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Down-regulation of insulin substrate receptor 1 and 2 in the liver may be a mechanism for insulin resistance during testosterone deprivation

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Abstract

Background and Objective: Type 2 diabetes is a global concern worldwide. Despite extensive studies on the physiological effects of diabetes on the testicular functions, the impact of testosterone deficiency on the glucose homeostasis remains to be clarified. This study was designed to investigate the effects of testosterone deprivation and its replacement with testosterone enanthate on the molecular mechanisms of insulin signaling pathway in the liver of rats.

Materials and Methods: We first established a rat model of testosterone deficiency by castration (CAS-S). Subsequently, the castrated rats were administrated by subcutaneous injection of testosterone (CAS-T). Thereafter, fasting blood glucose (FBG), insulin, and homeostasis model-insulin resistance (HOMA-IR) level was assessed. The testosterone and insulin levels were further analyzed by ELISA. The mRNA expression of insulin receptor (IR)-β, insulin receptor-substrate (IRS)1 and 2 as well as glucose transporter (GLUT) 2 in the liver was analyzed by q-RT-PCR assay.

Results: Our data showed that testosterone deprivation significantly increases FBG and HOMA-IR and down-regulates IRS-1 and IRS-2 mRNA expression in the liver. However, the mRNA expression of GLUT2 and IR-β was not affected. We also found that testosterone administration could improve the liver insulin resistance.

Conclusion: These findings suggested that testosterone deprivation can impact insulin signaling in the liver via suppressing expression of IRS-1 and IRS-2 mRNA and treatment with testosterone can improve the insulin resistance in the castrated rats. Further experimental and clinical pathways are needed to be assessed for clinical application of our finding.

Keywords: Androgen, Hepatic insulin resistance, Diabetes, IRS 1 and 2, Glucose transporter 2

1. Introduction

Type 2 diabetes (T2D) is a major concern in healthcare worldwide, affecting over 462 million people corresponding to 6.28% of the people living in the world. This chronic metabolic disorder has a significant impact on the person's life and health expenditures (1). Recent epidemiological and experimental studies have revealed that one of the factors affecting glucose homeostasis in males is hypogonadism and testosterone deficiency (2-4). The leading causes of hypogonadism are age-related decreases in endogenous androgen levels, androgen deprivation therapies, and disorders that damage the testicles or reduce gonadotropin stimulation (5). Studies show that about 18% of men over the age of 70 are deficient in testosterone (2). The characteristic of T2D is the hyperglycemia induced by insulin resistance and impaired insulin secretion (6). Some studies suggest that T2D is associated with chronic dysfunction and failure of some organs, such as the liver. The liver is an important organ in the regulation of metabolic processes such as glycogen storage and gluconeogenesis and it is one of the main targets for insulin (6). The most important effects of insulin on the liver are stimulation of glucose uptake, glycogen accumulation, and suppression of glycogenolysis and gluconeogenesis (7). In the liver, the expression of

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insulin receptor substrate (IRS)1 and 2 is high so that these factors interact with downstream molecules such as phosphatidylinositol 3-kinase for regulation of metabolism (7). The molecular studies show that among IRSs, IRS-2 is mostly related to insulin resistance, especially in liver tissue and human studies show that the IRS-2 genetic polymorphisms in patients with T2D play a role in the risk of T2D (8-10).

Some evidence shows a direct correlation between plasma testosterone and insulin sensitivity so that low testosterone levels are associated with an increased risk of T2D. In this regard, Schattner et al. reported that treatment with a steroidal anti-androgen called cyproterone acetate for six weeks raised blood glucose levels from 12 mg/dl to 880 mg/dl (11). Also, one study by Inaba et al. (2005) reported that non-diabetic 81-year-old men with prostate cancer that were treated with leuprolide acetate for seven months were hyperglycemic. Also, they showed that administration of MAB (leuprolide acetate & flutamide) for 11 months in 61-year-old patients with prostate cancer increases blood glucose levels (3). Another clinical trial on 200 patients examined the effect of testosterone treatment on hyperglycemia in T2D patients with hypogonadism and found that testosterone treatment improves blood glucose levels in a dose-dependent manner (12). However, the results of researches on the effect of testosterone on glucose homeostasis are mixed. For example, the results of a study by Grossmann et al. (2015) investigated the effects of testosterone treatment on the improvement of blood glucose control in the hypogonadal men with T2D and they showed that testosterone did not have an improving effect on blood glucose (13). Considering the mixed results of effects of testosterone on glucose homeostasis (12, 13) and the role of liver tissue in the regulation of metabolic processes, this study was designed to investigate the molecular mechanisms underlying the effects of testosterone on insulin signaling pathway in liver of rats.

