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A correlation between selenium and carnitine levels with hypo-osmotic swelling test for sperm membrane in low-grade varicocele patients

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Abstract. – OBJECTIVE: The hypo-osmotic swelling (HOS) test predicts membrane integrity by determining the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment. The aim of our study was to determine the correlation between selenium and carnitine levels in the seminal fluid with HOS test for sperm membrane in low-grade varicocele patients.

PATIENTS AND METHODS: Study numbered 64 examinees who suffered from low-grade varicocele and were divided into two groups, according to fertility potential and HOS test outcome. The study also included a control group of 64 healthy subjects, with no varicocele.

RESULTS: From the Shapiro-Wilk's test, it is clear that carnitine distribution differs significantly from normal (0.938, $p = 0.03$). In distribution of selenium, Kolmogorov-Smirnov test clearly shows statistically significant deviation from the normal curve ($z = 0.225$, $p < 0.000$), likewise Shapiro-Wilk's statistic (0.787, $p < 0.000$). According to the results, the second group had significantly higher levels of carnitine and selenium than the first group of examinees ($p < 0.05$); therefore, when we compared epididymal markers with HOS tests outcomes, we found significant differences between the two groups. There were no significant differences between second group and healthy subjects ($p > 0.05$).

CONCLUSIONS: HOS test outcome in varicocele patients is directly proportional to the carnitine and selenium levels, which could play a major role in both determining fertility parameters and in the treatment of its impairment. This result is important for sub-clinical varicocele in infertile patients with normal semen analysis, since there is no evidence of benefit from any treatment so far.

Key Words:
Selenium, Carnitine, Hypo-osmotic swelling test, Semen analysis, Fertility.

Introduction

The hypo-osmotic swelling (HOS) test predicts membrane integrity by determining the ability of the sperm membrane to maintain equilibrium between the sperm cell and its environment[1]. Sperm plasma membranes are particularly susceptible to oxidative stress (OS) through lipid peroxidation of polyunsaturated fatty acids, which affect the fluidity of the sperm plasma membrane[2]. The effectiveness of various antioxidants is variable in respect to improving semen parameters and pregnancy rates[3]. Oxidative stress impairs sperm parameters in varicocele, but its etiology and association with varicocele remain unclear[4]. It seems that varicocele can be a cellular source of reactive oxygen species (ROS) in the semen through an increased number of immature sperm cells and white blood cells[5]. Nevertheless, there is little information about selenium and carnitine concentration in patients with varicocele and its association with seminal parameters.

Epididymal spermatozoa of selenium deficiency exhibit a loss of motility and display various defects including abnormalities of the mitochondrial sheath and disorganization of the flagella fibers[6]. Carnitines play an important role in initiating sperm motility, regulating Sertoli cell function and protecting sperm against oxidative damage. Recently, carnitine has been used as a therapeutic in the treatment of male infertility[7].

The aim of our study was to determine the correlation between semen levels of selenium and carnitine by using an HOS test for sperm membrane in low grade varicocele patients.

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Patients and Methods

The study was performed on 64 patients who suffered from low - grade varicocele, with a normal semen analysis. Patients were divided into two groups according to fertility potential and HOS test outcomes. The first group was made up of 32 participants with negative or suspicious HOS test results. Infertility for more than one year was the main reason for their referral to our clinic, in order to conduct further analysis. The second group included 32 fertile male subjects with normal natural conception ability and positive HOS test outcomes, which were examined during a routine urological survey. The study also included a group of 64 healthy controls, with positive HOS test results and no varicocele formation or concomitant urological diseases. All 128 subjects were evaluated through a complete examination of their medical history, physical examination, HOS test and semen analysis for selenium and carnitine levels.

Inclusion and Exclusion Criteria

Eligibility requirements for examined patients included either subclinical (detectable only by ultrasound) or grade I varicocele (palpable during valsalva maneuver) with normal semen analysis. The study excluded patients with genital infections, hypogonadism, teratozoospermia and oligoasthenospermia, as well as patients with sperm autoimmunity and chromosomal aberrations.

