bovine albumin (Biological Industries; Cat# 02–023-5A). Subsequently, the cells were labeled with the following antibodies. Anti-mouse NK1.1 (murine NK cell marker) (Biogems; Cat# 83712–70), anti CD49a (MACS; Lot# 5150716246), anti

CD49b (MACS; Lot# 5150716256), anti-mouse lysosomal-associated membrane protein-1 (CD107a; NK1.1 cells cytotoxicity marker, eBioscience, Cat# 48–1071) and anti-IL-6 R (R&D; Cat# 48–1044) were used. All antibodies were incubated for 40 min at 4°C. pHSCs (106 cells/mL) were stained with rabbit anti-mice αSMA (R&D; IC1420P). The cells were washed with 0.5 ml staining buffer and fixed with 20 ml 2% paraformaldehyde. All stained cells were analyzed with a flow cytometer (BD LSR Fortessa™, Becton Dickinson, Immunofluorimetry systems, Mountain View, CA).

Statistical analysis

Statistical differences were analyzed with a two-tailed unpaired Student's t test (for comparisons between two groups) or one-way or two-way analysis of variance (ANOVA; one-way ANOVA with the Newman–Keuls post hoc test for comparisons among multiple groups) with GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA). A t-test of p value ≤ 0.05 is considered statistically significant and was calculated as the difference in means between two variables. A Mann-Whitney U test was performed to evaluate whether the mice metabolic panel elements (ALT, AST, Cholesterol, Triglyceride, and FBS levels) altered following testosterone treatment in both the acute and chronic liver injury groups. The correlation co-efficient r test and normality test [the Shapiro-Wilk test] was used (p value≤ 0.05 is considered statistically significant). The experiment was repeated three times, with each repetition consisting of ten sample replicates. Results are presented as mean ± SD or as average means of experimental replicates ± SD.

Results

Testosterone ameliorates inflammatory and fibrotic profiles of CCl_4 liver injury mice model

Liver sections from mice with acute and chronic CCl_4-induced liver injury were evaluated for liver injury and phenotypic changes after treatment with testosterone. Representative images of H&E staining (Figure 1A) and Sirius Red staining (Figure 1B) of liver sections depicting acute and chronic liver injury are shown. The H&E staining of CCl_4-treated livers revealed centrilobular hepatocytes that were swollen, along with extensive necrotic areas containing a high number of infiltrating inflammatory cells (white arrows left) and steatosis (white arrows right), indicating the presence of a chronic CCl_4 model. However, in mice treated with testosterone, there was a delay in the appearance of these histological findings, and a significant reduction in both microvascular and macrovascular steatosis was observed specifically in the chronic model. Sirius red staining of livers from the CCl_4

mice exhibited increased collagen deposition in perisinusoidal areas in both the acute and chronic CCl_4 models (black arrows), but the impact of collagen depositions was more pronounced in the chronic model. Treatment with testosterone resulted in a remarkable reduction in the dense fibrous tissue of the stained area as compared to the vehicle-treated mice. Figure 1C summarizes a detailed histology scoring system for H&E and fibrosis assessments (M. C. Wallace et al, 2015)(P. J. Scheuer, 1991)(P. Bedossa, 1996)(K. Ishak *et al.*, 1995). Biochemical markers were also assessed in our mice groups. Serum inflammatory profiles of ALT (Figure 1D) and AST (Figure 1E) were elevated in both acute and chronic CCl_4 model in favor of the later one. A significant amelioration in ALT levels of 2.1-folds, and 3.6-folds were achieved in the acute and chronic CCl4 model following testosterone treatment, respectively ($P<0.05$). Similar effects of testosterone treatment were achieved only in the chronic CCl4 model of 2.6-folds while no effects were seen in the acute model. To confirm liver fibrosis in CCl_4-induced mice, fibrosis markers were quantified by assessing liver αSMA (Figure 1F) and collagen III (Col III) (Figure 1G) using RT-PCR. The data showed a significant increase in αSMA and Col III levels in both the acute and chronic CCl_4 models compared to the vehicle group, with a 1.2-fold increase in the acute model and a 4.2-fold increase in the chronic model for αSMA, and a 1.2-fold increase in the acute model and a 3.2-fold increase in the chronic model for Col III ($p=0.002$). However, mice with liver fibrosis receiving testosterone treatment exhibited significant reductions in αSMA and Col III levels, with a 1.2-fold decrease and a 1.3-fold decrease, respectively ($P<0.03$), in the acute CCl_4 model, and a 2.2-fold decrease and a 2.3-fold decrease, respectively ($P<0.03$), in the chronic CCl_4 model. The results obtained from both RT-PCR and histology assessments were comparable and clearly indicated an improvement in liver injury, inflammation, and fibrosis in liver sections following treatment with testosterone. To elucidate effects of testosterone on alleviating histopathological findings of liver sections; we assessed for serum testosterone and estradiol levels. Testosterone could be metabolized to estradiol through aromatization (Roncati, L. *et al.*, 2016). In males, testosterone is the major source of plasma estradiol, the main biologically active estrogen, only 20% of which is secreted by the testes. Plasma estrone, 5% of which is converted to plasma estradiol, originates from tissue aromatization of, mainly adrenal, androstenedione (Vermeulen, A. *et al.*, 2002).

