Effect of L-carnitine and meloxicam treatment on testicular Leydig cell numbers of varicocelized rats

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Abstract  Objective: Varicocele is a pathobiological condition associated with abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the spermatic cord; it is one of the leading causes of male infertility. Although several studies have considered the relationship between varicocele and semen L-carnitine concentrations, no study on the effects of L-carnitine on testicular number of Leydig cells which are important in fertility of the individual has been reported.

Design: Prospective study.

Setting: Institute of the Embryo Research and Infertility Treatment, College of Medicine, AL-Nahrain University.

Materials and methods: Thirty male albino Wister rats divided into three groups, 10 animals each. The groups A and B underwent a left experimental varicocele induction. Group C was not operated on and served as control. Animals in group A (Treatment group) were treated with L-carnitine twice daily and meloxicam once every four days. Group B (Placebo group) and C (Control group) received placebo and distilled water, respectively; using the same treatment program of group A. Treatment in all cases was given orally by oral cavage, and continued daily for 12 weeks. At the end of treatment rats were sacrificed. Serial histological sections were then prepared and examined microscopically for number of testicular Leydig cells.

Main outcome measure: The number of Leydig cells was used to evaluate testicular changes:

Results: Histological examination shows new findings of significant improvement of decrease in the number of Leydig cells ($P < 0.05$) in treatment groups as compared to placebo.
1. Introduction

Varicocele is a pathobiological condition associated with abnormal tortuosity and dilatation of the veins of the pampiniform plexus within the spermatic cord; it is one of the leading causes of male infertility (1). The exact mechanism of impaired testicular function in patients with varicocele is not known. The most widely accepted concept is currently a varicocele-related increase of testicular temperature. Normally, the difference between the intraabdominal and scrotal temperature averages 2.2 °C. Varicocele can cause an increase in scrotal temperature by 2.6 °C, neutralizing the required temperature gradient. However, there is considerable overlap between the range of scrotal temperatures in infertile men with varicoceles and in normal fertile men (2). The varicocele-associated pathology mainly includes changes in testicular size, histology, impaired Leydig cell function (steroidogenesis), and sperm characteristics (spermatogenesis) (3). Previous studies were performed to evaluate the role of L-carnitine in spermatogenesis and seminal fluid parameters. In an attempt to characterize the protective action of Acetyl L-carnitine (ALC) using an in vivo test system, the recovery and maturation process of mouse spermatogenesis was investigated. Mice were exposed to irradiation to deplete the spermatogonia and then were given ALC. The sperm population in the mice that received ALC demonstrated a quicker recovery throughout the maturation process than the spermatozoa in those that did not receive ALC (4). Therefore, it appears that ALC could influence the early stages of spermatogenesis with consequent favorable effects on DNA repair and on proliferation of regenerating germ cells (4). Similarly, shortening of the spermatogenesis recovery time following hyperthermic injury was reported (5), which may be of clinical importance in humans as hyperthermia affects the reproductive capacity in cases of varicocele, one of the most common etiologies of male infertility (6).

Leydig cells express cyclo-oxygenase enzyme-2 (COX-2) in human pathological biopsies (7). Moreover, the increased number of testicular macrophages found in biopsies from testes of infertile patients (8) appears to be involved in the induction of COX-2 expression in human Leydig cells (7).

Studies performed in Brown–Norway rats have shown that COX appears to play a role in the decrease of the testicular production of testosterone that takes place during aging (9). Experiments carried out in adult golden hamsters establish that Leydig cells produce PGF2α, which exerts an inhibitory role on the expression of StAR and 17β-hydroxysteroid dehydrogenase, as well as the synthesis of testosterone induced by hCG/LH (10). Testicular function is impaired by chronic subcutaneous administration of high doses of PGE-2 and PGF-2α to rats and mice as indicated by a decrease in testosterone production and spermatogenesis (11–14), which may suggest that male fertility is ‘down-regulated’ by endogenous PGs present in the testis (15,16). In humans a high sperm density is associated with a low concentration of PGs (17,18). In mice, spermatogenesis is increased by prolonged treatment with acetylsalicylic acid or indomethacin (13).

In addition, Wang et al. (19) have shown that in the MA-10 murine Leydig tumor cell line, the inhibition of COX-2 stimulates steroidogenesis and the expression of the StAR protein, whereas the enhanced expression of COX-2 generates opposite effects. Sirianni et al. (20) have recently demonstrated that COX-2 inhibitors down-regulate aromatase expression and inhibit proliferation in the R2C rat Leydig tumor cell line.

