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VARICOCELECTOMY IMPROVES SPERM PARAMETERS, SPERM DNA INTEGRITY AS WELL AS THE OTHER CRITICAL SEMEN FEATURES

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Varicocele is a major entity defined within male infertility. In this report we have studied the influence of laparoscopic varicocelectomy on semen quality, biochemical parameters of seminal plasma and sperm DNA fragmentation. In this study, the semen samples from patients with left-side varicocele of grade II-III before and after laparoscopic varicocelectomy were compared to healthy individuals and separated into three groups. The volume of semen, sperm concentration (106/ml), motility (%), viability (%) and normal morphology (%) were assessed. Total antioxidant capacity (TAC), catalase (CAT), superoxide dismutase (SOD) and malondialdehyde (MDA) together with other biochemical substances in seminal plasma as alpha-glucosidase (α-Glu), fructose (Fr) and citric acid (CA) were determined by ELISA method. The spermatozoa activity including ion-transports through sodium, potassium ATPase (Na^+, K^+-ATPase) and calcium, magnesium ATPase (Ca^{2+}, Mg^{2+}-ATPase) were determined by using spectrophotometry. In addition, flow cytometry method for detection of sperm DNA fragmentation was used. The results showed, that three months after varicocelectomy such intervention led to significant postoperative improvement in volume of semen ($p<0.001$), total sperm count ($p<0.001$), sperm motility ($p<0.001$) and spermatozoa with normal morphology ($p<0.001$). We found decreased α-Glu levels due to varicocelectomy ($p<0.05$). There has been shown a high positive correlation between Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase activity with total number of spermatozoa ($p<0.05$). The TAC levels and DNA fragmentation values after varicocelectomy can be considered as significant indicators of good prognosis after surgical intervention. It has to be emphasized that α-Glu levels and total sperm count expressed statistically significant both positive and negative predictive values for semen assessment. Varicocelectomy may lead to significant improvement of semen quality although the observations must be correlated with clinical pregnancies observed thereafter.

Key words: *varicocele, sperm quality, biochemical parameters, DNA fragmentation, varicocelectomy, sodium, potassium ATPase, magnesium ATPase, spermatozoa, oxidative stress*

INTRODUCTION

Varicocele is a pathologic dilatation of the testicular pampiniform venous plexus, present in 15–20% of post-pubertal males (1). Varicocele can contribute to a low testosterone level, orchialgia and testicular atrophy (2). Varicocele has been considered to be one of the main causes of the male infertility. Approximately, 15% of the healthy male population and up to 40% out of the infertile men are believed to have clinical or subclinical varicocele (3). Semen oxidative stress (OS) and apoptosis markers are associated with both pathological grades as well as unilateral or bilateral form of varicocele (4, 5).

This entity can be associated with both abnormal semen parameters and male infertility. Varicocelectomy seems to improve semen parameters, positively influencing pregnancy rate and improving the reproductive potential of previously subfertile male patients (6). However, this concept is not completely free from controversy, since some studies have shown that varicocelectomy is ineffective in respect to the likelihood of pregnancy (7, 8). Varicocele may contribute to subfertility through a variety of reasons (1). There are several pathophysiological mechanisms by which varicocele may promote male infertility: 1) testicular hypoxia due to venous stasis (extended exposition to sperm antigens - immune activation, possibly antisperm antibodies synthesis); 2) hormonal dysfunction; 3) reflux of renal and adrenal toxic metabolites; 4) internal spermatic vein hypertension; 5) increased testicular temperature; 6) germ cell apoptosis (1, 3). Hypoxia, hyperthermia and impaired blood flow could result in severe chronic oxidative and nitrosative stress in varicocele patients (9).