Varicocele Diagnosis and Working Principle

The diagnostic of varicocele was confirmed by the color Doppler flow imaging system and 7.5 MHz linear-array transducer (Hewlett-Packard-Sonos 1000; Providian Medical Equipment LLC**,** Willovick, OH**,** USA)[8]. The currently valid varicocelce classification, which we used in our study, was taken from the European Association of Urology (EAU) guidelines on Male Infertility[9]. The difference in carnitine and selenium levels was searched for between the groups. The statistical evaluation of the results was performed by the "SPSS version 12 for Win" software package (SPSS Inc., Chicago, IL, USA). Standard statistical protocol was used for descriptive statistics, followed by the Shapiro-Wilk's test for establishing the differences between the two groups. The normality of distribution was checked by the Kolmogorov-Smirnov test. The receiver operating characteristic (ROC) curve was used to assess the predictive potential of the carnitine and selenium levels for the HOS test outcomes. *p* value below 0.05 was considered as significant, since below 0.01 was deemed as highly significant. The ROC curve provides us with an opportunity to find the cut-off score with an optimal ratio between sensitivity and specificity (or to maximize proportions between true positive ones and true negative ones). The Y axis represents sensitivity with values ranging from 0.0 to 1.0 (since proportions are considered). The X axis represents 1 – specificity or proportion of false positive ones. If using normalized units, the area under the curve (AUC) is equal to the probability that a classifier will rank a randomly chosen positive case higher than a randomly chosen negative one; e.g. AUC represents the accuracy of the test. The working principle for the HOS-test was according to the original protocol[10] which was as follows: after the liquefaction at room temperature, 0.1 ml of semen and 1.0 ml of hypo-osmotic solution (equal parts sodium citrate (150 mOsmol) and fructose (150 mOsmol) were mixed. After 60 minutes of incubation in a bathtub at 37 °C, a slide of the mixture was prepared and examined under a phase-contrast microscope (x450). Two hundred spermatozoa with swollen and tortuous tails were counted, and estimated by their percentage. A sperm sample with ≥ 60% of spermatozoa that had swollen and tortuous tails was considered to be a positive (normal) finding, from 50% to 60% of such spermatozoa was considered to be a suspicious finding, and below 50% to be a negative finding. At least five sperm samples were taken with a five-day abstinence rule, used in every case.

Reference values for HOS test[11]:

- Positive findings, more than 60 % of the bent tail
- Suspicious findings, curled tail between 50-60%,
- Negative test result, below 50 % drooping tail.

Selenium and carnitine testing in semen was done on an atomic absorption spectrophotometer Perkin Elmer 3300 (PerkinElmer Inc., Waltham, MA, USA) which was connected to a computer in the hydride generator MHS-10. A descriptive seminal fluid analyses was performed on all the samples according to the World Health Organization standard[12].

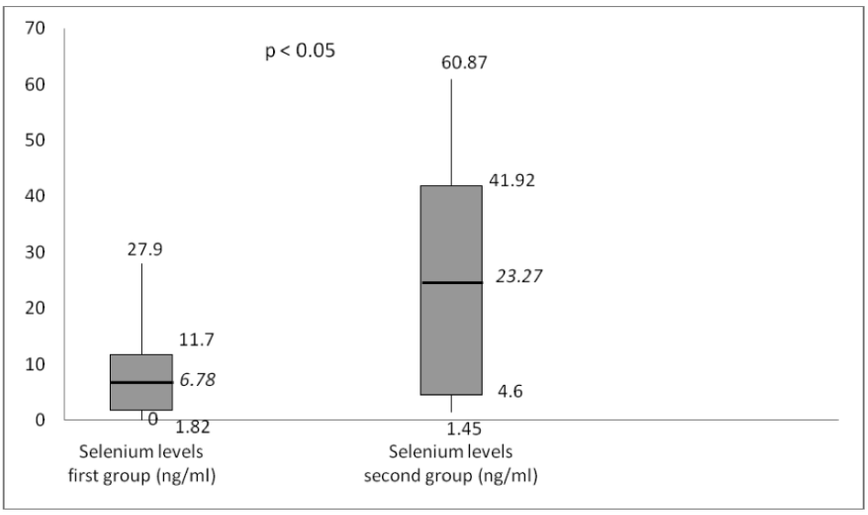


Figure 1. Comparison of selenium levels between two groups of low - grade varicocele patients, according to the HOS test outcome. Grey boxes represent mean value ± SD and vertical lines are minimum and maximum. Horizontal lines represent average values. *p*-values show significance of results between first and second group of examinees.

Each subject signed the acceptance form of the study protocol, in which the Ethical Principles for Medical Research Involving Human Subjects (The Helsinki Declaration) were clearly stated. They all signed the written consent form.