Based on these fundamental facts the present study was performed to evaluate the pharmacological effects of L-carnitine and meloxicam combination on testicular histology in experimental varicoceledized rats.

2. Materials and methods

2.1. Animals

Thirty mature healthy adult male albino Wister rats, with a body weight ranging from 250 to 300 g weighed by using mechanical balance (Tefesa, Germany). Rats were obtained from the colony of the animal house of the institute for embryo research and infertility treatment, AL-Nahrain University. They were kept in an air-conditioned room (22–24 °C) with an automatically controlled photoperiod (14 h light and 10 h darkness). Rats were fed the standard balanced pelleted diet prepared in the animal house of the institute and supplied with tap water “ad libitum”. Before the experimentation all rats were left for at least three weeks for adaptation, then numbered using ear or tail marking according to the international system used (21,22). For fertility test, each rat was placed in the same cage with three 10-week-old female albino Wister rats for adaptation and mating. After 21 days of the beginning of the mating period, the females were checked regularly for parturition. The male rats were considered as fertile if its mating resulted in at least one pregnancy and delivery. Then, only fertile male animals were selected to be used in this study.

2.2. Treatment protocol

Treatment was given to evaluate changes in the reproductive system of varicoceledized male albino Wister rats, treated with L-carnitine and meloxicam.

Rats were subdivided into three groups of 10 animals named A (Treatment group), B (Placebo group) and C (Control group). Animals of each group were housed in 5 cages (2 animals/cage). Group A and B underwent a left experimental varicocele by partial renal vein ligation. Group C not operated and served as control. Twenty four hours after varicocele induction in group A and B, group A was treated with intragastric L-carnitine powder (Ultimate Nutrition, USA) dissolved in distilled water (D.W), given at a dose of 84.5 mg/kg body weight twice
daily orally by oral cavage, and meloxicam oral liquid (Meta-
cam®, Veterinary product, Boehrnger Ingelheim, Germany), at a
dose of 0.6 mg/kg body weight given once every four days
by oral cavage. Group B was treated with intragastric starch
powder dissolved in D.W, while group C was treated with
D.W only, using the same treatment program of group A. Treat-
ment in all cases was continued for 12 weeks.

2.3. Operation

General anesthesia was induced by intramuscular (I.M) injec-
tion of (60 mg/kg) ketamine hydrochloride (Astrapin, Ger-
many) and (7 mg/kg) of xylazine (Rompum® 2%, Bayer,
Germany) (21). The upper left abdominal quadrant is ap-
proached through a midline laparotomy incision. The abdom-
inal contents are packed to the right in order to visualize the
left kidney, left adrenal vein, the left renal vein and the left
internal spermatic vein as it inserts into the left renal vein
(Fig. 1a and Scheme 1). Using careful blunt dissection, a tun-
nel is made in the fat and connective tissues surrounding the
left renal vein and then the vein is cleared of adhering tissue
in a position medial to the insertion of the left spermatic vein
and left adrenal vein. Partial occlusion of the left renal vein at
the point the vessel has been cleared of other tissue was per-
formed by the method of Jane-Dar et al. (23) at a point medial
to the junction with the adrenal and spermatic veins, so as to
reduce the renal vein to an external diameter of about 1 mm.
A 4-0 silk ligature was loosely placed around the left renal vein
at this site, and a metal guide wire of 0.85 mm in diameter was
placed on the left renal vein. The ligature was tied around the
vein over the top of the guide wire. The guide wire was then
withdrawn, and the vein was allowed to expand to the limits
of the ligature, which caused the vein diameter to decrease to
approximately 1/2 of its original diameter. This occlusion
causes increased intravenous pressure lateral to the obstruc-
tion, and the pressure is transmitted to the left spermatic vein,
this causes a varicocele to develop (Fig. 1b and Scheme 1).
Then the midline incision was closed in 2 layers with 4-0 silk
sutures, and local antibiotic spray of oxytetracycline HCl (Me-
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Figure 1 Illustration of the rat left renal vein and tributaries. (A)
Normal renal vein architecture. Vessel names are as in picture (B).
The adrenal vein (unlabeled) joins the renal vein superiorly. (B)
Renal vein after partial occlusion with a 4-0 silk ligature.
Intravenous pressure lateral to the ligature is increased.