High level of oxidative stress, which arises from the imbalance between antioxidants and reactive oxygen species (ROS) may be reflected in semen alterations and deterioration of sperm DNA integrity in men with varicocele (3, 10). Some

spermatozoa may induce high ROS production while being deficient in the anti-oxidant defense system, which may further increase their oxidative DNA damage. It has been well established that varicocele is usually associated with impaired spermatogenesis (11). Also, it has been well documented that increased ROS secretion resulting in oxidative stress, may seriously affect sperm function, motility, fertilization potential and the integrity of sperm genome (12). Oxidative stress can have dramatic consequences not only on spermatozoa, but also on early embryonic development (13). In varicocele patients there is a critical imbalance between pro-oxidant and antioxidant factors. The antioxidant action has been attempted in numerous clinical trials, however, might be questioned due to reductive imbalance (14)

It has been widely accepted that during inflammatory reaction, a broad spectrum of inflammatory mediators, including cytokines, are released from inflamed tissues, organs and migrated cells. Alternatively, recent evidence suggests that ROS levels can be also paradoxically increased by so called 'reductive stress', due to over-accumulation of reductants. Following the reductive stress this could be considered as the initiator of other damaging mechanism leading to DNA fragmentation (15).

Neutrophils and macrophages are the main cell types found in semen which can be detrimental for sperm through ROS overproduction and apoptosis initiation. Furthermore, ROS, released by leukocytes or granulocytes, exert harmful effects on human spermatozoa, causing a marked loss in sperm motility and morphology, and reduce sperm hyperactivation and oocyte penetration. During genital tract inflammation activated leukocytes in human seminal plasma are able to release elastase (PMN elastase), cathepsin G, collagenase, ROS, pro- and anti-inflammatory cytokines. Some investigations have suggested that varicocele-related infertility is mostly associated with cytokines release (16, 17). Cytokines may be mediators of oxidative stress and have the potential to affect redox equilibrium, as well as to influence sperm function by themselves (18, 19).

Spermatogenesis is an essential process that determines male gamete production and is tightly regulated by thousands of genes. In patients with varicocele, reproductive potential may vary and different clinical phenotypes can be observed, in some cases it may result in permanent infertility. Observed phenotypic variability suggests different genetic background, that regardless of the disease severity compromises fertility itself (20). Numerous genetic and epigenetic mechanisms have been reported in men with varicocele. Some studies on patients with varicocele reported an association between the disease and following genetic entities: 1) chromosomal abnormalities - including inversions, translocations, deletions and others (21); 2) Y-chromosome microdeletions; 3) gene copy number differences. Epigenetic mechanisms as DNA methylation, histone modifications and regulatory non-coding RNAs are essential for a number of biological processes, including genomic imprinting and X chromosome inactivation. Epigenetic markers, acquired during cell growth and differentiation, play a fundamental role in gametes functioning. There was reported that patients with varicocele demonstrated increased susceptibility to DNA damage when it was hypomethylated (20, 22).

The exact pathophysiological mechanism by which varicocele promotes male infertility still remains unclarified. An important role in male infertility caused by varicocele may also indicate ROS-associated autophagy and apoptosis. Autophagy is a process of reusing old and damaged cell parts. Varicocele activates autophagy in the testis which may proceed along with increased apoptosis of spermatogenic cells (23, 24).

Regarding our and the others findings, it could be concluded that several cell type residents in the male reproductive system, or migrated and/or chemoattracted ones have been involved in inflammatory reaction development, leading to fertility loss in patients with varicocele (11, 25, 26). Because of the multifactorial nature of varicocele, biomarkers which should be identified in the early stage of the disease are not well recognized, especially, in the prevention or treatment (conservative or surgical) of infertility in this group of patients (20).

Despite the extensive literature on varicocele, the exact mechanisms by which they can potentially affect spermatogenesis remain unclear due to variety of factors at operation (27). Varicocelectomy may improve spermatogenesis in patients with symptomatic varicocele. A meta-analysis explored the impact of three surgical approaches (high ligation, inguinal and subinguinal) on sperm count and motility. The results obtained showed that all three surgical approaches led to significant postoperative improvement of sperm parameters. Overall, we may take an assumption that varicocelectomy in adult population usually improves sperm concentration, sperm motility and sperm DNA integrity (12, 28, 29).

For that we aimed to characterize semen parameters, pro-antioxidants systems, sperm DNA fragmentation, ion transport systems and selected biochemical compounds in sperm/seminal plasma before and after varicocelectomy in order to identify possible novel biomarkers for varicocelectomy monitoring.

MATERIALS AND METHODS

We selected altogether 214 males with a primary, left-side varicocele, grade II–III (observation stage I–V I), from 19 to 33 years of age out of these 193 patients at 3 months after the laparoscopic varicocelectomy (observation stage II–VC II) and 25 healthy individuals (control group; C) were observed. All studied participants underwent careful semen analysis at the first stage of investigation. The follow up diagram has been shown to present patients subgroups designated to particular tests (*Fig. 1*).