Supplementary 1 display serum testosterone (A) and estradiol (B) following testosterone treatment. Low serum testosterone levels were significantly obtained following CCl_4 inductions as compared to untreated mice and were positively correlated with liver fibrosis severity. Testosterone treatment elevated serum testosterone levels and were comparable in all mice groups including the control group (untreated mice). In parallel, same pattern of estradiol serum levels were achieved in untreated mice

and have shown reductions in their levels along liver fibrosis severities. Testosterone treatment induced elevated estradiol levels and were positively correlated to liver fibrosis severity of chronic CCl_4 inductions (2.3-folds, p=0.0001). Estradiol levels although increased following testosterone treatment, they remained within the normal range (Ström JO. *et al.*, 2012) highlighting the importance of testosterone in delaying liver fibrosis.

Testosterone improves metabolic assessments of liver injury mice model

CCl_4 administration in C57BL/6J mice exacerbates high cholesterol levels and induces steatohepatitis changes in the liver. Previous research by Bassi et al. (2005) demonstrated that CCl_4 leads to increased hepatic lipid profiles of cholesterol, fatty acids, and triglycerides in both chronic and acute treatments in rats. Based on these findings, we utilized this model to examine the metabolic outcome markers of lipid and glucose profiles following treatment with testosterone. Our mice model exhibited perturbations in the metabolic profile of CCl_4-induced animals. In Figure 2, we observe elevated serum levels of cholesterol (Figure 2A), triglycerides (Figure 2B), C-peptide (Figure 2C), and fasting blood sugar (FBS) (Figure 2D) in both the acute and chronic treatments of CCl_4 mice, with a more significant impact in the chronic model. However, the liver injury mice treated with testosterone demonstrated lower serum levels of cholesterol, triglycerides, and C-peptide compared to the control groups receiving the vehicle, while also displaying a reduction in FBS levels (Figure 2D).

Testosterone decrease IL-6 concentrations liver injury mice model of CCl_4

Altogether, the above data indicate that testosterone has an antifibrotic effect, most probable due to their effects in ameliorating lipid and glucose profiles, both of which are risk factors contributing to fibrogenesis. Thus, our results indicate testosterone as a potential target for delaying and inhibiting liver injury through improving insulin sensitivity. To further explore the mechanism behind the antifibrotic effects of testosterone, we further assessed the inflammatory and immune contribution in alleviating liver injury. We assessed serum IL-6 levels, activity of isolated liver tissue-resident NK (trNK) cells, and the expression of IL-6 receptors on trNK cells. Testosterone exhibited immune-modulating properties, supported by in vitro evidence suggesting its potential to suppress the expression of proinflammatory cytokines such as TNFα, IL-1β, and IL-6, while enhancing the expression of the anti-inflammatory cytokine IL-10 (Arslan et al., 2016). Furthermore, testosterone displayed anti-inflammatory effects by significantly inhibiting adipose tissue formation and downregulating the expression of various adipocytokines, including leptin, TNF-α, IL-6, and IL-1, while positively correlating with adiponectin levels. Conversely, low testosterone levels were

associated with increased expression of inflammatory markers. In our research study, presented in figure 3 displayed both naïve mice treated and untreated with testosterone had comparable low levels of serum IL-6 of 65±10 pg/ml (p=ns). Serum IL6 showed an increase in their levels within liver injury severities of 180±24 pg/ml and 345±52 pg/ml in the acute and chronic models, respectively (p=0.002). Testosterone treatments exhibited a significant decrease of 2.4-fold (p=0.0001) and 2.3-fold (p=0.0003) in the acute and chronic models, respectively (p=0.002). Testosterone has an anti-inflammatory effect due to the reduction of inflammatory cytokines (V. E. Bianchi, 2019).

Testosterone treated CCl_4-mice showed liver recruitment of trNK cells and restored their activity

The immune system plays a crucial role in the process of tissue healing. Consequently, managing the immune system is essential for effective planning of the healing process. In our study, we examined liver tissue-resident NK cells (trNK) isolated from the different mice groups. NK cells have been shown to possess antifibrotic properties by eliminating activated hepatic stellate cells (HSCs) (Muhanna et al., 2008). However, it is believed that their functionality may be impaired in cases of advanced liver injury (Amer et al., 2020; Salhab et al., 2020). Figure 4A shows an inverse correlation between trNK secretions of IFN-γ and liver injury severity of 2-fold-decrease (p=0.003). Following testosterone treatment, trNK secretions of IFN-γ showed higher levels of 2.1 and 6.3 folds in the acute and chronic model, respectively. Regarding the correlation co-efficient r test, Figure 4A's Pearson's correlation coefficient (-0.675) provides evidence for a large inverse strength of association between the variables. Same data patterns were obtained using NK activation marker of CD107a. Moreover, to further associate trNK activatory effects following testosterone treatment with IL-6 receptor. Flow cytometry analysis was performed as indicated in Materials and Methods. Figure 4B shows a significant reduction of 1.6-fold and 2.5-fold in IL-6 receptor in the acute and chronic liver injury model, respectively, as compared to mice receiving the vehicle (p=0.0001).

