A Combination of Clomifene Citrate (CC) and L-Carnitine (LC) Significantly Reduces Spermatozoal Lipid Peroxidation (LP) in Obese Male Idiopathic Subfertile Normozoospermic Patients

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Abstract

Objective: To study the effect of a combination of CC and LC in decrease of spermatozoal LP and improvement sperm quality in obese male idiopathic subfertile normozoospermic patients.
Design: A prospective study.
Setting: Maternity and Childhood hospital.
Patient and Method(s): 35 proven obese male idiopathic subfertile normozoospermic patients were given CC and LC for 3 months ,10 subfertile patient did not received any medication except placebo.10 healthy proven fertile man served as control volunteers group. Their semen analyses and spermatozoal Malondialdehyde (MDA) assay were carried out as an index of spermatozoal LP before and after course of treatment**.**
Results: There is high statistically significant differences ($P<0.05$) in mean of MDA concentration in post-treated group when compared to non-treated group of obese male idiopathic subfertile patients and control group. Significant increase ($P<0.05$) in sperm vitality in combination post-treated group when compared to non-treated group of obese male idiopathic normozoospermic subfertile patients and control group.
Conclusion(s): This study suggests that a combination of CC and L-C reduced spermatozoal lipid peroxidation (MDA) with improving sperm vitality in obese male idiopathic subfertile normozoospermic patients.

استعمال دواء كلوميفين ستريت و ال كارنتين يقلل معنويا من اكسدة دهون الحيامن لدى مرضى البدانة عند عقم الدكوري الغير المفسر

الخلاصة

الاهداف : تقييم ثاثير استعمال دواء كلوميفين ستريت و ال كارنتين على أكسدة دهون غشاء بلازما الحيامين والقياسات النوعية للحيامن لدى مرضى البدانة في عقم ألذكوري الغير المفسر.
الطريقة :درسة مستقبلية ،اجريت على ٤٥ مريض من مجموع ٦٧ مصابين بالبدانة ولديهم حالة عقم ذكوري غير مفسر .تم اعطاء ٣٥ منهم (مجموعة المعالجة) من كلوميفين ستريت و ال كارنتين لمدة ثلاثة اشهر متتالية . ثم أخذ عينات الدم لفحص الهورمونات الخاصة بالغدد التناسلية والجنسية والسائل المنوي قبل و بعد اعطاء العلاج لغرض تقييم نوعية الحيامن وقياس مستوى اكسدة دهون الحيامن من خلال قياس مستوى المالوندايالهايدات في الحيامن والدي اعتبر كمؤشر لقياس اكسدة دهون الحيامن.اما عشرة مرضى العقم الاخرين لم يتم اعطائهم علاج عدا الوهمي .ان مجموعة السيطرة تالفت من احدى عشر رجل متطوع ومنجب وبصحة جيدة .
النتائج :اشارت نتائج الدراسة بعد مقارنتها بالمجموعة المعالجة وغير المعالجة مع مجموعة السيطرة بان المجموعة المعالجة كان لديها انخفاضا ملحوضا في معدل مستوى المالوندايالهايدات في الحيامن مما يؤثر ايجابيا في انخفاض مستوى على اكسدة دهون غشاء بلازما الحيامين ودلك من خلال عمل مضاد الاكسدة في ال كارنتين اضافة الى التحسين النوعي في الحيامن بسبب التاثيرات المزدوجة للعلاج مما قد تسبب في نقص اجهاد الحيامن والدي بدوره من المحتمل ان يزيد من فرص الاخصاب والحمل لدى مرضى البدانة عند العقم الدكوري الغير المفسر

الخلاصة :ان استعمال دواء كلوميفين ستريت و ال كارنتين تسبب في نقص ملحوض في معدل مستوى المالونديالدهايدات في الحيامن والدي اعطى مؤشرا على انخفاض مستوى الدهون اضافة الى التاثيرالعلاجي المزدوج والدي تسبب في تقليل اكسدة دهون الحيامن والتحسين النوعي للحبامن.

Introduction

The medical management of human infertility is unique area of clinical medicine. An elevated risk for subfertility among couples in which the male partner is obese, as a result of several studies pointed to an increased likehood of abnormal semen parameters among overweight men [1]. Obesity is therefore is associated with the higher incidence of male factor of unexplained infertility [2].