Thus in the next step of the study were examined 27 males with a primary, left-side varicocele, grade II–III (observation stage I), from 19 to 33 years of age: 15 patients at 3 months after the laparoscopic varicocelectomy (observation stage II) and 12 healthy normospermic volunteers (control group). The mentioned groups underwent TAC, CAT, SOD, seminal plasma MDA analysis, and sperm DNA-fragmentation assessment.

Ion-transport activity systems: Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase in spermatozoa were measured in 67 patients with primary, left-side varicocele, grade II–III (observation stage I–V I), in 62 patients at 3 months after the laparoscopic varicocelectomy (observation stage II–VC II), and 25 healthy normospermic volunteers (control group; C).

Selected seminal plasma biochemical parameters: α-Glu), fructose (Fr) and citric acid (CA) were measured in 27 patients with primary, left-side varicocele, grade II–III (observation stage I–V I), in 15 patients at 3 months after the laparoscopic varicocelectomy (observation stage II–VC II) and 12 healthy normospermic volunteers (control group; C).

The control group consisted of 25 healthy volunteers from 19-33 years of age, half of them revealed already offsprings the other half presented normospermia without any signs of disease or environmental hazards.

Experimental procedures

Semen analysis followed the WHO Laboratory Manual for the examination and processing of human semen (30). In this study we have analyzed following semen parameters: the volume, sperm concentration (10^6/ml), motility (%), viability (%) and normal morphology (%).

Catalase (CAT), superoxide dismutase (SOD), malondialdehyde (MDA), alpha-glucosidase (α-Glu), fructose (Fr), citric acid (CA) as well as total antioxidant capacity (TAC) were measured in seminal plasma samples. The level of MDA was assessed with the use of OxiSelect™ TBARS Assay Kit, enzyme-linked immunosorbent assay ELISA-method (Cell Biolabs, Inc., San Diego, CA, USA). CAT, SOD activities and TAC were performed with the use of CAT Assay Kit, SOD Assay Kit and Antioxidant Assay Kit, ELISA-method (Cayman Chemical Company, Ann Arbor, MI, USA). The sperm DNA fragmentation was determined by the TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labelling)/PI (propidium iodide) staining using the FlowTACS Apoptosis Detection Kit (Trevigen, Inc., Minneapolis, MN, USA) as previously described (31). Briefly, after fixation (1% formaldehyde) and permeabilization (0.1% solution of Triton X-100 in 0.1% sodium citrate), sperm pellets were incubated in Binding Buffer containing biotinylated dNTP, Mn^{2+}, and terminal deoxynucleotidyl transferase (TdT) enzyme for 45 minutes at 37ºC. Next, the sperm cells were resuspended in FITC-labeled streptavidin solution, and incubated for 20 minutes at room temperature. After the TUNEL labelling reaction, sperm cells were stained with PI to exclude necrotic sperm cells in the flow cytometry analysis. The percentage of TUNEL - positive sperm was assessed using the Cell LabQuanta SC MPL Analysis software (Beckman Coulter, Fullerton, CA, USA).

Spermatozoa activity of ion-transport action of sodium, potassium (Na^+, K^+-ATPase) and calcium, magnesium adenosine triphosphatases (Ca^{2+}, Mg^{2+}-ATPase) were determined spectrophotometrically in saponin permeabilized spermatozoa, recording the process of ATP hydrolysis by accumulation of inorganic phosphorus (P_i). 'Basal' spermatozoa Ca^{2+}, Mg^{2+}-ATPase activity was tested in a similar incubation environment, but in the presence of 1 mM ouabain - a selective inhibitor of Na^+, K^+-ATPase (32). Determination of Ca^{2+}, Mg^{2+}-ATPase activity in saponin permeabilized spermatozoa was performed using Costerin (33), Zvarych method (34). Ca^{2+}, Mg^{2+}-ATPase activity was measured by recording the process of ATP hydrolysis by accumulation of Pi (phosphorus inorganic). Ca^{2+}, Mg^{2+}-ATPase activity was expressed as nmoles of released Pi mg of protein per minute. The content of Pi was determined by Rathbun and Betlach (1969) method (35).