Results

The examined groups were homogenous in regards to the prevalence of varicocele grades, since any differences between them were non-existent. According to the results of the Kolmogorov-Smirnov test, the distribution of carnitine does not deviate significantly from normal (z = 0.110, $p = 0.054$), but the statistical significance was close to the limit value. From the Shapiro-Wilk's test, which was used for the samples, it is clear that this distribution differs significantly from normal (Shapiro-Wilk's statistic 0.938, $p = 0.03$). In the distribution of selenium, there is a statistically significant deviation present from the normal curve (z = 0.225, $p < 0.000$, and Shapiro-Wilk's statistic 0.787, $p < 0.000$). It is recommended to use non-parametric statistical methods according to the inquiry. Therefore, the differences between the groups in levels of carnitine and selenium were tested using the Mann-Whitney U test for independent samples.

Figure 1 represents selenium levels between the first and second group of patients, while Figure 2 represents carnitine levels in those two

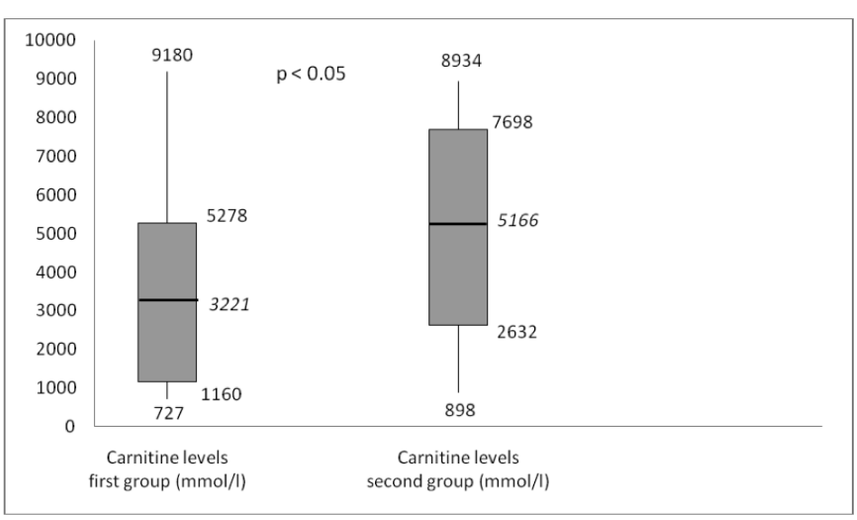


Figure 2. Comparison of carnitine levels between two groups of low - grade varicocele patients, according to the HOS test outcome. Grey boxes represent mean value ± SD and vertical lines are minimum and maximum. Horizontal lines represent average values. *p*-values show significance of results between first and second group of examinees.

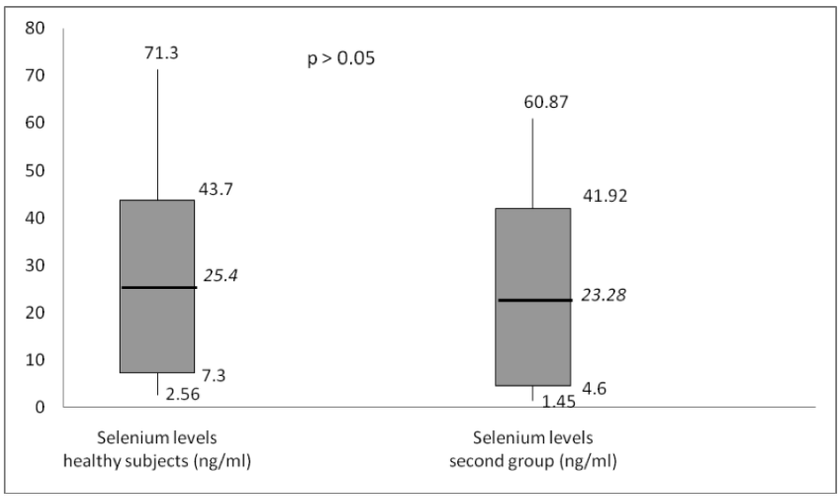


Figure 3. Comparison of selenium levels between second group of patients and healthy subjects, according to the HOS test outcome. Grey boxes represent mean value ± SD and vertical lines are minimum and maximum. Horizontal lines represent average values. *p*-values show significance of results between second group and control group of healthy subjects.

groups of patients. Figure 3 and Figure 4 represent the correlation between healthy subjects and the second group of patients. The data is shown as the mean values ± 1 SD, followed by their minimum and the maximum values. Results of the ROC curve are shown in Figure 5. Both values of the AUC do not deviate significantly from 0.5, although the significance of selenium is close to the border ($p = 0.084$). The cut-off score for carnitine is 1807, while sensitivity is 0.929, and the specificity is 0.389. The cut-off score for selenium is 3.315, for sensitivity 0.571, and specificity 0.833.