An individual can be defined as an overweight if their BMI is 25-30kg/m^2 ,and obese if their BMI exceeds 30kg/m2 [3]. A combination of an increasingly sedentary lifestyle and unfavorable diet in the western world has resulted in an increase number of overweight & obese children and adults. According to WHO, approximately 1.6 billion adult were classified as being overweight and 400 million were obese in 2005. Statisticians have predicted that, by 2015, approximately 2-3 billion adult will be overweight and 700 million will be obese [4]. Also gaining attention is the reported decline in semen quality and men reproductive potential over the past 50 years. The quality of semen has potentially declined with the consequent negative effect of poor semen quality of men fertility According to Carlson et al [5]. In contrast to extensive knowledge of the effects of obesity on female fertility , male factor infertility as a result of obesity has been overlooked ,even after discovery of a threefold increase in the incidence of obesity in patient with male factor infertility demonstrating the need for greater clinician awareness in this area [6].

Carnitines play an important role in regulating Sertoli cell function ,protecting sperm against oxidative stress ,reducing apoptosis of spermatogenic cells and inhibiting sperm aggregation[7]. The etiology cause in nearly one –third of male factor infertility is known .The percentage of men with idiopathic infertility account for 30% of male infertility.In15-20% of cases doctors are unable to find a specific cause of infertility, so its labeled as a cause of unexplained infertility [2,8].

L-carnitine and Acyl- L–carnitine are highly concentrated in epididymis and are important for metabolism and maturation. In a double-blind crossover trial infertile patients, receiving either L-carnitine or placebo a significant improvement in sperm quality (sperm concentration & forward motility) was observed in the L-carnitine group .In addition, the largest improvements were noted in men with the poorest semen quality [9]. In another study by the investigators, combination therapy with L-carntine and acetyl-L-carnitine was given to 60 infertile men and seminal outcomes were observed . Whether or not this form of supplementation can result in significant improvements in pregnancy rate remain unknown [10]. L- carnitine is required during oxidation of lipid for transport of fatty acid from the cytosol in to mitochondria for generation of energy. L-carnitine exerts a substantial anti-oxidation, anticytokines and anti-apoptotic action providing multi-mechanisms protective effects for the cells [11].

L-carnitine has antioxidant activity that combines both free radical –

scavenging and metal –chelating properties [12]. L-carnitine protects all membrane and DNA against damage induced by free oxygen radicals & has a pivotal role in the mitochondrial oxidation of long fatty acids which increase energy to cell [12]. Mitochondrial dysfunction may lead to incomplete detoxification of the free radicals, which may lead to oxidative damage to micromolecule s such as lipids, proteins and DNA. L-carnitine has free radical –scavenging activity and ability to scavenge superoxide anion and inhibits lipid peroxidation (LP), thereby, conferring protection against damage induced by hydrogen peroxide [13-15].

Sperm membranes play an important role in fertilization capacity .Sperm membranes harbor highest concentration of polyunsaturated fatty acids(PUFA) than other human cells, sperm with highest concentration of PUFA are thought to have the most normal morphology [9]. Reactive oxygen species (ROS) can cause instability of membrane permeability through effects on PUFA as these fatty acids are extremely sensitive to oxidative stress. Indeed, the most protective antiperoxidative mechanisms are thought to maintain cell membrane stability [10].

Clomifene citrate (CC) the logic underlying the use of antioestrogens lies in their ability to favorably alter the intrinsic hypothalamogondal hormone axis .The most popular of these drug is clomifene citrate, a selective oestrogen receptor modulator (SERM), by reducing hypothalamic and pituitary sensitivity to oestrogen, antioestrogens increase pituitary outcome of LH and FSH, thus stimulating both testosterone production and spermatogenesis [16]. Despite of at least 20 clinical trials with CC for male infertility in the last 30 years ,there is still debate about the value of antioestrogens for male infertility .A WHO study of 190 couples found CC to have no significant effects on pregnancy rate or semen characteristics [16], while an older Cochrane meta-analysis of 10 controlled studies involving 738 men showed beneficial hormonal effects, but no effect on pregnancy rate [17]. However ,there is emerging literature to suggest that CC may play an important role in a subgroup of infertile patients to overcome acquired hypogonadotropic hypogonadism stimulate the pituitary level due to prolactinemia, sickled cell disease, diabetes mellitus [18]. Other selective oestrogen receptor modulator drugs, including tamoxifene citrate, act in similar fashion to CC [19].

