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The Biochemical Efficacy of Insulin Resistance on Sex Hormones in Obese Male Rats

KEYWORDS: Insulin Resistance, Sex Hormones, Obesity, Male, Testosterone

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ABSTRACT *OBJECTIVE: Insulin resistance has been associated with low total and free testosterone and increased the risk for type2 diabetes in males. Truncal obesity, a case accompanied by increased insulin resistance, is also associated with low total and free testosterone levels. In this study we evaluated if the insulin resistance primarily related to low total and free testosterone in obese males, and the effect of treatments for hypercholesterolemia on the levels of sex hormones.*

RESEARCH DESIGN AND METHODS: Fifty male rats, their weights between 150-200 gm. were divided into5groups: one as control fed on low fat diet, groups (2, 3, 4, and 5) fed on high fat diet. Group 2 serve as obese without treatment addition. Different treatments for hypercholesterolemia were added to groups (3, 4, and5) as Ezetimibe, Simvastatin, and Simvastatin+Ezetimibe respectively .Hormonal parameters include: Total testosterone, free testosterone, FSH, and insulin were measured. Also biochemical parameters include: FBS and cholesterol were measured. Insulin sensitivity index was measured by was calculated using homeostasis model assessment (HOMA) formula.

RESULTS: Low level of total testosterone was associated with obesity (0.9 ±0.07, and 2.98 ±0.07 in control), also free testosterone was associated with it (0.3 ±0.03, and 0.75 ±0.02 in control).Insulin sensitivity index was negatively correlated with obesity (r = - 0.425, p= 0.05).And have a positive correlation with testosterone (r =0.540, p=0.001) in obese males. Significant improvement in total testosterone and free testosterone level found among the treated groups when compare with obese rats but still lower than control.

CONCLUSIONS: We conclude that insulin resistance associated with lowering in total and free testosterone depends on the obesity. So obesity has negative relations with total and free testosterone, and positive with insulin resistance. We also conclude that combination of simvastatin + ezetimibe record the lowest level of cholesterol and FBS and highest improvement in insulin sensitivity index, total, and free testosterone levels among treated groups.

Introduction:
Insulin resistance is defined as the impaired ability of cells to response to the action of insulin in transporting glucose (sugar) from the bloodstream into muscle and tissues. In humans, insulin resistance develops with obesity and type 2 diabetes (non-insulin-dependent). (Shepherd and Kahn, 1999) .Type 2 diabetes (non-insulin dependent diabetes) is characterized by insulin resistance and is usually linked with abnormality in secretion of insulin. Generally, about 90–95% of cases of diabetes are of this type of diabetes. (Zimmet 1999; Zimmet et al 2001).Obesity increase resistance to the cellular actions of insulin, characterized by losing the ability of insulin to inhibit glucose output from the liver and to promote glucose uptake in fat and muscle. (Saltiel and Kahn 2001; Hribal et al. 2002). The association between obesity and insulin resistance is likely a cause and effect relationship since human and animal studies indicate a directly proportional between weight and insulin sensitivity. (Sims et al. 1973; Freidenberg et al. 1988; Bak et al. 1992).

Most studies have shown an inverse correlation between Total Testosterone (TT) and levels of fasting insulin in males (Simon D, et al. 1992 , Pasquali R, et al 1991, Zumoff B, et al 1990). Furthermore, men with insulin resistance states such as obesity (Pasquali R, et al 1991, Glass AR, et al 1977). And type 2 diabetes (T2D) (Barrett-Connor E et al 1992 , Andersson B, et al 1994) have significantly lowering in levels of Testosterone than non diabetic and normal weight and control males. To date, the mechanism of the association between lowering Testosterone levels and insulin resistance in men has not been elucidated. It has been suggested that the inverse correlation between Testosterone and insulin resistance is due to obesity (Abate NA, et al 2002, Tsai EC, et al 2004). Given that the latter is associated with both insulin resistance and low levels of SHBG. If this hypothesis is correct, the reducing in levels of Testosterone seen with increasing obesity level in men should be referenced by low levels of SHBG alone, and the Free Testosterone should be normal. However, many studies have shown that total and free T levels reduced in proportion to the level of obesity (Zumoff B, et al 1990, Isidori AM, et al 1999). The recent reports indicate that obese males have a reduction in sperm quality, and cause particular changes in shape and molecular structure of germ cells in the testis and mature sperms. (MacDonald AA, et al.2010, Teerds KJ, et al.2011, Du Plessis SS, et al.2010).