2. Materials and Methods

2.1. Animals

Twenty-eight adult male Wistar rats (190-200 g) were purchased from Kermanshah University of Medical Sciences (Kermanshah, Iran) and kept in the animal room of Razi University under 22-25°C, 12/12 light-dark cycle with 50% relative humidity. The animals had unlimited food and water supply. All animal procedures were approved by the Animal Ethics Committee of Razi University (Kermanshah, Iran, permit number: IR.RAZI.REC.1399.017).

2.2. Study design

The male rats were randomly divided into three groups: I) Normal control (NC) group that had no treatment; II) Castrated (CAS-S) group. This group served as a testosterone deprivation model. They were castrated by bilateral orchidectomy surgery. Their treatment was followed by subcutaneous injection of sesame oil as solvent of testosterone enanthate (TE) once per day for 10 weeks; III) Castrated rats that were treated with testosterone (CAS-T) group. These animals were castrated surgically and treated by subcutaneous injection of a dose of 25 mg/kg of TE, once per day for ten weeks (14). TE was dissolved in sesame oil which was made ready immediately before injection. These animals were treated with subcutaneous injection of TE dissolved in the sesame oil (25 mg/kg/day). After ten weeks of treatment, the animals were fasted overnight and were euthanized by intraperitoneal injection of ketamine and xylazine. Then, the blood samples were drawn by cardiac puncture. A piece of the liver tissue of each rat was removed, immediately was frozen in liquid nitrogen, and stored at ‑80˚C until further processing.

2.3. Total testosterone, fasting blood glucose, insulin, and homeostasis model-insulin resistance (HOMA-IR) measurement in the serum

The sera were separated from clotted blood samples by centrifugation at 3000 rpm for 10 minutes at 4°C to separate serum (15) and stored at -20°C. Then, the total serum testosterone levels were measured using a rat testosterone enzyme-linked immunoassay (ELISA) kit (Cusabio, Wuhan, China). The intra-assay coefficient of variation for the testosterone kit was < 15% and the inter-assay coefficient of variation (CV) was < 15%.

Also, the insulin levels of sera samples were measured using a rat insulin ELISA kit (Mercodia, Sweden). The coefficient of variation within assay of kit was 3.1% and its coefficient of variation between assays was 4.4%. The fasting blood glucose (FBG) concentrations were measured using a commercial kit (Zistchemi, Tehran, Iran) according to the instructions. The equation (1) was applied to estimate the insulin resistance (HOMA-IR) (16).

Equation (1) HOMA-IR= fasting insulin× fasting glucose/405

2.4. Reverse transcription quantitative polymerase chain reaction (qPCR)

The TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was utilized to isolate the total RNA from liver tissue samples. Total RNA was treated with RNase-free DNase I (Ferementase, California, U.S.A.). Afterwards, the total RNA was reversely transcribed to complementary DNA (cDNA) using reverse transcriptase enzyme (Ferementase, California, U.S.A.) according to the manufacturer's instructions.

The qPCR was conducted using Green-2-Go qPCR Mastermix (Bio basic, Canada) and the Corbett Research RG 3000 thermal cycler (CR CORBETT, Australia) in duplicate reactions to assess the target genes mRNA expression. Cycling conditions were as follows: Denature at 94˚C for 30 sec; 40 cycles of 95˚C for 5 sec and 60˚C for 34 sec. The primer sequences are listed in Table 1. The primers were purchased from Sinaclon (Tehran, Iran). The expression of target genes was normalized to that of the GAPDH gene. The comparative CT method was used to calculate relative gene expression (17).