Aim

To study the possible effects of a combination of CC and L-carnitine in decrease of MAD as an index of lipid peroxidation and semen quality improvement in idiopathethic obese male subfertile with normozoospermia.

Patients and Methods

This is a prospective study was carried out at Maternity and Childhood Hospital at period between January 2006 to July 2007. The study is consisted of total number 67 normozoosperpmic subfertile men with a primary factor of idiopathic infertility (barren marriage) of more than one year. All the participated patients were selected to be obese according to their body mass index (BMI) measurement, more than 30kg/m² in respect to WHO criteria [3]. Patients with proven normal sperm parameters and infertility factor that might interfere with fertility–related origin viz: Hypogonadotropic hypogonadism, varicocele, cryptorchidisim, venereal disease, leucocytospermia, drug and hormonal

therapy, abnormal sexual function (erectile dysfunction and impotence) and any patient who had difficulties in semen collection by masturbation or coitus interrupts were excluded.

Out of the total number of patients only 45 patients were randomly assigned to submit this study ,divided into two groups: group one :- is consisted of 35 patients that represents the treated group .All patients in this group were given a combination of clomifene citrate Asia pharmaceutical industries, 50mg daily for a period of three consecutive months and L-carnitine (USP) Ultimate Nutrition INC Farmington, CT 06034USA at dose 1000 mg twice daily for a period of three consecutive months as well. The second group is: - composed of 10 patients that represent the non-treated group that received placebo and served as a control group to treated group. The third group: - is consisted of 11 healthy, normal man with proven fertility volunteers (donors) that initiated a successful pregnancy within 1-2 years, regarded as a control group for both treated and nontreated groups .All scheduled patients in this study were accept with verbal consent, their actual agreement to contribute, take drugs, complete their investigations follow up during the whole period of study.

Quantitation of seminal granular leucocytes:-in semen specimen were assessed according to Endtz test [21]. The second aliquot was used for a preparation of sperm homogenization buffer for measurement of Malondialdehyde (MDA) as an index of a spermatozoal lipid peroxidation All investigations were carried out before and after period of three months treatment for whole participants.

Semen collection:

Sample of semen ejaculate were collected from all married patient by masturbation technique or coitus interrupts after 3-5 days of sexual abstinence. All ejaculate samples were collected at laboratory and brought within 20 min. into a clean aseptic vails.After ejaculation the specimen was placed in an incubator at 37C° for 30min to allow liquefaction .Each specimen was divided into aliquot. The first part was used to examine in details all macroscopic and microscopic examinations according to WHO criteria [20].

Sperm preparation

All masturbated semen samples liquefied after 30 minutes at 37 °C, spermatozoa were separated from seminal plasma by centrifugation at 500 x rpm for 30 minutes. The supernatant was precisely measured by a graduated centrifuge test tube and discarded. The supernatant was used for enzymatic measurements [22]. Homogenizing buffer added to the pellet fraction. Homogenizing buffer consisted of (11.9 gms of manitol, 4.8 gms of sucrose, 0.09 gms of EDTA in 250 ml of distilled water adjust the pH to 7.4 with tris-base. Homogenized buffer was kept in refrigerator at 4°C. The samples were hand homogenized and were subsequently centrifuged for 10 minutes at 3000 rpm. Cooled 0.9 ml of Triton X – 100 (0.1%) was added to each 0.1ml of pellet obtained from the sample, the samples were centrifuged again at 8000 rpm for half an hour in a centrifuge.

Determination of Malondialdehyde (MDA):-was determine by thiobarbituric acid (TBA) assay by Mihra and Uchiyama [23].

Principal : Malondialdehyde react with thiobarbituric acid (TBA) to form a pink coloured product.