Our results undoubtedly indicate the impact of testosterone as a potential therapy as they exhibit antifibrotic and anti-inflammatory effects by reducing αSMA mediated by increased NK activity and reductions in their IL-6 receptor and could be of beneficial influence for patients with advanced liver injury.

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Discussion

In this study, we investigated the potential beneficial effect of testosterone on hepatic histopathological, immunological, and biochemical changes in a CCl_4-induced mice model. Several studies have showed a relationship between testosterone and liver injury (A. Al-Qudimat *et al*., 2021), even though testosterone treatment improves liver injury and metabolic syndrome, and type two diabetes mellitus are well-documented (S. Sun *et al*., 2021), the testosterone effects on hepatic injury are still limited. In the present study, we conducted an experimental prospective study on the effect of testosterone treatment on liver injury through assessed liver enzyme, metabolic and oxidative stress and real time PCR for liver and prostate. Their study used a model of CCl_4- encourage hepatotoxicity and showed precautionary and therapeutic management of these substances (E. M. B. El Naggar *et al*., 2015). In addition, previous studies showed that testosterone has an anti-inflammatory effect and improves liver injury (D. E. Ochayon *et al.* 2021).

Lipids and glucose are major risks in the pathogenesis of liver injury and are associated with morbidity due to diabetes and atherosclerosis. Free cholesterol activates HSCs, and the addition of cholesterol to a high-fat or methionine/choline-deficient diet leads to the accumulation of free cholesterol in HSCs, which accelerates experimental liver fibrosis (K. Tomita *et al.*, 2014). High hepatocyte lipid droplet accumulation in the livers propagates liver injury and causes a storm of pro-inflammatory cytokines that can lead to steatosis and hepatocyte injury (L. Chin *et al.* 2020). Previous studies have demonstrated that testosterone has effects on various enzymatic pathways involved in fatty acid metabolism, glucose control, and energy utilization. These effects can be tissue-specific, with different outcomes observed in different fat depots, muscles, and the liver. Testosterone treatment has been shown to have beneficial effects on obesity-related measures, partially attributed to its direct metabolic actions on adipose tissue and muscles, as well as potentially increasing motivation and energy levels, leading to more active lifestyles in obese individuals (Kelly et al., 2015).

CCl_4 is well-known for its hepatotoxic effects1. CCl_4 disrupts the structural integrity of hepatocyte membranes and causes cellular death. This damage triggers inflammation and activates signaling pathways involved in tissue repair (Liu F. *et al*., 2021). Moreover, CCl_4 generates reactive oxygen species (ROS) in the liver, leading to oxidative stress causing damage to cellular components, including lipids, proteins, and DNA. In addition, it activates immune cells, such as Kupffer cells and infiltrate neutrophils, which release pro-inflammatory cytokines and chemokines (Khan HA *et al*., 2017). This inflammatory response contributes to tissue damage and can exacerbate liver injury5. Prolonged or repeated exposure to CCl_4 can lead to liver fibrosis, a condition characterized by excessive

accumulation of scar tissue in the liver. CCl_4 promotes the activation of hepatic stellate cells, which are responsible for producing excessive extracellular matrix components, leading to fibrosis development (Wang T *et al*., 2021). Since liver diseases are typically multifactorial and involve a combination of genetic, environmental, and lifestyle factors, one of the limitations of the CCl_4 mice model is the lack of fully capture the complexity of human liver diseases.

In our study, we observed improvements in metabolic markers such as cholesterol, triglycerides, C-peptide, and fasting blood sugar levels following testosterone treatment. These findings suggest that testosterone may contribute to improved liver histology and potentially slow down the progression of liver fibrosis by targeting the metabolic profile. Lipid and glucose dysregulation are major risk factors in the development of liver injury. Hepatic lipid accumulation can lead to systemic metabolic dysfunction by upregulating the expression of gluconeogenic peroxisome proliferator-activated receptor (PPAR) ligands, resulting in hyperglycemia, ketosis, and hyperlipidemia (Geng et al., 2015). Hepatic insulin resistance, characterized by impaired insulin-mediated suppression of glucose output from the liver, contributes to increased blood glucose levels (Jiang et al., 2020). Our data supports the notion that testosterone administration leads to lower levels of cholesterol, LDL, triglycerides, and glucose compared to the non-treated group, indicating its potential role in regulating these metabolic parameters.

The present study revealed that there is a statistically significant relationship between testosterone hormone and liver injury through assessing injury markers of serum AST and ALT are indicators of hepatocellular injury. Several studies have demonstrated that high ALT and AST levels are correlated with a higher risk of liver fibrosis. High liver enzymes frequently signify liver cell inflammation or damage. Liver cells that are inflamed or wounded leak more substances into the bloodstream than usual, including liver enzymes, causing liver enzyme levels in the blood to rise (J. J. Corrales *et al.*, 2006). In our present study, a significant decrease in ALT and AST was observed when administrating testosterone exceeding those of level at baseline of untreated group.