Table 1. Sequences of primers used in qRT-PCR experiments

SYBR Green RT-PCR Primers	Sense primer (5' toward 3')	Anti-sense primer (5' toward 3')
GAPDH	AAG TTC AAC GGC ACA GTC AAG G	CAT ACT CAG CAC CAG CAT CAC C
GLUT2	AGAATTCCGGAAGAAGAGTGGT	TCCGCTTGGTGGTTTGCTACGAC
IRS-2	TGGATCAGGTATCTGGGGTAGAGG	GACAGTGGTGGTAGAGGAGAAGGT
IRS-1	TGTTGAGAGCGGTGGTGGTAAG	ATGAGTAGTAGGAGAGGACAGG
IR-β	AGAGTGAAGAGCTGGAGATGGAG	TTCATGTTGGTGTAGGGGATGTGT

2.5. Statistical analysis

All data were expressed as the mean ± standard error of the mean (SEM). All the analyses were performed with SPSS (IBM SPSS 20 statistic, San Diego, CA, USA). Statistical analysis was done using one-way ANOVA followed by Tukey's post hoc test. The p-values less than 0.05 were considered statistically significant.

3. Results

3.1. The total testosterone levels in the blood

To generate an animal model of testosterone deprivation, the rats were castrated, which mimicked the testosterone deficiency in the hypogonadal individuals. Compared with the total testosterone level of the NC group (2.88 ± 0.02 ng/ml), the total testosterone levels of CAS-S (1.5 ± 0.03 ng/ml) and CAS-T (2.52 ± 0.10 ng/ml) rats were significantly decreased (P = 0.000 and p=0.004, respectively) (Fig. 1a) that show the injection of 25 mg/kg/day of TE was not able to restore the levels of this hormone so that the testosterone levels of CAS-T rats were not significantly different from the levels of testosterone in the CAS-S (p=0.000) (Fig. 1a).

3.2. The fasting blood glucose, insulin, and HOMA-IR levels

Significantly higher levels of FBG in the CAS-S group (99.13±0.99) compared with the NC group (81.69±0.99) indicated that the FBG levels were increased by testosterone deprivation (p=0.000) (Fig. 1b). The FBG levels of CAS-T group (60.71± 0.61 mg/dl were lower than those of NC group (81.69± 0.99 mg/dl) (P = 0.000). The FBG levels of CAS-T groups were significantly lower than the levels of CAS-S group; P = 0.000). The insulin levels (12.34 ± 0.35 µIu/ml for the CAS-S vs. 6. 1 ± 0.11 µIu/ml for the NC; P = 0.000) appeared to have elevated in the castrated rats and the testosterone replacement did not suppress this increase (9.27 ± 0.17 µIu/ml for the CAS-T; P = 0.000 vs. the NC and the CAS-S) (Fig. 1c). The level of HOMA-IR significantly increased in the CAS-S group (3.02 ± 0.075) compared to the NC group (1.23 ± 0.031 mg/dl) (P=0.000) (Fig. 1d) and testosterone replacement suppressed this increase (1.39 ± 0.032 for the CAS-T; P =0.000 vs. the CAS-S). Also, HOMA-IR in the CAS-T group was not statistically different from the NC group (p=0.086) (Fig. 1d).