Fr concentration was studied with the use of Fructose Test (spectrophotometric test for quantifying Fr in human seminal plasma samples; FertiPro N.V., Beernem, Belgium). CA levels were studied with the use of CA test (spectrophotometric test for CA quantification in human seminal plasma samples; FertiPro N.V., Beernem, Belgium). Neutral α-Glu activity was measured with the use of EpiScreen Plus™ (*in vitro* diagnostic method by use of quantitative ELISA; FertiPro N.V., Beernem, Belgium).

Statistical analysis

Statistical calculations were performed by using the software package STATISTICA 10.0 (StatSoft Inc. Tulsa, OK, USA), 'Free statistical calculators' MedCalc, MedCalc Software. The Shapiro-Wilk test was used to check the normal distribution of the evaluated parameter. Statistical significant differences among the studied subgroups at the I^{st} and II^{nd} observation stages, as well as versus healthy control group were determined by non-parametric Mann-Whitney U test - as following: V I vs. VC II, V I and VC II vs. C. For these tests, U was a criterion for sample numbers - U [n1; n2]. Median (Me), lower quartile (LQ) and upper quartile (UQ) were also determined. The numerical results were presented as Me (LQ; UQ). For the estimation of statistical significance hypothesis we have not only used p value, but also the confidence interval (CI). CI were calculated for a number of indicators with a probability of 95%. The indicator was submitted in the format [L-U], where L is lower and U is the upper limit of the CI. A special kind of logistic regression - the receiver operator characteristic curve (ROC-analysis) was used to assess the diagnostic significance of the applied methodology. The ROC analysis operates with 2 classes of