Our data indicate improved liver histology after testosterone treatment; thus, lowering the progression of liver injury could be partly achieved by targeting the metabolic profile. otherwise, in our study we evaluated testosterone effect of selected real time PCR result present collagen and alpha smooth muscle in mice model have liver injury (acute and chronic injury). Moreover, our data showed a decrease in collagen and αSMA levels following testosterone treatment. Previous research has shown that the severity of liver fibrosis in humans is associated with increased levels of αSMA and collagen. During

the progression of fibrosis, the extracellular matrix (ECM) composition changes, and activated hepatic stellate cells (HSCs) play a role in inhibiting ECM degradation by secreting higher amounts of αSMA and collagen. The levels of αSMA and collagen were found to be significantly higher in cases of chronic fibrosis compared to acute or absent fibrosis (Munsterman et al., 2018), consistent to our generated data.

Our study provided compelling evidence for the amelioration of liver injury and the improvement of liver histology in terms of inflammation and fibrosis following testosterone treatment. These beneficial effects were accompanied by a decrease in the expression of IL-6 receptors on liver-resident NK cells and an increase in NK cell activity. These results suggest that the anti-inflammatory and anti-fibrotic effects of testosterone may be mediated, at least in part, through its impact on NK cells. Targeting the immune system, particularly NK cells, may be a promising strategy for delaying liver injury.

Conclusion

Our study findings provide evidence for the therapeutic potential of testosterone in reducing liver injury and improving the histology of inflammation and fibrosis in the liver. This improvement was attributed to a decrease in NK IL-6 receptors, resulting in increased NK cell activity. These results highlight the immune-modulatory effects of testosterone, which are associated with its anti-inflammatory and anti-fibrotic properties. This suggests that testosterone may serve as a valuable approach in the treatment of liver conditions characterized by inflammation and fibrosis. It would be interesting to explore the practical implications of testosterone to fully assess its efficacy and safeness for future clinical trials in humans.

Data Availability Statement

The study's original findings and contributions are detailed in the article itself. For any additional inquiries or information, it is recommended to contact the corresponding authors of the study. They will be able to provide further clarification and address any specific questions related to the research.

Author Contributions

JA contributed to the study design, manuscript writing, and revision. AS contributed to practical work, and revision. HS contributed to implementation of the experiments and data analysis. YA contributed

to perform and analyzed all statistical data. All authors contributed to the article and approved the submitted version.

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NA

Conflict of Interest

The authors state that the research was conducted without any commercial or financial relationships that could be perceived as a potential conflict of interest. This declaration suggests that the study was carried out without any external influences that could compromise the integrity or objectivity of the research findings.

Legends

Figure 1: Testosterone alleviates histopathological and biochemical findings of liver fibrosis. Liver injury was induced in C57/BL male mice for 2 and 4 weeks and compared with naive counterparts. Testosterone was administered via *i.p* injection for 1 week and 2 weeks, starting at week 1 and 2 of the acute and chronic CCl_4 models, respectively, as described in the Materials and Methods. Representative images of immunohistochemical liver staining sections of (A) H&E and (B) Sirius red, shown at an original magnification of 10x. The quantification of liver histology assessments is presented in (C) as the average ± SD for each group (6 mice per group). Serum markers of liver injury (D) ALT and (E) AST were measured. mRNA markers of liver fibrosis of (F) αSMA and (G) collagen III were assessed. Each experiment was repeated three times to ensure reliability and reproducibility. [**p=0.01, *** p=0.005, ****p=0.0001]. A Mann-Whitney U test was performed to evaluate whether the liver injury mice marker (ALT, AST), are altered by testosterone treatment in both the acute and chronic CCl_4-injected groups. The results demonstrated significant values in both groups. Therefore, the null hypothesis is rejected. Data is normally distributed (alpha=0.05).

Figure 2: Testosterone improved the perturbed metabolic profile in CCl_4-induced animals. Metabolic markers of lipid and glucose profile of serum levels of (A) cholesterol; CHOL, (B) triglyceride; TRG, (C) C-peptide, and (D) fasting blood sugar; FBS were assessed following sixteen hours of fasting. Each measurement was repeated three times and data represented as mean ± SD [*p=0.01, *** p=0.005, ****p=0.0001]. A Mann-Whitney U test was performed to evaluate whether the metabolic panel

elements (Cholesterol, Triglyceride, C-peptide and FBS levels) are altered by testosterone treatment in both the acute and chronic CCl_4-injected groups. The results demonstrated significant values in both groups. Therefore, the null hypothesis is rejected. Data is normally distributed (alpha=0.05)

Figure 3: Testosterone displays an inflammatory effect by reducing inflammatory cytokine**.** Pro-inflammatory cytokine levels of IL-6 were measured in all groups in triplicates. Data were analyzed using a Quantibody Q-Analyzer and an Excel-based program; results are presented in pg/ml. Data show mean ± SD. [*p=0.04, ** p=0.012].