Male Obesity usually expresses a unique hormonal profile described as "hypogonadotropic hyperestrogenic hypogonadism." Both total and free testosterone levels are reduced in obese males. Total body fat content, subcutaneous fat, and intra abdominal fat have been associated with reduction in levels of total and free testosterone. (Haffner SM,,et al 1993, Tsai EC, et al. 2004). Central obesity in particular appears to be associated with a decrease in levels of circulating androgen directly proportional to the degree of obesity. (Giagulli VA,et al. 1994). We have reexamined the effect of the insulin resistance on levels of total and free testosterone in obese males. And also study the ameliorating effect of different drugs on the side effect of induced obesity in male rats. The experimental data were collated in years 2011–2012.

Material and Methods:
Rats :study was carried out on a healthy ,adult male Sprague Dawley rats, weighing between 150-200 gm were used,

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they were housed in separate metal cages under controlled environmental and nutritional conditions (20° - 25°C). The animals were divided randomly into five groups; Each group consisted of 10rats.

Diet : Two pelleted chows were prepared, The highly fat diet (HFD) contained 20 g of fat/100 g of diet (19 g of oil of butter and 1 g of oil of soybean to supply fundamental fatty acids) and supply 19.34 kJ/g of diet, include 7.74 kJ/g as fat. The low-fat diet (LFD) contained 3 g of oil of butter and 1 g of oil of soybean /100 g of diet and supply 16.12 kJ/g of diet include 1.29 kJ as fat. In addition to the essential amount of minerals and vitamins required for rats in case of HFD and LFD (Table1).

ITEM	L.F Diet	H.F Diet
g/kg of Diet		
Casein, high nitrogen	140	164
Cornstarch	455.7	303.1
Dyetrose	155	115
Sucrose	100	89.9
Cellulose	50	58.6
Butter oil	30	190
Soybean oil	10	10
Mineral	35	41.0
Vitamin	10	11.7
L-Cystine	1.8	2.1
Choline bitartrate	2.5	2.9
Mineral	10	11.7

Table1.show the compositions of High fat Diets (HFD), and Low fat Diets (LFD).

Experimental Design:
The duration of the experiment is 12 weeks. After 4 weeks from starting of the experiment, the treatments for hypercholesterolemia were added to the water to different groups as the following design:

Group 1(Control): received low fat diet chow, and distilled water (n=10). Group 2(Obese): received high fat induced diet, and distilled water (n=10).Group 3: received high fat induced diet, and received (Ezetimibe) at dose (20mg/Kg/day) after 4 weeks from starting 0f experiment and for 8 weeks (n=10). Group4: received high fat induced diet, and received (Simvastatin) at dose (20mg/Kg/day) after 4 weeks from starting 0f experiment and for 8 weeks (n=10). Group 5: received high fat induced diet, and received (Ezetimibe + Simvastatin) at dose (20mg/Kg/day) for each one after 4 weeks from starting 0f experiment and for 8 weeks (n=10).

Biochemical analysis:
After 8 Weeks from addition of treatment, the blood samples collected after overnight fasting (10-12 hr) from the medial canthus of eye using micro-hematocrite tubes. At the same time of the day, Sera were obtained after at least 30 min clotting by centrifugation at 2000 rpm for 15 min. Sera were removed and frozen at -20°C. After collection of all specimens, levels of testosterone were determined by Enzyme-linked immunosorbant assay (ELISA) according to (O'Donnell L, et al.1994). Free testosterone by technique of (ELISA) according to (McCann D, et al.1985). FSH was measured by (ELISA) according to (Ulloa-Aguirre A;et al 1998). Plasma insulin was measured by Enzyme-linked immunosorbant assay (ELISA) according to (Carlsson, et al 2010). In case of serum cholesterol levels was examined using an enzymatic assay with a serum Auto-analyzer (Roche Co, Switzerland). Also, Fasting Blood Sugar (FBS) was examined by enzymatic assay assay with a serum Auto-analyzer (Roche Co, Switzerland). Insulin sensitivity index was calculated by formula as: Insulin sensitivity index (ng/dl): Fasting Glucose (mg/dl) / Insulin (ng/ml). By Homeostasis model assessment (HOMA) (Vuguin P, et al .2001, Bastard J.P, et al. 2001).