Procedure: Sperm homogenate (500 ml) was added to 3 ml of 1% phosphoric acid, 1.0 ml of 0.6% TBA and 0.15 ml of 2.0% butylated hydroxytoluene (BHT) in 95% methanol. The samples were heated in

a boiling water bath for 45 minutes, cooled and 4.0 ml of butanol was added. The butanol phase was separated by centrifugation at 3000 rpm. All values were expressed as nmoles MDA / mg of protein using spectrophotometer Cecil – 1011 England in measurements.

Calculations:

$$\text{The concentration of MDA nmol / mg} = \frac{A}{L \times Eo} \times D \times 10^6$$

A = Absorption
L = light bath
Eo = Extension coefficient 1.56×10^5 m^{-1}. cm^{-1}
d = dilution factor 6.7

Results

Table 1 demonstrated a comparison of all sperm variables and spermatozoal Malondialdehyde (MDA) concentration in pretreated and post-treated group of obese male idiopathic subfertile patients (n-35).The presented results depicted that there was a high statistically significant difference ($P < 0.05$) in active sperm motility%, immotile sperm% and malondialhyde (nmol/mg of protein) concentration. The results were (61.92±1.40 VS 67.65±1.09), (19.96±1.17 VS 11.24±1.15) and (1.46±0.16vs $0.74±7.17\times10^{-2}$) respectively. While, there was significant difference ($P < 0.05$) in sperm count ×10 6 /ml, sluggish sperm motility %, sperm viable %, normal sperm morphology % and abnormal sperm morphology. A probable statistical cause of high significance of results is attributed to the effect of treatment on the value of sperm variable, whereas, the significant difference is related to the effect of treatment on post-treated values. And insignificant statistical values ($P >0.05$) refer to close similar values between pre-treated and post-treated values.

Radioimmunoassay (RIA):

5ml venous blood was drawn from whole participated idiopathic obese male subfertile patients (treated and non treated) groups and healthy fertile donors (control) group at 9A.M.with gauge 21 needle, placed on a rack for 30 min for clotting. Blood samples were centrifuged 3000 rpm for 15min to allow separation of serum .The serum was aspirated and kept at -20°C frozen until usage .RIA for luteinsing hormone (LH), follicular stimulating hormone (FSH) and testosterone hormone (T) were assessed ,using immunotech a Beckman Coulter company (cat.# 2125) immunoradiometric assay kit, reference 1M 2125-1M 3301 and minigammacounter, LKB-Wallac (Gamma counter).

Statistical analysis; Data were analyzed using inbuilt functions within the statistical package of SPSS UK version 10 surrey UK .University of continuous variables among the groups was performed .Analysis of variance (ANOVA) and least significant deference between means at level of significance 0.05. All hypothesis testing two tailed, $P < 0.05$ was considered statistically significant.

Table 1 Shows a comparison of each mean sperm variables of treated group between (pre and post treatment) of obese male subfertile normozoospermic patients (n=35) for 3 months period. Data are presented as mean ± (SEM).

Sperm variable	Mean		P value
	Pre	Post	
Sperm count x10^6/ml	61.34 ±3.88	63.53 ±3.64	0.02*
Sperm concentration 10^6 /semen volume	147.07 ±15.40	158.83 ±13.43	0.18
Sperm active motility %	61.92 ±1.40	67.56 ±1.09	0.00**
Sperm sluggish motility %	18.32 ±1.41	20.80 ±1.51	0.21*
Sperm immotility %	19.96 ±1.17	11.24 ±1.15	0.00**
Sperm viability %	65.20 ±1.59	69.08 ±1.36	0.01*
Normal sperm morphology %	63.28 ±1.72	66.84 ±1.52	0.01*
Abnormal sperm morphology %	36.56 ±1.72	33.16 ±1.52	0.01*
Malondialdehyde nmol/mg	1.46 ±0.16	0.74 ±7.17 x10^{-2}	0.00**

**** Highly significant value P < 0.05 when value 0.00 (2-tailed)**
*** Significant value less than P < 0.05**
Insignificant value P > 0.05

group, the results were (1.31±0.02 vs 0.74±0.12) respectively. While there was statistically significant difference (P< 0.05) in an active sperm motility%, sluggish motility% and immotile sperm %.The results were (61.00±2.24 vs 65.61±1.88, 19.00±1.59 vs 24.12±1.83 and 20.00±1.87 vs 16.92±1.74) consecutively.