Figure. 4: Testosterone ameliorates liver injury by reducing NK IFN γ and improving liver trNK activity**.** (A) ELISA showed secreted IFN-γ in mice in al mice groups. Flow cytometry analysis data demonstrated trNK (B) CD107a and (C) IL-6R percentages. (Each experiment was repeated three times and data represented average mean ± SD. [* p=0.05, **p=0.01, *** p=0.005, ****p=0.0001].

Supplementary 1: Testosterone treatment elevated serum testosterone and estradiol levels. Serum (A) testosterone and (B) estradiol following testosterone treatment are shown using ELISA. Each experiment was repeated three times and data represented as average mean ± SD. [* p=0.05, **p=0.01, *** p=0.005, ****p=0.0001].

Reference

Yassin A, AlRumaihi K, Alzubaidi R, Alkadhi S, Al Ansari A. Testosterone, testosterone therapy and prostate cancer. Aging Male. 2019 Dec;22(4):219-227.

Kelly DM, Jones TH. Testosterone: a metabolic hormone in health and disease. J Endocrinol. 2013a;217:R25–R45. [Google Scholar]

Morgentaler A, Traish A. The History of Testosterone and the Evolution of its Therapeutic Potential. Sex Med Rev. 2020 Apr;8(2):286-296.

Natale C, Carlos C, Hong J, Khera M, Baum N, Raheem OA. Testosterone Therapy After Prostate Cancer Treatment: A Review of Literature. Sex Med Rev. 2021 Jul;9(3):393-405.

Sinclair M, Grossmann M, Gow PJ, Angus PW. Testosterone in men with advanced liver disease: abnormalities and implications. J Gastroenterol Hepatol. 2015 Feb;30(2):244-51.

Yurci A, Yucesoy M, Unluhizarci K, Torun E, Gursoy S, Baskol M, Guven K, Ozbakir O. Effects of testosterone gel treatment in hypogonadal men with liver cirrhosis. Clin Res Hepatol Gastroenterol. 2011 Dec;35(12):845-54.

Al-Qudimat A, Al-Zoubi RM, Yassin AA, Alwani M, Aboumarzouk OM, AlRumaihi K, Talib R, Al Ansari A. Testosterone treatment improves liver function and reduces cardiovascular risk: A long-term prospective study. Arab J Urol. 2021 Aug 9;19(3):376-386.

Wei Y, Bingyu W, Lei Y, Xingxing Y. The antifibrotic role of natural killer cells in liver fibrosis. Experimental Biology and Medicine. 2022;247(14):1235-1243

Fasbender F, Widera A, Hengstler JG, Watzl C. Natural Killer Cells and Liver Fibrosis. Front Immunol. 2016 Jan 29;7:19.

Zhang Y, Wu Y, Shen W, Wang B and Yuan X: Crosstalk between NK cells and hepatic stellate cells in liver fibrosis (Review). Mol Med Rep 25: 208, 2022.

M. C. Wallace et al., “Standard operating procedures in experimental liver research: thioacetamide model in mice and rats.,” Lab. Anim., vol. 49, no. 1 Suppl, pp. 21–29, Apr. 2015.

15

P. J. Scheuer, “Classification of chronic viral hepatitis: a need for reassessment.,” J. Hepatol., vol. 13, no. 3, pp. 372–374, Nov. 1991.

P. Bedossa and T. Poynard, “An algorithm for the grading of activity in chronic hepatitis C. The METAVIR Cooperative Study Group.,” Hepatology, vol. 24, no. 2, pp. 289–293, Aug. 1996.

K. Ishak et al., “Histological grading and staging of chronic hepatitis.,” J. Hepatol., vol. 22, no. 6, pp. 696–699, Jun. 1995.

Roncati, L., Manenti, A., Pusiol, T., Piscioli, F., Barbolini, G., & Maiorana, A. (2016). Testosterone Aromatization to Estradiol in Course of Ovarian Functioning Brenner Tumor Associated With Endometrial Carcinoma and Endometriosis (Roncati-Manenti Triad). International journal of gynecological cancer: official journal of the International Gynecological Cancer Society, 26(8), 1461–1464.

Vermeulen, A., Kaufman, J. M., Goemaere, S., & van Pottelberg, I. (2002). Estradiol in elderly men. The aging male: the official journal of the International Society for the Study of the Aging Male, 5(2), 98–102.

Ström JO, Theodorsson A, Ingberg E, Isaksson IM, Theodorsson E. Ovariectomy and 17β-estradiol replacement in rats and mice: a visual demonstration. J Vis Exp. 2012 Jun 7;(64):e4013. doi: 10.3791/4013. PMID: 22710371; PMCID: PMC3471296

A. M. Bassi *et al.*, “Protein and m-RNA expression of farnesyl-transferases, RhoA and RhoB in rat liver hepatocytes: action of perillyl alcohol and vitamin A in vivo.,” *J. Biomed. Sci.*, vol. 12, no. 3, pp. 457–466, 2005.

M. S. Arslan *et al.*, “Effects of Ecballium Elaterium on Proinflammatory Cytokines in a Rat Model of Sepsis.,” *J. Investig. Surg. Off. J. Acad. Surg. Res.*, vol. 29, no. 6, pp. 399–404, Dec. 2016.