Scheme 1 The renal vein and tributaries of mature male rat
showing the Internal Spermatic Vein (Sp.), Renal Vein (R), Left
Adrenal Vein (A), Left Kidney (K), Site of ligation (S). Picture
A = Left Internal Spermatic Vein before partial ligation of the
renal vein. Picture B = Left Internal Spermatic Vein immediately
after partial ligation of the renal vein. Picture C = Left Internal
Spermatic vein that was distended 12 weeks after partial ligation
of the renal vein.

doxy Spray, Holland) was applied (24,25). Animals were killed
12 weeks after the operation.
2.4. Macro and microscopical examination of the reproductive system

At the end of the treatment rats were sacrificed. The abdominal wall was opened longitudinally and the whole left sided reproductive system was quickly removed and immersed in a Petri-dish filled with normal saline at 37 °C. Then under the dissecting microscope (Wild, Switzerland) both testis and epididymis were quickly dissected out at the vaso-epididymal junction from one end and from the surrounding non-testicular tissues from the other end, being cleared from the surrounding non-testicular and non-epididymal tissues. Testicular tissues were then taken for subsequent histological examination (26). Serial sections were prepared and examined microscopically using eyepiece micrometer (27).

Testis was processed totally for preparation of serial histological sections, six serial sections were prepared. Histological sections, 5 μm thick, were stained with hematoxylin and eosin and a minimum of 50 cross-sections were evaluated per each slide (28).

2.5. Parameters used in the studying of histological sections

The fixed testicular tissue sections were processed using the routine histological technique of tissue preparation for histology (29–31). The number of Leydig cells per cross-section was used to evaluate testicular changes:

2.6. Microscopic photography

Using light microscope, photographs of histological sections were taken, using Konica film. For the photography of the genital system of rats, digital camera was used.

3. Statistical analysis

Collected data were analyzed using SPSS version 10.0 for windows (SPSS, Chicago, Illinois, USA). Differences of means between groups were examined by paired and unpaired t-test, P-value < 0.05 was considered as statistically significant. Descriptive analysis of Means and SD was calculated on all clinical and Experimental variables. Variance analysis for repeated measures was used to evaluate any significant variation in testicular number of Leydig cells between control, placebo and treatment groups. Multiple comparisons of independent series of data were done using ANOVA and Fisher’s least significant difference (LSD) tests.

4. Results

All data are presented in Table 1. The present work evaluates and compares the histological and morphometrical analysis of testicular number of Leydig cells of varicocelized mature adult male rats receiving treatment with those varicocelized and non-varicocelized rats receiving placebo and distilled water, respectively.

A statistical representation and histological appearance of leydig cells number were shown in (Fig. 2 and Schemes 2–4). The numbers of Leydig cells were:

- Highly and significantly (P < 0.0001) decreased in treatment group as compared with placebo group (16.20 ± 1.81 vs. 30.50 ± 3.02).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Statistical analysis of histological and morphometrical analysis of testicular and epididymal tissue samples of varicocelized placebo and treatment groups in comparison to the control group (n = 10 rats/group).</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>Control group</td>
</tr>
<tr>
<td>No. of leydig cells</td>
<td>13.50 ± 3.022</td>
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Values are means ± standard deviation (SD). ANOVA and LSD tests. Means carrying different small letters are significantly different (P < 0.05).
The cytoplasm of the Leydig cells shows abundant smooth endoplasmic reticulum, well-developed Golgi complexes, and numerous mitochondria (32,33). Most of the enzymes involved in the synthesis of testosterone are located in the smooth endoplasmic reticulum and mitochondria of interstitial cells (34). L-carnitine transports fatty acids to the mitochondria, where they undergo β-oxidation leading to the generation of metabolic energy in the form of ATP needed by the cells to perform their functions (35–37). In human, decreasing circulating testosterone level in varicocele patients, affects the testicular morphometry and function of both Sertoli and Leydig cells, displaying ultrastructural changes and various maturation defects in the process of spermatogenesis from the germinal cells.