Statistical Analysis:
Analysis was carried out by using SPSS program for windows version 20 (SPSS, Chicago, USA). All results are presented as "Mean ± SE", One-way analysis of variance (ANOVA) was used for comparison between different groups and treatments. Differences were considered statistical significance at ($P < 0.05$).

Results:
In Figure 1, Table 2, the results illustrate mean of weight gain (g) Using body weight as the sole indicator of "obesity" between different groups of rats. Weight gain degree was highly significant increased in obese group which fed on high fat diet when compared to their corresponding control (group 1), ($P < 0.001$).Treated groups (3, 4, and5) which also fed on high fat diet and received treatments for hypercholesterolemia record lowering in obesity degrees when compare with obese group, but still higher than control group. Among the treated groups the lowest degree of obesity found in (group5) as (252 ±1.92) followed by (group3) as (263±1.51), then (group 4) as (270 ±1.94).

The results also compare the different levels of (FBS) and insulin among groups. The highest level of both (FBS) and insulin were found in obese group (Group 2) with highly significance increasing when compare with control. The levels of (FBS) and insulin in treated groups which received treatments for hypercholesterolemia were lower than the level in obese group, but still higher than control one. Among the treated groups the lowest level of both (FBS) and insulin were found in (group5) as (72.6 ±3.69, and 17.1 ±0.69) respectively. Followed by (group3) as (82.8 ±4.06, and 20.8 ±0.89) respectively. Then (group 4) as (102.4 ±1.9, and 23 ±0.71) respectively.

Consequently, the level of (insulin sensitivity index) was highly significantly decreased in obese group (group 2), when compare to control (group1), ($P < 0.001$). Also the levels of (insulin sensitivity index) in treated groups were higher than obese with a non significance between them and control. Among the treated groups the highest level of (insulin sensitivity index) was found in (group4) as (4.46 ±0.183) .Followed by (group5) as (4.26 ±0.365) .Then (group 3) as (4.04 ±0.301).

Figure 2, Table 2.Total and free testosterone have negative correlation with obesity degree that appear when the levels of total and free testosterone in obese rats were lower than control. treated groups have an improvement in levels of them when compare with obese but still lower than control. Among the treated groups the highest level (Total testosterone) was found in (group5) as (2.58 ±0.106), followed by (group3) as (2.1±0.11), then (group 4) as (1.74 ±0.049). In addition, among the treated groups the highest level of (free testosterone) was found in (group5) as (0.58 ±0.022), followed by (group4) as (0.56 ±0.025), then (group3) as (0.54 ±0.022). in focus on group treated with simvastatin record that simvastatin effect on lowering the level of total testosterone but not free testosterone.

In addition, level of follicular stimulating hormone (FSH) (ng/ml) has a positive correlation with obesity degree that appear when the levels of it in obese rats was higher than control. Levels of follicular stimulating hormone (FSH) in treated groups were lower than in obese rats, but there is a non significance between levels of (FSH) in these groups when compare with control group. Among the treated groups the lowest level (FSH) was found in (group4) as (0.68 ±0.011), followed by (group5) as (0.74 ±0.022), then (group 3) as (0.78 ±0.022).

The highest level of the cholesterol (mg/dl) was shown in obese rats group due to the highly fat diet with a highly significance increase when compare with control. The levels of cholesterol were decreased between treated groups which received treatments for hypercholesterolemia when compared to obese group (group 2), but still higher than control (group 1). Among the treated groups the lowest level of cho-

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lesterol level was found in (group 5) as (101.4 ±2.357), followed by (group4) as (118.4 ±4.38), then (group3) as (130.8 ±3.42).

Table 3 is the summary of the correlation analysis between different parameters.as the level of (insulin sensitivity index) was negatively correlated with obesity degree, and (FSH). and positively correlated with total and free testosterone. our results showed that both of total and free testosterone have negative correlation with obesity, and cholesterol level.so the insulin resistance is associated with lowering in total and free testosterone in obese males.