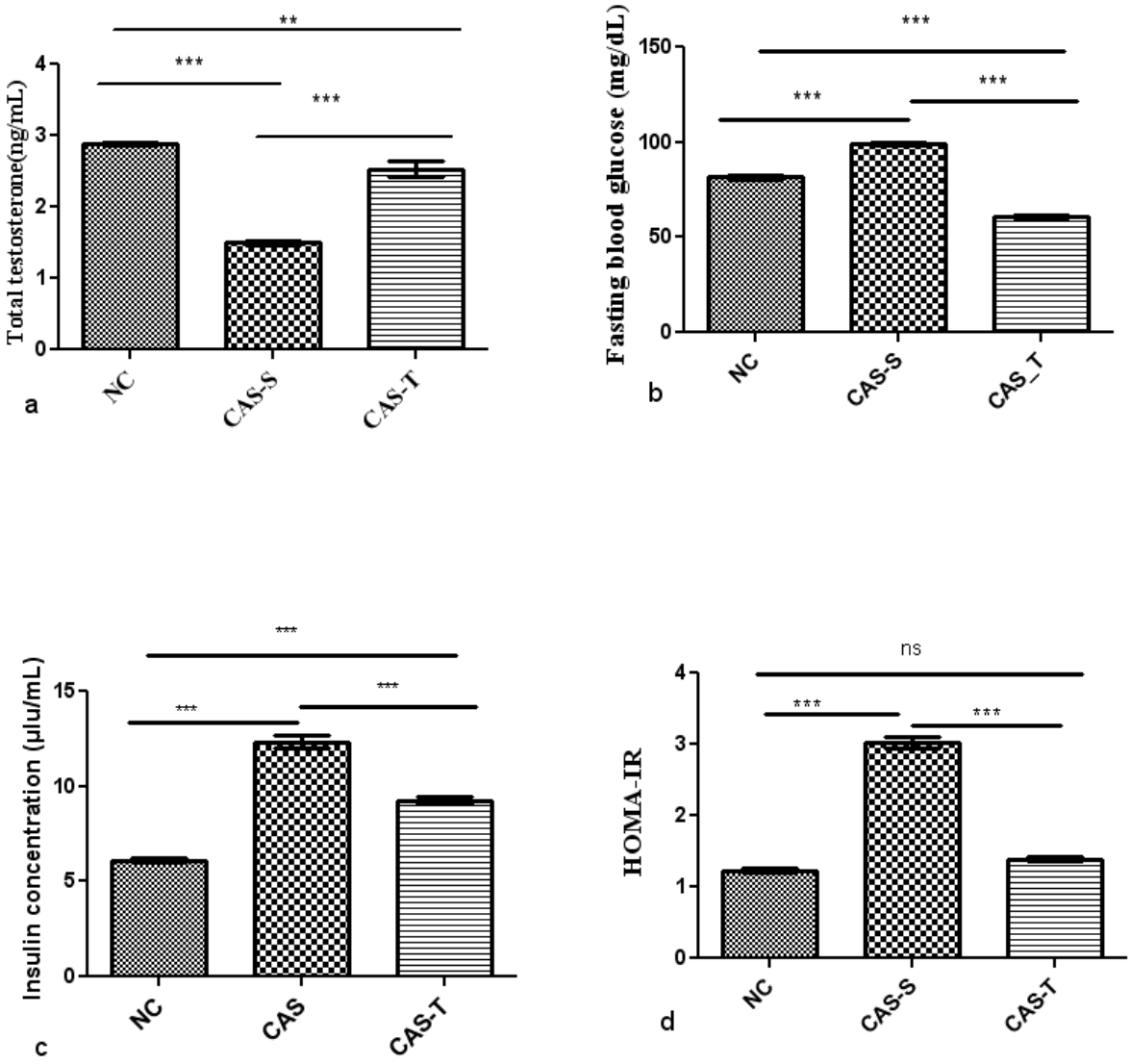

Fig 1. Effects of testosterone on the total serum testosterone (a), fasting blood glucose (b), insulin concentration (c) and HOMA-IR (d). NC group, Normal control rats that had no treatment; CAS-S group (Castrated group): the rats of this group were castrated surgically and then treated by subcutaneous injection of sesame oil as the solvent of testosterone enanthate (TE) once per day; CAS-T group, castrated rats that treated with testosterone: these animals were castrated surgically and were administrated by subcutaneous injection of 25 mg/kg of TE dissolved in the sesame oil once per day. Statistics were done by one-way ANOVA followed by Tukey's post hoc test. P values less than 0.05 were considered statistically significant. ***: P < 0.001; ns: non-significant difference.

3.3. The mRNA expression of GLUT2, IRS-1, IRS-2, and IR-β in the liver

Ten weeks after the castration surgery, expression levels of GLUT2 and IR-β mRNA in the liver of the CAS-S group were not significantly altered compared with controls (0.1665 ± 0.04158 for GLUT2 and 0.13596 ± 0.2125 for IR-β in the CAS-S group vs. 0.4581 ± 0.1181 for GLUT2 and 0.4529335±0.1158 for IR-β in the NC group; P =0.062 and p= 0.065, respectively) (Fig. 2a and b).