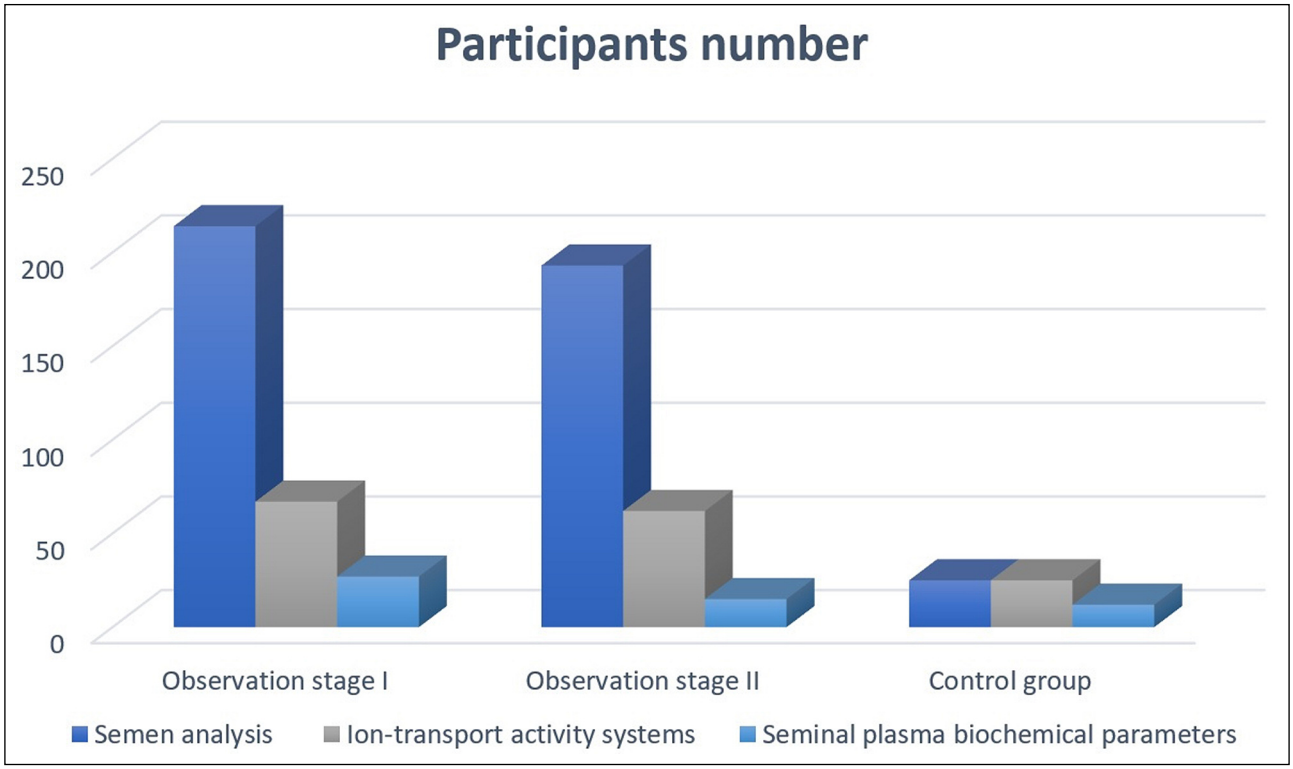

Fig. 1. Three subgroups of males evaluated by assigned assays for semen parameters monitoring.

events - with positive and negative results. The share of truth positive cases - tests 'sensitivity' (Se), and in the truth negative cases - 'specificity' (Sp) were estimated. The ROC curve showed to be dependent from the number of correctly classified positive results from incorrectly classified negative results. The area under the curve - AUC (area under curve) assessed the quality of the model in the following scale: 0.9–1.0 - excellent, 0.8–0.9 - very good, 0.7–0.8 - good, 0.6–0.7 - average and 0.5–0.6 - unsatisfactory. The level of statistical significance with respect to AUC=0.5 was (p <): ′ - 0.05, ″ - 0.025, ‴ - 0.003, * - 0.001. For AUC indicators, the CI was calculated with a probability of 95%, the comparison of which strictly established the statistical significance of the ROC curves differences.

During ROC analysis, the optimal independent cut-off or optimal cut-off values (OC) were determined in the investigated independent variables, which characterized the rate of sensitivity and specificity. The predictive value of positive test result (PPV - positive predictive value) and negative ones (NPV - negative predictive value) were also evaluated. We also determined positive LR - the likelihood ratio of a positive test result and a negative one for the optimal OC. Spearman rank order (r) correlations were used to determine the relationship between the studied parameters.

RESULTS

Semen parameters, that demonstrated numerous significant differences, were measured before and after laparoscopic varicocelectomy in patients with left-side varicocele of grade II–III (*Table 1*).

In patients at the Ist stage of observation, all measured semen parameters were statistically significant lower than in control group: volume, total sperm count, motility (a + b), viability and normal morphology ($p<0.001$). According to the semen analysis at the IInd stage of observation, volume did not change substantially and remained below the normal value while concentration, motility, viability as well as normal morphology returned almost to normal values (not showing statistically significant differences for motility and morphology versus control). In our view, the observed significant increase of semen parameters after surgical treatment is a good prognosis for subsequent fertility, but mechanisms which potentially affect spermatogenesis still remain unknown. Therefore we have continued the study of biological factors possible involved in varicocele etiopathogenesis.

ROC-analysis of semen parameters value demonstrated the statistical significance for PPV for all studied semen parameters (as volume of ejaculate, sperm concentration, motility, viability and morphology) but both Positive Predictive Value (99.5%) as well as Negative Predictive Value (100%) ($p<0.001$) were significant only for total number of spermatozoa.

Next, we have examined the major parameters of pro-/antioxidants system in seminal plasma in patients with left-side varicocele grade II–III before (V I) and after operation (VC II) (*Table 2*).

Table 1. Seminal parameters in patients with left-side varicocele of grade II–III at different observation stages and in healthy controls.

Parameter	**Ist stage V I** (n=214)	**IInd stage VC II** (n=193)	**Control C** (n=25)	**U**			**Statistical Differences**
				VC II vs. C	**V I vs. VC II**	**V I vs. C**	
	Me (LQ; UQ)			**193 vs. c25**	**214 vs. 193**	**214 vs. c25**	
Volume (ml)	2.8 (1.9; 3.5)	3.11 (2.64; 3.65)	4 (2.4; 5.9)	1742.5	15986	1559.5	V I vs. C, p<0.001 VC II vs. C, p<0.023 V I vs. VC II, p<0.001
Total sperm count 10^6	23.35 (14.8; 35.8)	45.5 (37; 58.5)	72 (97.5; 50.4)	895	4558.5	12	V I vs. C, p<0.001; VC II vs. C, p<0.001; V I vs. VC II, p<0.001;
Motility (%)	23.25 (15.4; 30.3)	44 (36; 52)	46 (39; 62)	1847.5	4504	273.5	V I vs. C, p<0.001; V I vs. VC II, p<0.001;
Viability (%)	44.25 (29.9; 59.5)	59.8 (50.3; 69)	73 (65; 81)	980.5	11416	639	V I vs. C, p<0.001; VC II vs. C, p<0.001; V I vs VC II, p<0.001;
Normal morphology (%)	5.05 (3.1; 8.3)	27.1 (17.5; 45.6)	30 (15; 59)	2255.5	2016	328.5	V I vs. C, p<0.001 V I vs. VC II, p<0.001