V. E. Bianchi, “The Anti-Inflammatory Effects of Testosterone.,” *J. Endocr. Soc.*, vol. 3, no. 1, pp. 91–107, Jan. 2019.

N. Muhanna *et al.*, “Activation of hepatic stellate cells after phagocytosis of lymphocytes: A novel pathway of fibrogenesis.,” *Hepatology*, vol. 48, no. 3, pp. 963–977, Sep. 2008.

Amer J, Salhab A, Noureddin M, Doron S, Abu-Tair L, Ghantous R, Mahamid M, Safadi R. Insulin signaling as a potential natural killer cell checkpoint in fatty liver disease. Hepatol Commun. 2018

Feb 14;2(3):285-298.

Salhab A, Amer J, Yinying L, Safadi R. 25(OH) D3 alleviate liver NK cytotoxicity in acute but not in chronic fibrosis model of BALB/c mice due to modulations in vitamin D receptor. BMC Gastroenterol. 2020 Apr 10;20(1):102.

A. Al-Qudimat *et al.*, "Testosterone treatment improves liver function and reduces cardiovascular risk: A long-term prospective study.," *Arab J. Urol.*, vol. 19, no. 3, pp. 376–386, 2021.

S. Sun *et al.*, "Testosterone and Estradiol as Novel Prognostic Indicators for HBV-Related Acute-on-Chronic Liver Failure," *Front. Med.*, vol. 8, no. September, 2021.

E. M. B. El Naggar *et al.*, "Hepatoprotective and proapoptotic effect of Ecballium elaterium on CCl4-induced hepatotoxicity in rats.," *Asian Pac. J. Trop. Med.*, vol. 8, no. 7, pp. 526–531, Jul. 2015.

D. E. Ochayon and S. N. Waggoner, "The Effect of Unconventional Cytokine Combinations on NK-Cell Responses to Viral Infection," *Front. Immunol.*, vol. 12, no. March, pp. 1–13, 2021.

K. Tomita *et al.*, "Free cholesterol accumulation in hepatic stellate cells: mechanism of liver fibrosis aggravation in nonalcoholic steatohepatitis in mice.," *Hepatology*, vol. 59, no. 1, pp. 154–169, Jan. 2014.

Liu F, Sun C, Chen Y, Du F, Yang Y, Wu G. Indole-3-propionic Acid-aggravated CCl4-induced Liver Fibrosis via the TGF-β1/Smads Signaling Pathway. J Clin Transl Hepatol. 2021 Dec 28;9(6):917-930.

Khan HA, Ahmad MZ, Khan JA, Arshad MI. Crosstalk of liver immune cells and cell death mechanisms in different murine models of liver injury and its clinical relevance. Hepatobiliary Pancreat Dis Int. 2017 Jun;16(3):245-256.

Wang T, Zhou X, Kuang G, Jiang R, Guo X, Wu S, Wan J, Yin L. Paeoniflorin modulates oxidative stress, inflammation and hepatic stellate cells activation to alleviate CCl4-induced hepatic fibrosis by upregulation of heme oxygenase-1 in mice. J Pharm Pharmacol. 2021 Mar 6;73(3):338-346.

L. Chin, N. D. Theise, A. E. Loneker, P. A. Janmey, and R. G. Wells, "Lipid droplets disrupt mechanosensing in human hepatocytes.," *Am. J. Physiol. Gastrointest. Liver Physiol.*, vol. 319,

no. 1, pp. G11–G22, Jul. 2020.

D. M. Kelly and T. H. Jones, “Testosterone and obesity.,” *Obes. Rev. an Off. J. Int. Assoc. Study Obes.*, vol. 16, no. 7, pp. 581–606, Jul. 2015.

Y. Geng, K. N. Faber, V. E. de Meijer, H. Blokzijl, and H. Moshage, “How does hepatic lipid accumulation lead to lipotoxicity in non-alcoholic fatty liver disease?,” *Hepatol. Int.*, vol. 15, no. 1, pp. 21–35, Feb. 2021.

S. Jiang, J. L. Young, K. Wang, Y. Qian, and L. Cai, “Diabetic-induced alterations in hepatic glucose and lipid metabolism: The role of type 1 and type 2 diabetes mellitus (Review).,” *Mol. Med. Rep.*, vol. 22, no. 2, pp. 603–611, Aug. 2020.

J. J. Corrales, M. Almeida, R. Burgo, M. T. Mories, J. M. Miralles, and A. Orfao, “Androgen-replacement therapy depresses the ex vivo production of inflammatory cytokines by circulating antigen-presenting cells in aging type-2 diabetic men and partial androgen deficiency,” *J. Endocrinol.*, vol. 189, no. 3, pp. 595–604, 2006.

I. D. Munsterman *et al.*, “Extracellular matrix components indicate remodelling activity in different fibrosis stages of human non-alcoholic fatty liver disease.,” *Histopathology*, vol. 73, no. 4, pp. 612–621, Oct. 2018.

