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ORIGINAL ARTICLE

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

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KEYWORDS

L-carnitine plus meloxicam;
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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 gavage, 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.

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Conclusion: It is concluded that L-carnitine plus meloxicam treatment appears to have a beneficial effect in decreasing, restoring and maintaining the number of testicular leydig cells in experimental varicelized rats close to that control of non-varicelized rats.

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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 PGF2 α , which exerts an inhibitory role on the expression of StAR and 17 β -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-2 α 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 varicelized 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 varicelized 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 gavage, and meloxicam oral liquid (Metacam®, Veterinary product, Boehringer Ingelheim, Germany), at a dose of 0.6 mg/kg body weight given once every four days by oral gavage. 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. Treatment in all cases was continued for 12 weeks.

2.3. Operation

General anesthesia was induced by intramuscular (I.M) injection of (60 mg/kg) ketamine hydrochloride (Astrapin, Germany) and (7 mg/kg) of xylazine (Rompum® 2%, Bayer, Germany) (21). The upper left abdominal quadrant is approached through a midline laparotomy incision. The abdominal 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 tunnel 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 performed 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 obstruction, 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-

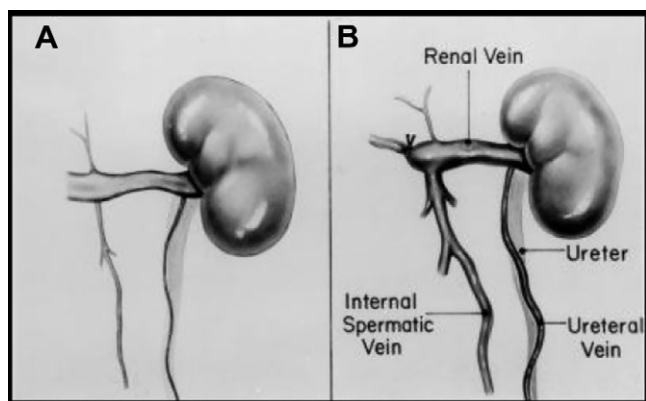
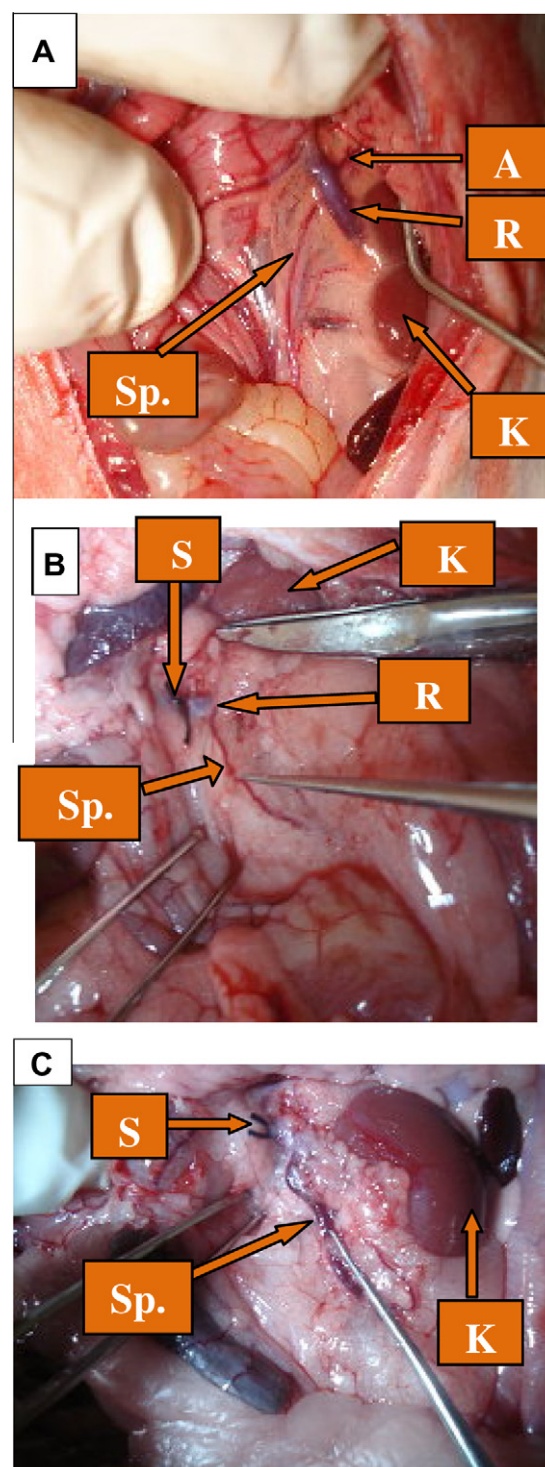


Figure 1 Illustration of the rat left renal vein and tributaries. (A) Normal renal vein architecture. Vessel names are as in picture (B). The adrenal vein (unlabeled) joins the renal vein superiorly. (B) Renal vein after partial occlusion with a 4-0 silk ligature. Intravenous pressure lateral to the 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 varicoceleized mature adult male rats receiving treatment with those varicoceleized and non-varicoceleized 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).