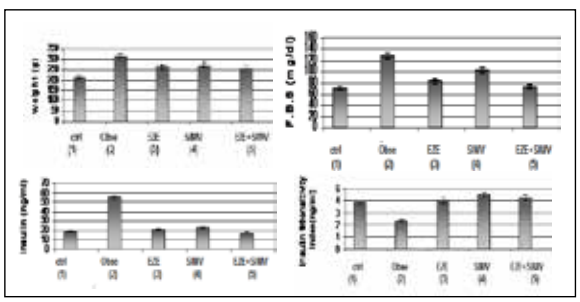

Fig 1. Means of the level of obesity, F.B.S, insulin, and insulin sensitivity index. in different groups of rats after 8 weeks (all values expressed as Mean±SE).

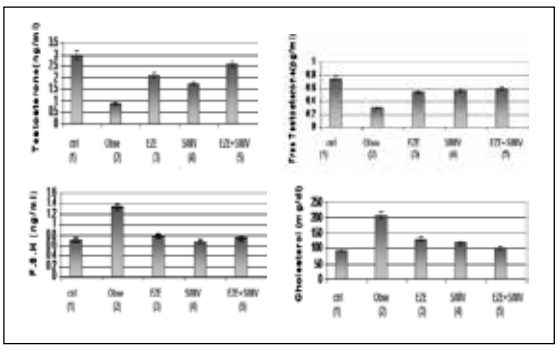

Fig 2. Means of the plasma level of testosterone, free testosterone, F.S.H, and cholesterol. in different groups of rats after 8 weeks (all values expressed as Mean±SE).

Discussion:

The results record increasing in weight gain (obesity) by using high fat diet. Groups (3,4,and 5)which received treatments for hypercholesterolemia record reducing in body weight .Most decreasing in body weight found in (group3&5) which received ezetimibe, the reason due to Ezetimibe acts through selective inhibition of intestinal cholesterol absorption. (Van Heek M,et al.1997, Van Heek M, et al.2000, Van Heek M, et al.2001, Davis HR, et al 2000). Also ezetimibe prevents dietary and biliary cholesterol uptake and transport across the intestinal wall. (Van Heek M, et al.2000). Also the results show the obesity have strong negative correlation with insulin sensitivity index and strong positive correlation with (FBS) and insulin that due to the increasing the supply of lipids which raises the level of free fatty acid (FFA) and lead to development of insulin and type 2 diabetes(Boden1997; Mc Garry 2001).

Tested parameters	Control (G1)	Obese (G2)	Ezetimibe (G3)	Simvastatin (G4)	Eze +Simva (G5)	P Value
Weight (g)	212^{c} ±1.51	311^{a} ±20.8	263^{b} ±1.51	270^{b} ±1.94	252^{b} ±1.92	<0.001
F.B.S (mg/dl)	70.0^{d} ±1.3	127 ^{a} ±3.57	82.8^{c} ±4.06	102.4^{b} ±1.9	72.6^{d} ±3.69	< 0.001
Insulin (ng/ml)	18.26^{c} ±0.87	55.1 ^{a} ±3.03	20.8^{b} ±0.89	23^{b} ±0.71	17.1^{c} ±0.69	< 0.001
Insulin Sensitivity Index level (ng/ml)	3.86^{a} ±0.237	2.34^{b} ±0.15	4.04^{a} ±0.301	4.46^{a} ±0.183	4.26^{a} ±0.365	< 0.001
Testosterone ng/ml)	2.98^{a} ±0.07	0.9^{e} ±0.07	2.1^{c} ±0.11	1.74^{d} ±0.049	2.58^{b} ±0.106	< 0.001
Free Testosterone (pg/ml)	0.75^{a} ±0.02	0.3^{c} ±0.03	0.54^{b} ±0.022	0.56^{b} ±0.025	0.58^{b} ±0.022	< 0.001
FSH (ng/ml)	0.7^{b} ±0.03	1.33 ^{a} ±0.109	0.78^{b} ±0.022	0.68^{b} ±0.011	0.74^{b} ±0.022	< 0.001
Cholesterol (mg/dl)	95.0^{d} ±2.167	209.6 ^{a} ±3.98	130.8^{b} ±3.42	118.4^{c} ±4.38	101.4^{d} ±2.357	< 0.001