Discussion

Comparing the epididymal markers with HOS test outcomes, significant differences were found between the first and second group of examinees. Worse HOS test results correlated with lower levels of carnitine and selenium, and *vice versa*. The HOS test is not strict in predicting sperm fertility, but the test should approximate answers to spermatozoids maturity. According to the results of the ROC curve that are shown in Figure 5, both values of the AUC do not deviate significantly from 0.500, although

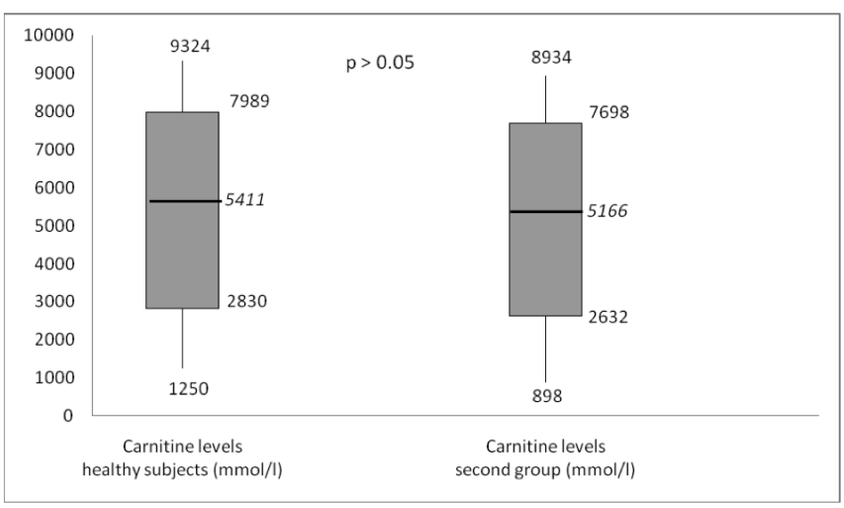
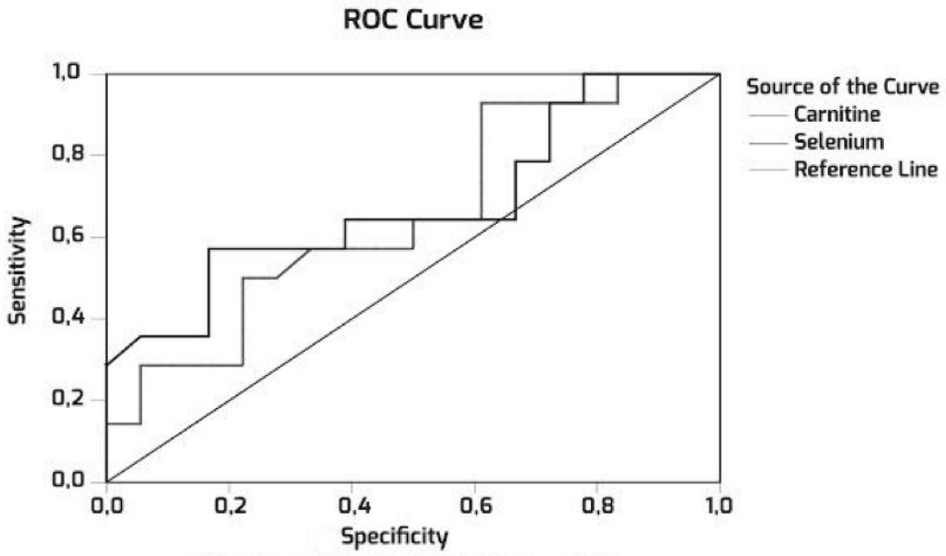


Figure 4. Comparison of carnitine levels between second group of patients and healthy subjects, according to the HOS test outcome. Grey boxes represent mean value ± SD and vertical lines are minimum and maximum. Horizontal lines represent average values. *p*-values show significance of results between second group and control group of healthy subjects.



Test result variables	AUC	SE	p value	95%CI	
				Lower bound	Upper bound
Carnitine	0,653	0,098	0,144	0,460	0,846
Selenium	0,681	0,099	0,084	0,486	0,875

Figure 5. Comparison of the predictive potential of the carnitine and selenium levels for the HOS test outcomes. ROC curve and test results variables (AUC- area under curve; SE – standard error; CI –confidence interval). Dark black line represent selenium and light black line carnitine values. Diagonal serments are produced by ties.