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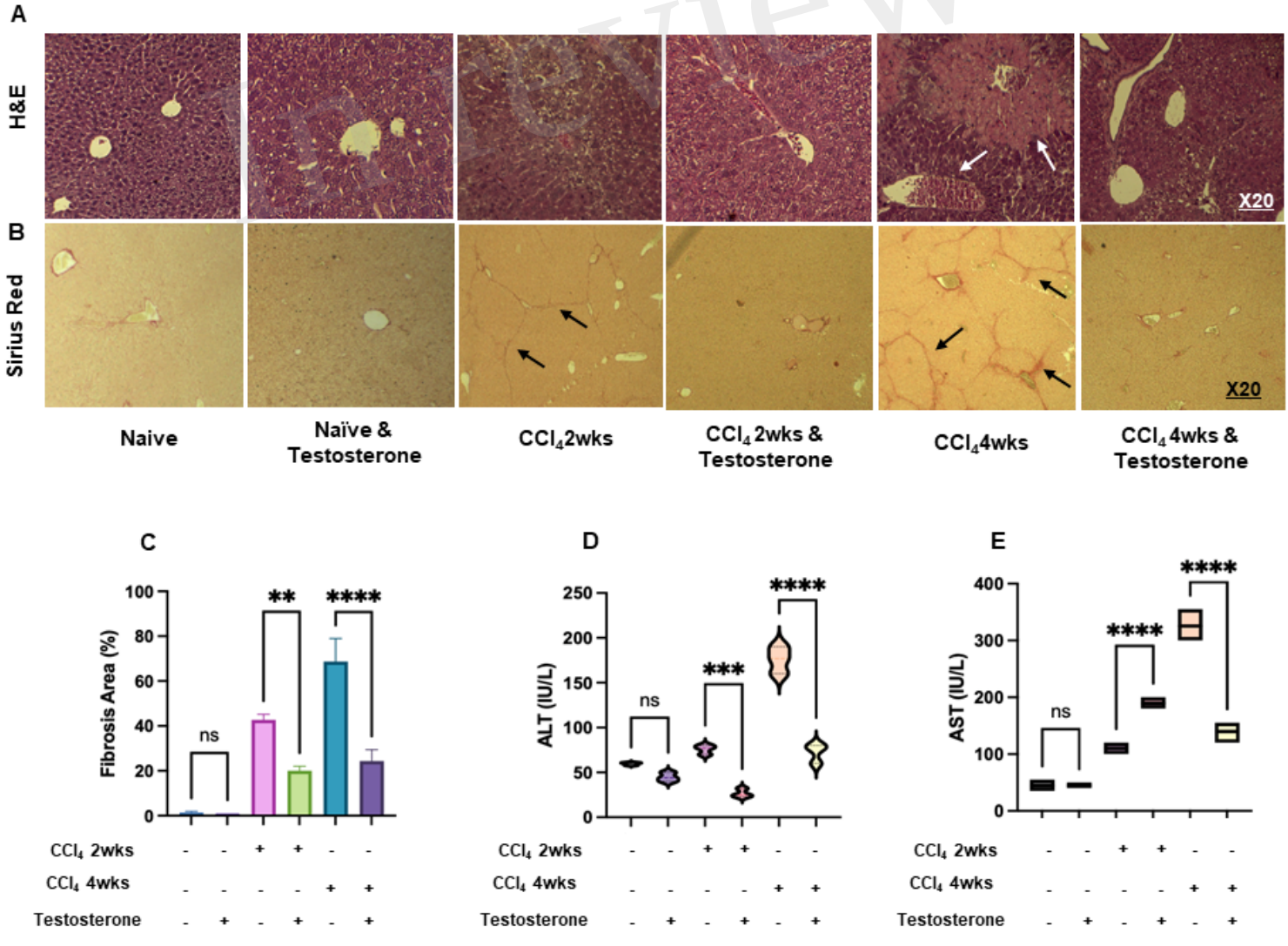
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CCl4 2wks
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Testosterone

Figure 2.TIF

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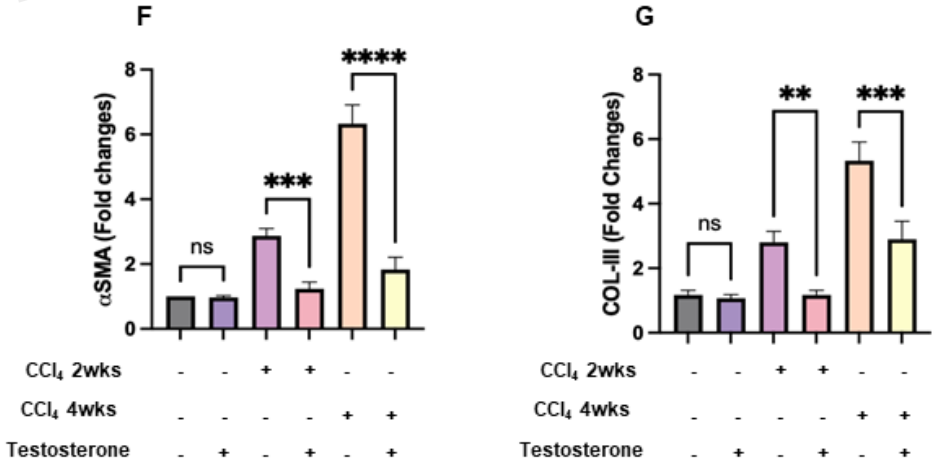