The GLUT2 and IR-β mRNA expression levels in the CAS-T group were not significantly altered compared with those in the NC group (0.3095124 ± 0.06389 for GLUT2 and 0.3619168 ± 0.0.09948 for IR-β in the CAS-T group vs. the NC group; P = 0.424, and P =0.956, respectively) (Fig. 2a and b). The IRS-1 mRNA expression levels of the CAS-S group (0.13825 ± 0.04212) were lower than those of NC group (0.56156±0.1053) (P = 0.021). The IRS-1 mRNA expression levels of the CAS-T group (0.409626 ± 0.1198) were not significantly different from the levels of the CAS-S and NC groups; P = 0.149 and P =0.514, respectively) (Fig. 2c). The qPCR

analysis showed that the IRS-2 mRNA expression in the liver tissue of castrated rats was significantly reduced (0.00095 ± 0.000252 for CAS-S vs. 0.003883 ± 0.00652 for NC; P = 0.001). Also, the rats of the CAS-T group exhibited significantly lower IRS-2 mRNA expression levels in the liver (0.00157 ± 0.000274 for CAS-T vs. NC; P = 0.007). In the CAS-T group, the expression levels of IRS-2 mRNA were not altered vs. CAS-S; P = 0.583) (Fig. 2d).

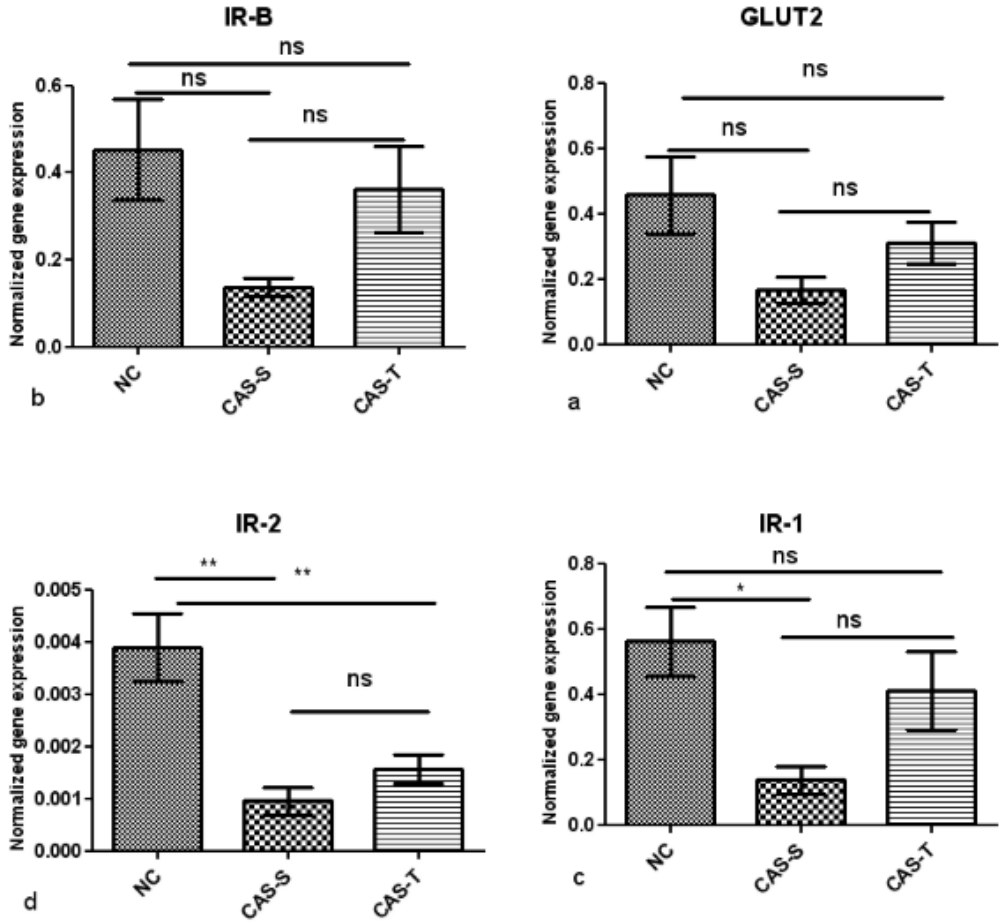

Fig 2. Effects of testosterone on the expression levels of GLUT2 (a), IR-β (b), IRS-1 (c), and IRS-2 (d) mRNA expression.in the rat liver. The expression of target genes was normalized to that of the GAPDH gene. NC group, Normal control rats that had no treatment; CAS-S group (Castrated group): the rats of this group were castrated surgically and then treated by subcutaneous injection of sesame oil as solvent of testosterone enanthate (TE) once per day; CAS-T group, Castrated rats that treated with testosterone: these animals were castrated surgically and were administrated by subcutaneous injection of 25 mg/kg dose of TE dissolved in the sesame oil once per day. Statistics were by one-way ANOVA followed by Tukey's post hoc test (n=5). P values less than 0.05 were considered statistically significant. *: P < 0.05, **: P < 0.01; ns: non-significant difference.