the significance of selenium is close to the border ($p = 0.084$). When the value of carnitine is 1807, the sensitivity is 0.929 and the specificity is 0.389. The cut-off score for selenium is 3.315, for sensitivity 0.571, and specificity 0.833. As shown in Figure 1 and Figure 2, the second group had significantly higher levels of carnitine and selenium than the first group ($p < 0.05$). This result clearly indicates the importance of micronutrient influence on fertility potential, and therefore points to a possible cause of infertility in low-grade varicocele patients. When we compared the healthy subjects with the second group, there were no differences in the HOS test outcomes, nor significant changes regarding selenium or carnitine seminal levels, although in healthy controls these values were higher. This suggests that in low grade varicocele subjects, higher selenium and carnitine levels lead to normal fertility, opposite to lower levels of those micronutrients, which lead to inappropriate HOS test results and infertility. According to several researches[13,14], antioxidant levels are significantly lower among men with varicocele, which is probably related to the negative varicocele influence on HOS test results, sperm motility and vitality[15]. Those results support the conclusions of our study and require a possible pursuit for controlled clinical trials regarding antioxidant supplementation in infertile men with varicocele. Nevertheless, the studies did not indicate an antioxidant specimen type, which makes comparison with our research incomplete. A recent report[16] indicates the positive influence of active selenium and carnitine micronutrient compound on semen analysis and pregnancy rates, which supports our attitude that selenium and carnitine play a crucial role in improving fertility, through the improvement of spermatozoa membrane integrity and its morphology, which can be presented in the form of a positive HOS test. Selenium in seminal plasma also correlates with good spermatozoa concentrations, motility, and morphology, and that decrease in selenium concentration, in varicocele patients, was associated with detriment of seminal parameters[17], which is comparable to our results of decreasing sperm membrane integrity in patients with lower selenium concentration, even though seminal parameters remain normal. Compared to patients with varicocele grades II and III, and the control group with no

varicocele[18], it is indicative that HOS test results and sperm quality were significantly lower in the varicocele group of patients. This data suggests that a reduction of fertilization capacity in men during varicocele can result from damage to both the testis and the epididymis, but unlike our study, they did not include selenium and carnitine as parameters of epididymal function and they included patients with grade III varicocele, which is opposite to our study with low grade varicocele participants. We have proved that sub-clinical varicocele can be prone to decrease in selenium and carnitine levels, which lead to negative HOS test outcomes and infertility, but, can also have a similar outcome as a group of healthy males, with normal fertility potential. The true mechanism that determines those differences is unknown. Similar findings were shown by Andrade-Rocha[19], indicating the negative varicocele influence on HOS test outcomes, but without a comparison on the correlation between antioxidant specimens and HOS test results, which is the cornerstone of our work. A statistically significant, positive correlation between seminal plasma total carnitine concentration with total sperm count and the percentage of normal forms was revealed[20], with suggestion that the determination of seminal carnitine levels may be a useful test in the evaluation of male infertility. Bearing in mind that our study showed a positive correlation between carnitine levels and HOS test outcomes, it adds significance to our data regarding the evaluation of male infertility and not only in its management.

As we can see, there is no contemporary research regarding the correlation between selenium and carnitine levels according to HOS test findings in low grade varicocele patients, which makes our study innovative and important. Our study also points out on the significance of treating infertile patients with low grade varicocele, but normal semen analysis, who were not prone to surgical treatment or any other according to EAU recommendation[21]. The increase in selenium and carnitine levels in seminal plasma is related to the improvement in HOS test outcomes, which finally leads to fertility improvement. Our conclusion is supported by recent research[22] which revealed a significant improvement in spermatozoa morphology and maturity after three months of treatment with micronutrient supplementation containing selenium and L-carnitine.

Conclusions

Selenium and carnitine could play major roles in both determining fertility parameters and in treating its impairments. This is especially important, for sub-clinical varicocele, in patients with normal semen analysis, because there isn't any evidence of benefit from any treatment. Our result can change this attitude, relying on the strong correlation between selenium and carnitine leves, HOS test results and fertility potential in low grade varicocele patients. Future researches should investigate the efficacy of selenium and carnitine antioxidant supplementation in improving the clinical condition in infertile men with varicocele.

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Conflict of Interest

No part of this paper has been presented, published, or submitted for publication elsewhere in this or in any other language.
This clinical study was conducted in accordance with the principles laid down in the WMA Declaration of Helsinki along with the strict respect of patient's rights and clinical study protocol. Patient confidentiality and data security is guaranteed.
Authors did not have any support funding of the paper. The authors of this paper hereby declare that have not received nor shall receive any financial benefits from publishing the paper, neither they have received any financial incentive from a third party. We, the author and co-authors, hereby declare that we are not in any situation which could give rise to a conflict of interest.

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