Table 2 shows a comparison of all variables and malondialdehyde (MDA) concentration (nmol /mg of protein) between treated (n=35) non-treated group (n=10) for 3months period of obese male idiopathethic subfertile patients .The results implied that there was a high statistical significant difference (P < 0.05) in MDA concentration (nmol /mg of protein) between treated group and non-treated

Table 2 Demonstrate comparison of each sperm mean variable between treated (n=35) and non-treated group obese male idiopathethic subfertile normozoospermic patients for 3 months period. Data are presented as mean ±(SEM).

Sperm variable	Mean		P value
	Non-treated	treated	
Sperm count x10^6/ml	45.53 ±5.43	46.43 ±5.13	0.51
Sperm concentration 10^6 /semen volume	114.08 ±15.97	126.75 ±15.32	0.11
Sperm active motility %	61.00 ±2.24	65.61 ±1.85	0.01*
Sperm sluggish motility %	19.00 ±1.59	24.00 ±1.83	0.21*
Sperm immotility %	20.00 ±1.87	16.92 ±1.74	0.05*
Sperm viability %	63.46 ±2.42	64.15 ±2.14	0.67
Normal sperm morphology %	63.46 ±2.96	65.38 ±2.56	0.37
Abormal sperm morphology %	36.53 ±2.96	34.61 ±2.56	0.37
Malondialdehyde nmol/mg	1.31 ±0.20	0.74 ±0.12	0.00**

**** Highly significant value P < 0.05 when value 0.00 (2-tailed)**
*** Significant value less than P < 0.05**
Insignificant value P > 0.0

Data of ANOVA demonstrated a comparison of all sperm variables among the treated group (n=35, non-treated group n=10) of obese male idiopathethic subfertile patients and normal healthy fertile donor of control group n=11. The present data showed that there was a statistically significant differences (P< 0.05) in sperm sluggish motility %, sperm immotility %, sperm viability % table(3). Generally, the results of malondialdehyde concentration (nmol/mg of protein) implied a high statistically significant difference (P<0.05) when compared control group to treaded group and non-treated group table (3). The high statistical difference refers to low values of the treated group when compared to control and non-treated value.

Table- 3 ANOVA comparison of each sperm variable among treated and non-treated group with control of obese male idiopathethic subfertile patients for 3 months period. Data are presented as mean ± (SEM).

Variables / Post treatment	Group	Mean	P value
Sperm count x10^6/ml	Control Non treated treated	50.78 46.20 63.53	0.20 0.06
Sperm concentration 10^6 /semen volume	Control Non treated treated	183.11 161.02 171.12	0.31 0.23
Sperm active motility %	Control Non treated treated	66.71 65.21 67.56	0.44 0.36
Sperm sluggish motility %	Control Non treated treated	17.57 16.50 20.80	0.75 0.34**
Sperm immotility %	Control Non treated treated	16.43 17.86 11.24	0.40 0.08*
Sperm viability %	Control Non treated treated	68.57 64.07 79.08	0.05* 0.01*
Normal sperm morphology %	Control Non treated treated	61.42 64.64 66.84	0.30 0.93
Abnormal sperm morphology %	Control Non treated treated	38.57 35.35 33.16	0.30 0.92*
Malondialdehyde nmol/mg	Control Non treated treated	0.88 0.54 0.22	0.09 0.01**

**** Highly significant value P < 0.05 when value 0.00 (2-tailed)**
*** Significant value less than P < 0.05**
Insignificant value P > 0.05

Discussion

From the preceding literature, it is evident that obesity is an influencing factor of male infertility and the medical management of human infertility is unique area of clinical medicine [24].

Many experts believe that overweight and obesity can be taken to reverse both unhealthy consequences associated with obesity and negative impacts on male infertility [25, 26]. The increase in the incidence of obesity has a substantial societal heath impact; contrasting reports have been published whether overweight % obesity affect male fertility [1].