4. Discussion

In the present study, we castrated the animals to evaluate the effects of testosterone deprivation on the glucose hemostasis and expression of insulin resistance-related genes *IR-β*, *IRS-1*, *IRS-2* as well as *GLUT2* in the liver tissue.

Castrated animals are an accepted model for men with hypogonadism (18). In the line of previous studies (14, 18), our results showed that in the castrated rats, the testosterone levels were significantly decreased, indicating the successful establishment of testosterone deprivation. Besides, in order to testosterone replacement, we treated the castrated animal with dose 25 mg/kg TE. In the present study, the FBG levels of castrated rats were increased. Like our results, finding of a study by Xia et al showed that the levels of FBG in the testosterone deficient group were significantly increased when compared to those in the control group (14). Also, another study by Christoffersen et al showed that the FBG and HbA1c concentrations were increased in the castrated rats 10 weeks after castration (19). The higher FBG shows the higher production of glucose by liver (19). Also, our finding of insulin concentration is in the line of finding of a previous study by Xia et al that showed the fasting insulin levels in the testosterone deficient group were significantly raised when compared with the control

rats (14). However, these results aren't consistent with the finding of one study that reported that ten weeks after orchidectomy surgery in the castrated rats, the insulin concentration did not affected (19).

Here, testosterone replacement with 25 mg/kg TE significantly decreased the FBG and improved insulin resistance (HOMA-IR levels). In the line of our findings, the results of one study showed that testosterone replacement with testosterone propionate was able to lower the FBG, insulin, and HOMA-IR levels and improved glucose homeostasis in the castrated rats (14). In the line our finding, one study on patients with obesity-related secondary male hypogonadism (including a subgroup with T2D) reported that treatment with testosterone undecanoate for 6–29 months improved glycemic control and beta-cell function (20).

Although various studies indicated that there is a reduction in the insulin sensitivity in the testosterone deficiency condition the molecular mechanisms involved in this process are unknown. The liver is an important organ in the regulation of glucose homeostasis and exerts its effects through glycogen synthesis, glycogenolysis, and gluconeogenesis. Finding a study showed that the insulin signaling in liver is important in the control of glucose homeostasis. They revealed that the loss of direct insulin action in the livers tissue of mice causes significant insulin resistance, serious glucose intolerance as well as the inability of insulin to stop production of glucose and regulation of gene expression by the liver (21). The deregulation of glucose homeostasis leads to hyperglycemia and insulin resistance that in turn contributes to the pathogenesis of T2D (22, 23). A decrease or loss of insulin action in liver tissue results in irregularly increased glucose production through gluconeogenesis and glycogenolysis, and lipogenesis (24). Animal and human studies show that in the T2D condition, selective insulin resistance is seen in the liver tissue so that insulin not only isn't able to suppress gluconeogenesis but also it continues the lipogenesis process. Thereby, this situation leads to simultaneous hyperglycemia and steatosis (7). The disturbance of insulin signaling in the liver can suppress insulin clearance, hepatic glucose uptake, and lipolysis, and as a result, it leads to dyslipidemia (24). Considering importance of liver in glucose homeostasis, in the present study, we used q-RTPCR analysis to investigate the expression of insulin resistance-related genes such as *IR-β*, *IRS-1*, and *IRS-2* in the liver tissue and our results showed that testosterone deprivation significantly down-regulate the *IRS-2* and *IRS-1* mRNA expression. The *IRS-1* is closely involved in glucose metabolism but IRS-2 is more related to fat metabolism and *IRS-2* is an important component in the biological pathways (25). Current results suggest that testosterone deprivation can affect the insulin signaling at the *IRS-2* and *IRS-1* mRNA expression. Particularly, as mentioned above, IRS-2 is responsible for insulin resistance at the liver level (8-10). Also, in the castrated rats treated with TE, the TE injection (25 mg/kg) increased the *IRS-2* slightly, but the levels of *IRS-2* mRNA in these animals remained significantly decreased compared to the controls. After treatment of castrated rats with TE, the mRNA levels of *IRS-1* were significantly increased compared to the normal rats.