Table 2. Show the mean of tested parameters of different groups

The excess free fatty acids (FFA) are ultimately stored in non-adipose tissue lead to increase intracellular lipids that cause insulin resistance. So we can says that FFAs play a major role in effecting insulin resistance.(Boden et al 2001; Kuhlmann 2003). Initiation of insulin resistance or loss the activity of insulin is the primary production of type 2 diabetes. Inhibition of transportation of glucose by FFA has been found to be linked to insulin-mediated signals. (Dresner et al 1999; Yu et al 2002; Chavez et al 2003). This suggests that the reduction in insulin activity leading to insulin resistance is associated with insulin signalling defects. Molecules of Insulin signalling involved in metabolic and mitogenic action may also have an important role in cellular insulin resistance (Zick 2001; White 2002; Greene et al 2004). A few recent researches show that some (protein kinase C) PKC isoforms may have a regulation effect on insulin signalling. The activity and levels of expression of a few PKC

Table3.Bivariate correlations between tested variables

	WEIGHT	F.B.S	Insulin	Insulin Sensitivity Index	Testosterone	Free Testosterone	F.S.H	Cholesterol
WEIGHT	1	0.728 **	0.648 **	- 0.425 *	- 0.819**	- 0.717**	0.644**	0.739**
F.B.S	0.728 **	1	0.838**	- 0.432*	- 0.916**	- 0.801**	0.749**	0.844**
Insulin	0.648 **	0.838**	1	-0.821**	- 0.827**	- 0.820**	0.848**	0.939**
Insulin Sensitivity Index	- 0.425 *	- 0.432*	-0.821**	1	0.540**	0.544**	- 0.711**	-0.749**
Testosterone	- 0.819**	- 0.916**	- 0.827**	0.540**	1	0.869**	- 0.738**	-0.872**
Free Testo	- 0.717**	- 0.801**	- 0.820**	0.544**	0.869**	1	-0.732**	- 0.889**
F.S.H	0.644**	0.749**	0.848**	- 0.711**	- 0.738**	-0.732**	1	0.880**
Cholesterol	0.739**	0.844**	0.939**	-0.749**	-0.872**	- 0.889**	0.880**	1

***correlation is significant at 0.05,**

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****correlation is significant at 0.01 (highly significant).**

isoforms are linked to insulin resistance. Lipid infusion in humans and rats decrease insulin-stimulated glucose elimination in muscle tissue concomitantly with the activation of certain PKC isoforms. (Griffi n et al 1999; Yu et al 2002; Boden and Shulman 2002).

Recently, the researches explain that FFA-induced insulin resistance is linked to insulin receptor gene down regulation, in which a PKC isoform have a significant role (Dey et al 2005). The association between obesity and insulin resistance syndrome and cardiovascular risk is not only related to the level of obesity but also related to distribution of body fat. Consequently, individuals with higher degrees of central adiposity develop this syndrome more than the syndrome which found in case of peripheral body fat distribution. By losing of weights in groups (3, 4, and 5) which received treatments for hypercholesterolemia we found decreasing in level of (FBS) and insulin and increasing in insulin sensitivity index. Weight loss is tightly linked with decreasing in insulin concentration and an increasng in insulin sensitivity in adult males. (Su HY,et al.1995). Specifically found in groups (3&5) which received ezetimibe because of prevention of extra uptake of cholesterol as previously discussed but still higher than control group.

Levels of Total and Free Testosterone are strong negatively correlated with obesity degree and insulin resistance. (Pitteloud N,et al 2005). Males with insulin resistance cases such as obesity (Pasquali R, et al 1991, Glass AR, et al 1977). And type 2 diabetes (T2D) (Barrett-Connor E et al 1992, Andersson B, et al 1994) have significantly lower Total and Free Testosterone levels than normal weight control groups. It has been suggested the inverse correlation between Testosterone and insulin level is due to obesity (Abate NA, et al 2002, Tsai EC, et al 2004).The reason due to the adipose tissue produce signals, which are known to modulate action of insulin, also have an important role in regulation of gonadal function. Leptin is a regulator of reproductive function, which is strongly associated with insulin resistance. (Chehab FF, 1996, Farooqi IS,et al 1999, Chan JL,ei al 2003). Several studies have proved that leptin is inversely proportional with serum Total and Free Teststerone levels (Luukkaa V, et al 1988, Wabitsch M, et al 1997, Haffner SM,et al 1997), and an excess of circulating leptin has been involved in the causing of low Total and Free Testosterone levels in obese male(Isidori AM, et al 1999). Leptin receptors are found in Leydig cells, and leptin cause inhibition for hCG-stimulated Testosterone secretion from Leydig cells in rats at similar concentrations in obese males. (Caprio M, et al 1999). There is a significant improvement in testosterone in groups which received treatments for hypercholesterolemia when compare with obese group that due to decreasing in fat accumulation and body weight. Also we record a strong positive correlation between insulin sensitivity index and Leydig cell function assessed by the Total and Free Testosterone levels response to physiological stimulation with hCG. that the low levels of testosterone seen in insulin-resistant men cannot be explained by low levels of SHBG alone, but, rather, represent a defect in function at one levels or more levels of the axis of HPG .((Pitteloud N,et al. 2005).