Cabler's study referred the corresponding decrease in male infertility and fecundility in parallel to obesity and obesity should be considered as an etiology of male infertility [24].Obesity increases the risk of hypogonadtropic hypogonadism.Animal models indicator that obesity causes liptin insensitivity in the hypothalamus ,leading to decrease Kiss1 expression

,which in turn ,alters the release of gonadotrophine releasing hormone GnRH [1].Aromatase inhibitors are an option for obese males facing infertility problems ,especially if they have elevated estrogen and lowered testosterone level [27]. All above mentioned findings support our results table (1) that presented an improvement in most sperm variables of post treatment group inspite of that our idiopathic obese patient patients have normal gonadotropic gonadal hormone level.

Also, corroborate with other studies mentioned that treatment with aromatase inhibitors lead to normalization of patients testosterone , LH,FSH hormone levels and normalization of spermatogenesis[28, 29].Generally ,our findings are consistent with the logic underlying use of antioestrogens that in their ability to favorably alter intrinsic hypothalamus gonadal hormone axis and clomifene citrate is the a popular drug of selective estrogen receptor modulator. [17] and not consistent with [16].

On the other hand ,the present results of high significant decrease (P< 0.05) of lipid peroxidation ,malondialdehyde concentration between pre-treated and post –treated group table (1) may also interpret the participation of antioxidant effect of L-carnitine on decrease spermatozoal lipid peroxidation which utilize MDA concentration as an index of sperm oxidative stress status .Our data agree other studies [9,10]. And also supports Abdulrazik 's study that mentioned L-carnitive is required during oxidation of lipids for transport of fatty acids from the cytosol to mitochondria for generation of energy[11]. L- carnitive exerts a substantial antioxidant, anticytokines and anti-apoptotic activity providing multi mechanisms proactive effects for cells [11].Interestingly, a study mentioned L-carnitive has antioxidant activity that combines both free radicals - scavenging and metal-chelating properties is consistent with our data as well [12].

The results of table (2) consolidate the data in table (1) in regard to high statistical significant decrease (P <0.05) in malondialdehyde concentration (nmol/mg of protein) between nontreated & treated groups of idiopathic obese subfertile patients .The present result corroborates with other findings [11,12] who found out a statistical effect of L-carnitine on decrease reactive oxygen species that result in decrease of spermatozoal lipid peroxidation (MDA) as an index of sperm oxidative stress ,this is explained by improved sperm vitality% obese in idiopathic subfertile patients is consistent with other studies which substituted to a combination of L- carnitine and acetyl L- carnitine treatment after failure to respond of treatment patient to integrated therapy of empirical medicine as a trial of antioxidant activity of L-carnitine [7,26].

The main event in table (3) that demonstrated ANOVA comparison of means of sperm variable and MDA concentration with both non-treated and treated groups of idiopathic male obese subfertile patients and donor volunteers control group ,obviously, depicted a high statistical significant difference (P<0.05) of MDA concentration among non treated , treated group and control group .Our data of pretreated group may give an explanation of a higher incidence of obesity -associated male subfertility that initiate a direct and /or indirect adverse mechanisms of obesity on idiopathic obese male subfertility and infertility .These results corroborate with [11,12,30]. Interestingly, our present study

interprets a high significant decrease of MDA concentration in treated group when compared to nontreated and control group to the antioxidant effect of L-carnitine that play a role in decrease spermatozoal lipid peroxidation ,that is indicated by determination of MAD as an index of decrease of sperm oxidative stress .These results agree with [12]. However, till now, the etiological cause in nearly one-third of male infertility is unknown, the presence of men with idiopathethic infertility who have been successfully treated by empirical therapeutics modalities is not high [31].

Generally, because of the intricate mechanisms that link obesity, male idiopathic subfertility and infertility inspite of clear shortage of explorations of those underlying mechanisms with lack of ineffective therapeutic interventions.The present study calls for more greater clinician awareness of the effect of obesity on fertility,better understanding of underlying mechanisms and eventually avenues for mitigation or treatment and in our opinion however, this approach may be of value in clinical application in field of male obese idiopathic subfertility.

In conclusion, this study suggests that a combination of CC and L-carnitine may have a significant value in improving sperm quality and decrease spermatozoal lipid peroxidation that may minimize spermatozoal oxidative stress of idiopathethic obese male subfertility.

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