The histology of the varicocele testis shows that all the cell types and compartments of the testis can be involved (38,39). The effects of varicocele on Leydig cells are indicative of hyperplasia (40,41). Importantly, these histologic changes are also characteristic of changes seen in the adolescent with a varicocele (42,43). In the present study, the increase in Leydig cell numbers might be relative as a result of reduction of the volume of the seminiferous tubules. It is known that varicocele causes impairment of spermatogenesis and this might cause reduction in tubular volume. Another explanation is that induced varicocele might have impaired Leydig cell function and testosterone production. Testosterone reduction may cause increase in LH levels and this might cause true Leydig cell hyperplasia. Increased mitochondrial production of enzymes that is responsible for the synthesis of testosterone in Leydig cells by L-carnitine in treated varicocelezed rats, may explain to us the histological picture of a significant decrease in the number of Leydig cells as compared to the placebo treated animals.

In addition to that, recently it is found that cyclooxygenase-2 (COX-2), a key enzyme in the biosynthesis of prostaglandins (PGs), is present in the testicular interstitial cells (Leydig cells) of reproductively active Syrian hamsters (Mesocricetus auratus) (44). PGF₂α, presumably acting through PGF₂α receptors located in Leydig cells and through a mechanism involving down-regulation of StAR (the protein involved in the regulation of cholesterol transport to the inner mitochondrial membrane) and 17β-HSD (enzyme that converts androstenedione into testosterone) expression, leads to the inhibition of LH/hCG-stimulated testosterone production (44). Thus, the testicular PGF₂α system working in concert with the primary effect of gonadotropins on the hypothalamic-pituitary axis represents a local inhibitory control of steroidogenesis in Syrian hamsters. Although COX-2 is not detected in human testicular biopsies with no evident morphological changes or abnormalities, it is expressed in testes from men with impaired spermatogenesis and male infertility (45). COX is also induced in testicular cancer (46). Moreover, COX-2 represents a potential key factor in the age-related reduction of testosterone production because an increased COX-2 expression in Brown–Norway rats during aging, concomitantly with the decreased testicular production of testosterone, has been recently described (9). In this context, COX-2 inhibition enhances steroidogenesis and StAR gene expression in MA-10 mouse Leydig cells, whereas its overexpression leads to the opposite (19). In the present study, varicocelezed rats, show a histological picture of a highly significant increase in number of Leydig cells. Such increase in number of Leydig cells leads to the augmentation of COX-2 expression and high production of PGF₂α which results in more testosterone inhibition and finally severe deterioration of spermatogenic process. Therefore, L-carnitine plus meloxicam treatment of varicocelezed rats, shows a histological picture of a significant decrease in number of Leydig cells as compared to the placebo treated animals. Therefore, such a significant decrease in number of Leydig cells produce marked inactivation of COX-2 expression and no excess PGF₂α production which results in no testosterone inhibition and finally no deterioration of spermatogenic process. Also such improvement in testosterone production returns back the negative feed-back effect of testosterone on pituitary gland relative to normal gradually which results finally in decreasing LH production from the pituitary gland and this may lead in turn to reduce Leydig cells
hyperplasia, because testosterone reduction due to Leydig cells dysfunction in varicocele may cause increase in LH levels and this might cause true Leydig cell hyperplasia.

On the other hand, since L-carnitine plus meloxicam treatment of varicoceleized animals results in a highly significant decrease in the number of Leydig cells mainly due to L-carnitine administration, it is still significantly increased as compared to the control of non-varicoceleized animals with normal number of Leydig cells. And this may be due to the varicocele itself is still present, as well as the relative decrease in intratesticular testosterone and the association of compensatory mechanism in varicoceleized animals in order to compensate the relative decrease in the intratesticular testosterone by increasing the number of Leydig cells to increase the production of testosterone in order to compensate the decrease in this hormone.

Further studies are warranted to study the clinical effect of L-carnitine plus meloxicam on semen quality including sperm density, sperm count, motility, and morphology and on the serum hormonal level of testosterone in infertile patients with varicocele. Also needed are biological studies of the effect of L-carnitine and meloxicam on the testicular interstitial cells to increase the production of testosterone in order to control non-varicoceleized animals with normal number of Leydig cells. And this may be due to the varicocele itself is still present, as well as the relative decrease in intratesticular testosterone and the association of compensatory mechanism in varicoceleized animals in order to compensate the relative decrease in the intratesticular testosterone by increasing the number of Leydig cells to increase the production of testosterone in order to compensate the decrease in this hormone.

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References

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