V I - patient with varicocele; VC II - patient after varicocelectomy; C - control; U - Mann-Whitney U test - statistically significant differences for values obtained in V I patients before (I) and after surgery VC II (II), and versus healthy controls (C); Me - median; LQ - lower quartile; UQ - upper quartile; p - statistical significance between values obtained in observation stages I and II and versus healthy control.

We have observed the statistical significant differences ($p<0.001$) between the values of TAC in patients before (V I) and after (VC II) surgical treatment (*Table 2*). The levels of MDA (the prooxidant), CAT and SOD (the antioxidant enzymes), showed no statistically significant differences for analyzed groups before and after surgical treatment. ROC values demonstrated both statistically significant positive and negative predictive values at level of 100 % for TAC only ($p<0.001$).

Next we have studied sperm DNA fragmentation at two observational stages (V I and VC II), assuming, that its level could be critically important for reproductive success.

In patients with varicocele before operation (V I) sperm DNA fragmentation level was statistically significant and 7.6-fold higher than in control males ($p<0,001$). While in observation stage after varicocelectomy (observation stage II–VC II) it was indicated statistically significant increased sperm DNA fragmentation level only 1.5-fold higher than in control group ($p<0.001$) (*Fig. 2*). Statistically significant differences were found also for groups before and after varicocelectomy (V I vs. VC II, $p<0.001$).

ROC values obtained have shown by demonstrating TUNEL assay of sperm DNA fragmentation with statistical significance both for positive (100%) as well as for negative predictive values (85.7%).

Next, we have examined adenosine triphosphatase system activities before and after the laparoscopic varicocelectomy (observation stages V I and VC II).

In patients with varicocele (V I) at the Ist stage of observation, the level of Na^+,K^+-ATPase was statistically significant (at 2.0-fold) lower than in control group, while after

Table 2. Values of pro-/antioxidant compounds detected in seminal plasma of patients with left-side varicocele grade II–III before (V I) and after operation (VC II), and compared to healthy controls (C).

Parameter	**TAC** (μM)	**CAT** (nM/min/mL)	**SOD** (U/mL)	**MDA** (μμM/mL)
Stage	Me (LQ; UQ)			
V I (n=27)	1393.5 (1183; 1491)	12.64 (8.79; 17.75)	2.9 (2.53; 3.48)	3.19 (2.45; 3.52)
VC II (n=15)	1726 (1514; 1931)	12.71 (9.23; 16.23)	2.43 (2; 3.26)	3.04 (2.8; 3.26)
Control C (n=12)	2091 (1999.5; 2274.5)	9.28 (8; 11.35)	2.85 (2.3; 3.15)	2.6 (1.8; 3.4)
V I vs. VC II $U_{[27; 15]}$	66	167	129.5	181
V I vs. VC II	p<0.001	–	–	–
V I vs. C $U_{[27; 12]}$	0	94	122	119
V I vs C	p<0.001	–	–	–
VC II vs. C $U_{[15; 12]}$	15	49	77	67
VC II vs. C	p<0.001	–	–	–

V I - patient with varicocele; VC II - patient after varicocelectomy; C - control; TAC - total antioxidant capacity; CAT - catalase; SOD - superoxide dismutase; U - Mann-Whitney U test - statistically significant differences among indices obtained in patients before - V I (I) and after surgery - VC II (II), and versus healthy controls (C). MDA - malondialdehyde; Me - median; LQ - lower quartile; UQ - upper quartile; p - statistical significance of changes in parameters obtained in two observation stages and versus healthy control.