This result is consistent with the results of measurement of insulin and HOMA-IR that show testosterone deprivation caused an increase in the insulin and HOMA-IR levels. Previous studies showed that the levels of the mRNA expression of *IRS-2* depend on the insulin levels so that with an elevation the insulin concentration, *IRS-2* expression is down-regulated (7). However, the *IRS-1* expression remains unaltered by changing insulin levels (7, 26, 27). It is likely, down-regulation of *IRS-2* be due to the increase of insulin levels in the castrated rats.

GLUT2 is the main glucose transporter in the liver cells. This transporter translocates glucose across plasma membranes of hepatocytes in two directions for efflux of the glucose produced from hepatocytes during gluconeogenesis or glycogenolysis as well as for uptake of glucose from blood into hepatocytes. The elevation of glucose and insulin levels causes up-regulation of hepatic GLUT2 (28). According to the results of current study, testosterone deprivation didn't have a significant effect on the *GLUT2* mRNA expression in the liver. This finding suggests that the insulin resistance observed with testosterone deprivation isn't related to the alteration of *GLUT2* mRNA expression in the liver tissue. Also, in the castrated rats treated with TE, the *GLUT2* mRNA expression in the liver didn't alter when compared with normal controls. This suggests that testosterone deprivation or treatment with 25 mg/kg TE didn't have a significant effect on the expression of this gene and the maybe *GLUT2* mRNA expression in the liver didn't depend on the testosterone levels.

Compared with the normal controls, the similar levels of *IR-β* mRNA were observed in the all groups, which mean it is likely the testosterone level didn't affect the *IR-β* mRNA expression.

The current study, suggests that testosterone deprivation has actions on the glucose homeostasis as well as hepatic *IRS-2* and *IRS-1* mRNA expressions and testosterone replacement was able to lessen insulin resistance (HOMA-IR) and increase the *IRS-1* mRNA expression in castrated rats but, the hepatic *IRS-2* mRNA expression didn't recover to that of normal controls. Also, here, the levels of *GLUT2* and *IR-β* were not affected by alteration of testosterone levels. Considering the role of skeletal muscles and adipose tissue in the glucose homeostasis, testosterone may exert its effects on the glucose homeostasis through acts on thesetissues and in this regard further research are needed. Because of the roles

hyperglycemia and insulin resistance in the development of complications associated with male hypogonadism and androgen deprivation therapy and progression of T2D, the efficient monitoring of the patients under androgen therapy for presence of hyperglycemia and insulin resistance and medical care targeting hyperglycemia and insulin resistance are very vital for the successful treatment of these patients.

Conclusion

Taken together, we demonstrated that testosterone deprivation Taken together, we demonstrated that testosterone deprivation exerts a significant effect on HOMA-IR, insulin concentration, and FBG levels as well as mRNA expression of IRS-2 and IRS-1 in the liver tissue. These findings indicate that down-regulation of IRS-2 and IRS-1 in the insulin signaling pathway may be a mechanism by which testosterone deprivation affects the liver function and predispose men with testosterone deficiency to insulin resistance and T2D. Also, we showed that testosterone replacement may effectively ameliorate hyperglycemia and insulin resistance castrated rats. Further clinical studies are needed to answer these questions: How much decrease of testosterone levels can affect the liver expression of IRS-2 and IRS-1 levels in the liver and how much decrease in the liver expression of IRS-2 and IRS-1 levels can induce clinical manifestations of insulin resistance and diabetes in humans? The present rat model of hypogonadism showed that testosterone deprivation might cause insulin resistance at the liver level by induction of a decrease in mRNA expression of IRS-1 and IRS-2. Study of the genes and molecular pathways in different tissues involved in the metabolism of glucose such as skeletal muscles and adipose tissue are needed to understand which molecular pathways may be influenced by testosterone. The clinical application of our finding is still somewhat limited because the safety of testosterone therapy has not yet been fully clarified. However, these results may provide insight for special medical care of hypogonadal men with T2D or men who receive androgen deprivation therapy. More research is necessary to verify this information before judgment about the clinical application of present findings.

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