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Figure 2

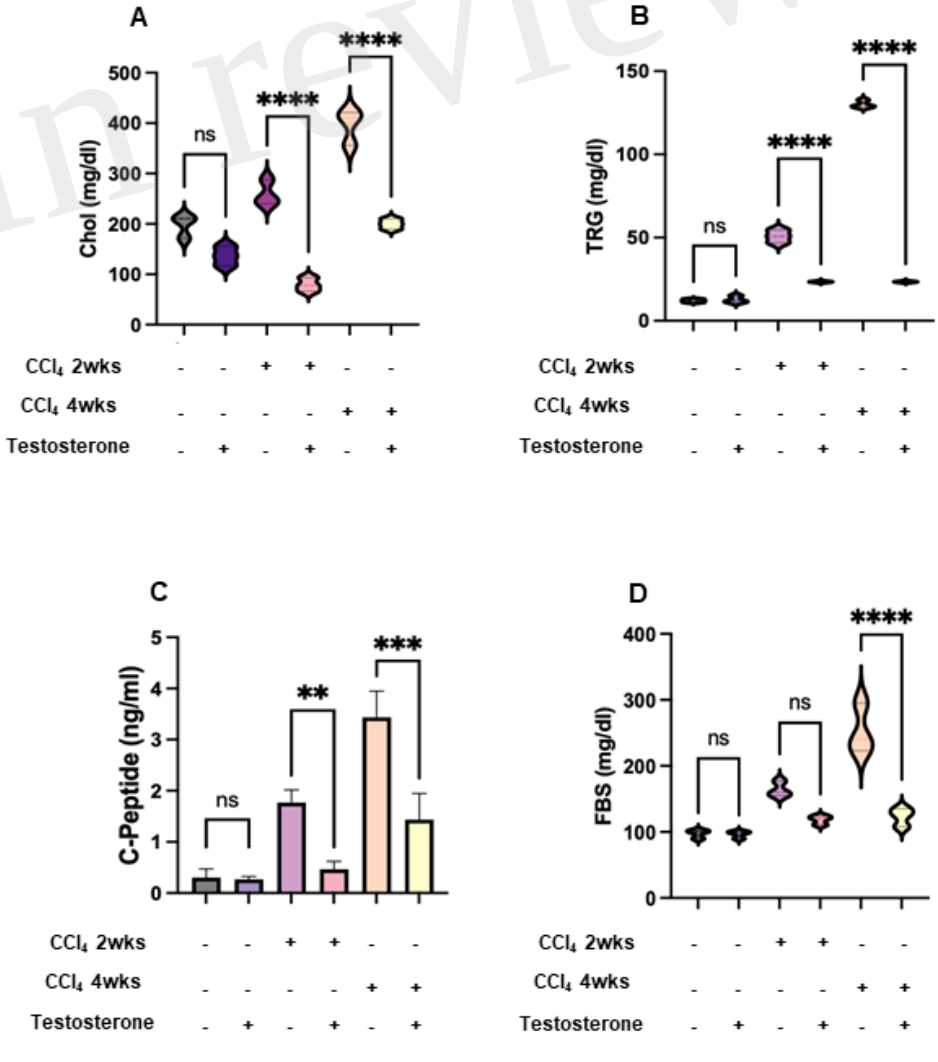

Figure 4.TIF

Figure 3

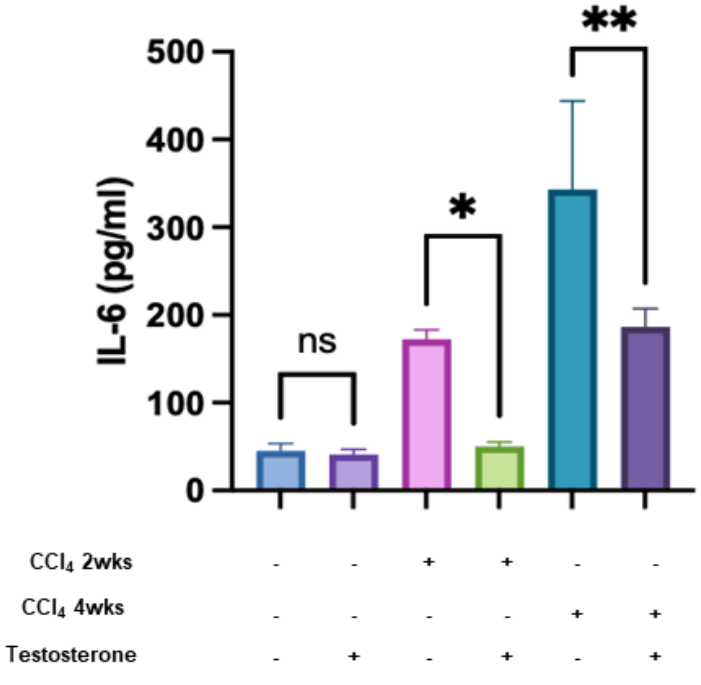

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Figure 4