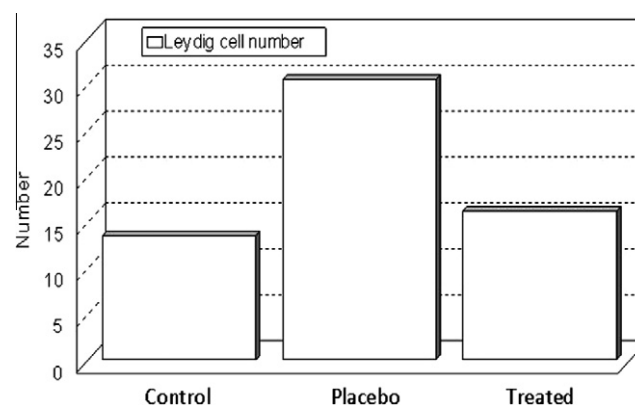
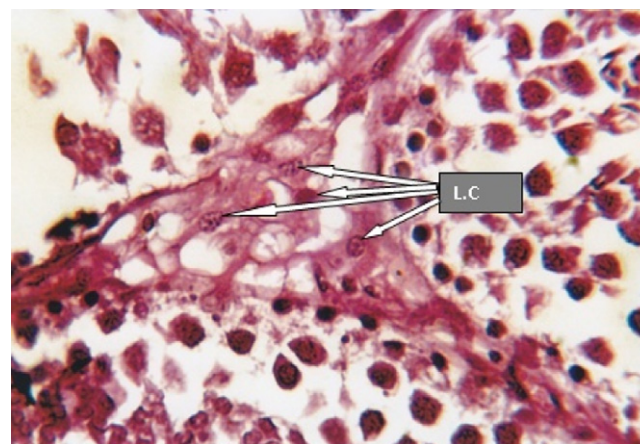


Figure 2 Testicular changes in number of leydig cells of varicoceleized rats receiving placebo and treatment as compared to Control animals. ANOVA and LSD tests.

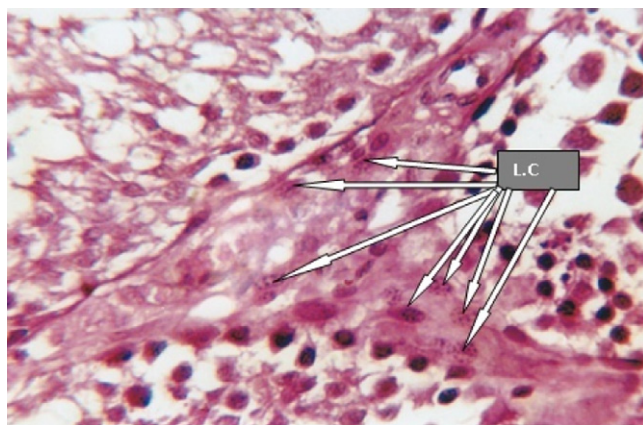


Scheme 2 Cross section through testicular tissue of mature male rat (control group) showing the interstitial “Leydig cells” (L.C). (E&H ×400).

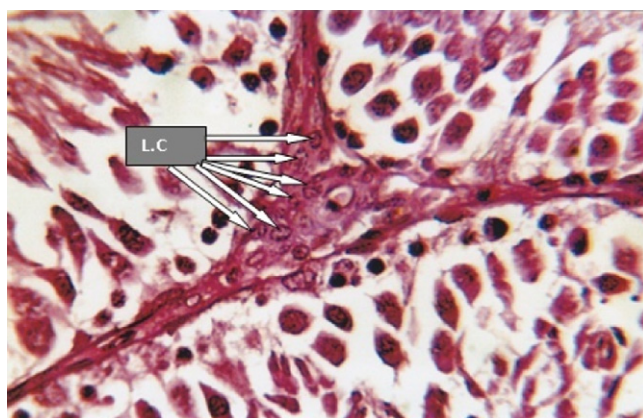
Table 1 Statistical analysis of histological and morphometrical analysis of testicular and epididymal tissue samples of varicoceleized placebo and treatment groups in comparison to the control group ($n = 10$ rats/group).

Experimental histological study Parameter	Control group	Placebo group	Treatment group
No. of leydig cells	13.50 ± 3.022 a	30.50 ± 3.0 b	16.20 ± 1.81 c

Values are means ± standard deviation (SD). ANOVA and LSD tests. Means carrying different small letters are significantly different ($P < 0.05$).



Scheme 3 Cross section through Testicular tissue of mature varicocele male rat (placebo group) showing the interstitial “Leydig cells” (L.C). (E&H $\times 400$).



Scheme 4 Cross section through Testicular tissue of mature varicocele male rat (treatment group) showing the interstitial “Leydig cells” (L.C). (E&H $\times 400$).

- Significantly ($P < 0.03$) increased in treatment group as compared with the control group (16.20 ± 1.81 vs. 13.50 ± 3.02).
- Highly and significantly ($P < 0.0001$) increased in placebo group as compared to the control group (30.50 ± 3.02 vs. 13.50 ± 3.02).

5. Discussion

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 varicocele 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). $\text{PGF2}\alpha$, presumably acting through $\text{PGF2}\alpha$ 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 $\text{PGF2}\alpha$ 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, varicocele 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 $\text{PGF2}\alpha$ which results in more testosterone inhibition and finally severe deterioration of spermatogenic process. Therefore, L-carnitine plus meloxicam treatment of varicocele 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 $\text{PGF2}\alpha$ 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

hyperplecia, 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 varicocele animals result 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-varicocele 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 varicocele 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 each L-carnitine and meloxicam on the testicular interstitial cells, using molecular and cellular studies on single intracellular functions or organelles.

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