All treated groups record an improvement in levels of total and free testosterone. Using the combination of (Simvastatin + Ezetimibe) as a treatment for hypercholesterolemia record a highest improvement in the levels of both total and free testosterone among treated groups that due to presence of 2 different pathways of treatments .which also record the lowest level in cholesterol and be near to cholesterol level in control (non significance). Discuss as the following: a) Simvastatin which inhibit HMG-CoA reductase (the enzyme which converts HMG-CoA into mevalonic acid, a cholesterol precursor), also have a capacity to reduce the endogenous cholesterol synthesis. (Vaughan C.J., et al 2000).

b) Ezetimibe which inhibit the absorption of intestinal cholesterol, and prevents dietary and biliary cholesterol uptake and that cause weight loss. (Van Heek M,et al.1997, Van Heek M, et al.2000, Van Heek M, et al.2001, Davis HR, et al 2000). So this reduction in cholesterol level causes improvement in levels of total and free testosterone by the negative correlation between them. Because of the high level of cholesterol, pre-diabetes or high blood pressure causes hardening and stiffness of the arteries that lead to arteriosclerosis, which decreases flow of blood to the testicles to damage the testicles and lower testosterone. High blood pressure and high cholesterol cause decreasing in total and free testosterone level, so men with low level testosterone are at increased risk for heart attacks. By the time a men have low levels of total and free Testosterone, they may already have significant arteriosclerosis (Fogari R, et al 2002). And also this recorded improvement in level of total and free testosterone was positively correlated with severely improvement of insulin sensitivity index (Chan JL,ei al 2003).

Group 4 which received simvastatin as treatment record a decreasing in Total Testosterone level but not effect on Free Testosterone (Dobs AS, et al 2000). This result can be discussed as the Cholesterol is the substrate for biosynthesis of testosterone, and theoretically, hydroxymethyl- glutaryl-CoA reductase inhibitors such as simvastatin affect serum testosterone levels. Recent studies have shown that simvastatin can reduce testosterone production with simvastatin treatment (Hyyppa MT, et al 2003, Rossato M, et al 1993). An alternative hypothesis is that simvastatin causes a reduction in SHBG with consequent reductions in Total Testosterone. Production of SHBG is in the liver, which is the primary site of action of the statins.(Hampl R, et al 1996).

FSH was positive correlated with obesity in males, also has a strong negatively correlation with Total and Free Testosterone level. That according to researches indicated increasing in levels of FSH, and LH with increasing body weight .that due to decreasing in levels of Total and Free Testosterone with increasing in body weight as previously discussed. When Total and Free Testosterone levels are low, gonadotropin-releasing hormone (GnRH) is secreted by the hypothalamus which in turn stimulates the pituitary gland to produce FSH and LH. These two later hormones stimulate the testes to synthesize testosterone. Finally increasing the levels of testosterone through a negatively feedback loop act on the hypothalamus and pituitary to inhibit the releasing of GnRH and FSH/LH respectively. That recorded in most studies have suggested higher levels of FSH and LH with increasing weight and BMI. (Jensen et al. 2004; Aggerholm et al. 2008).

Conclusion:

Insulin resistance plays an important role in the pathogenesis of many human diseases, such as diabetes, obesity, cardiovascular disease (CVD), hypertension, nonalcoholic fatty liver disease, stroke, certain forms of cancer, and reduction of male fertility. A condition of truncal obesity associated with increased insulin resistance is also accompanied by low testosterone levels. Obesity has been shown to inversely affect on levels of total and free testosterone, by reduction of spermatogenesis. We also conclude that combination of simvastatin + ezetimibe record the lowest level of cholesterol and FBS and highest improvement in insulin sensitivity index, total, and free testosterone levels among treated groups.

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