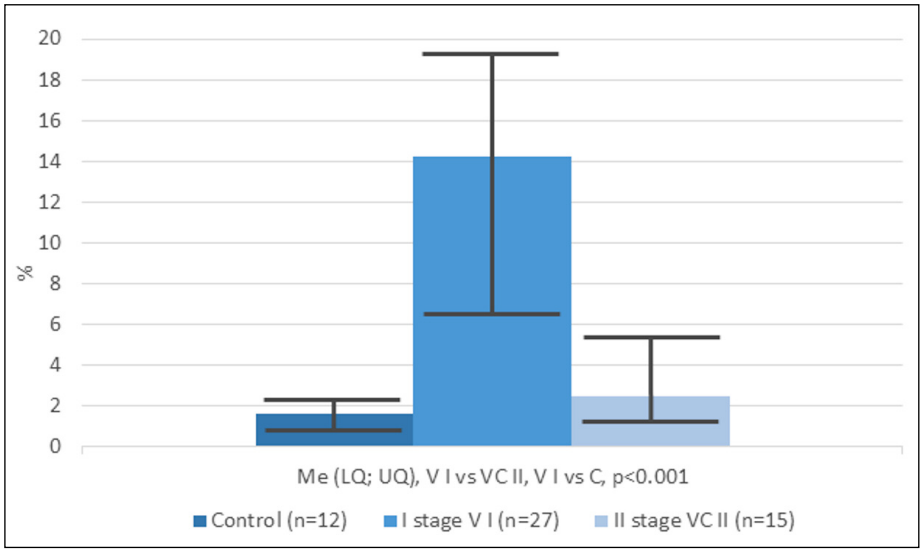

Fig. 2. DNA fragmentation level (TUNEL) in varicocele patients grade II-III of V I and VC II populations before and after surgery versus healthy controls.

surgical treatment (IInd stage of observation; VC II) the level of Na^+, K^+-ATPase increased in statistically significant fashion and returned to normal values (*Fig. 3*).

In patients with varicocele at the Ist stage of observation,(V I) the level of Ca^{2+}, Mg^{2+}-ATPase was statistically significant lower than in the control group ($p<0.001$), and after surgical intervention (IInd stage; VC II) level of Ca^{2+}, Mg^{2+}-ATPase/s increased in statistically significant fashion exceeding the values obtained at the Ist stage (V I) of observation (before surgery) returning almost to normal values (*Fig. 3*). The optimal scenario for functional activity of spermatozoa would be to obtain high concentration of K^+ ions and the low concentration of Na^+ and Ca^{2+} ions inside of spermatozoa. This status can be achieved through the action of both: Na^+, K^+-ATPase, which secretes Na^+ extracellularly and K^+ intracellularly together with and Ca^{2+}, Mg^{2+}-ATPase, which produces Ca^{2+} ions intracellularly.

The return to normal levels of Na^+/K^+-ATPase and/or close to normal levels by Ca^{2+}, Mg^{2+}-ATPase after surgical intervention seems to be important for spermatozoa function in terms of capacitation or acrosomal reaction (36). This has been shown by indicated in Table 3 positive correlations of both ATP-ases with total number of spermatozoa as well as in case of Na^+/K^+-ATPase also with sperm motility.

Summing up data from *Fig. 3* and *Table 3* there was demonstrated a statistically significant improvement in the Na^+, K^+-ATPase and Ca^{2+}, Mg^{2+}-ATPase systems correlating with semen features most importantly with sperm cell number and its motility.

ROC-analysis have shown statistically significant predictive values (both positive 98.5% and negative 92.3%) for Na^+/K^+-ATPase ($p=0.03$) while positive predictive value only for Ca^{2+}, Mg^{2+}-ATPase activity (100%).

We have also examined the concentration of selected biochemical substances in seminal plasma (SP) before (V I) and after varicocelectomy (VC II). Substances represented activity of reproductive glands, *i.e.* α-Glu has been considered as prominent secretory factor of epididymis while Fr and CA as important secretory components of seminal vesicle and prostate, respectively.

Data from *Table 4* demonstrated a statistically significant (3.0-fold) decreased concentration ($p<0.013$) of α-Glu after varicocelectomy of which level normalized almost to values in

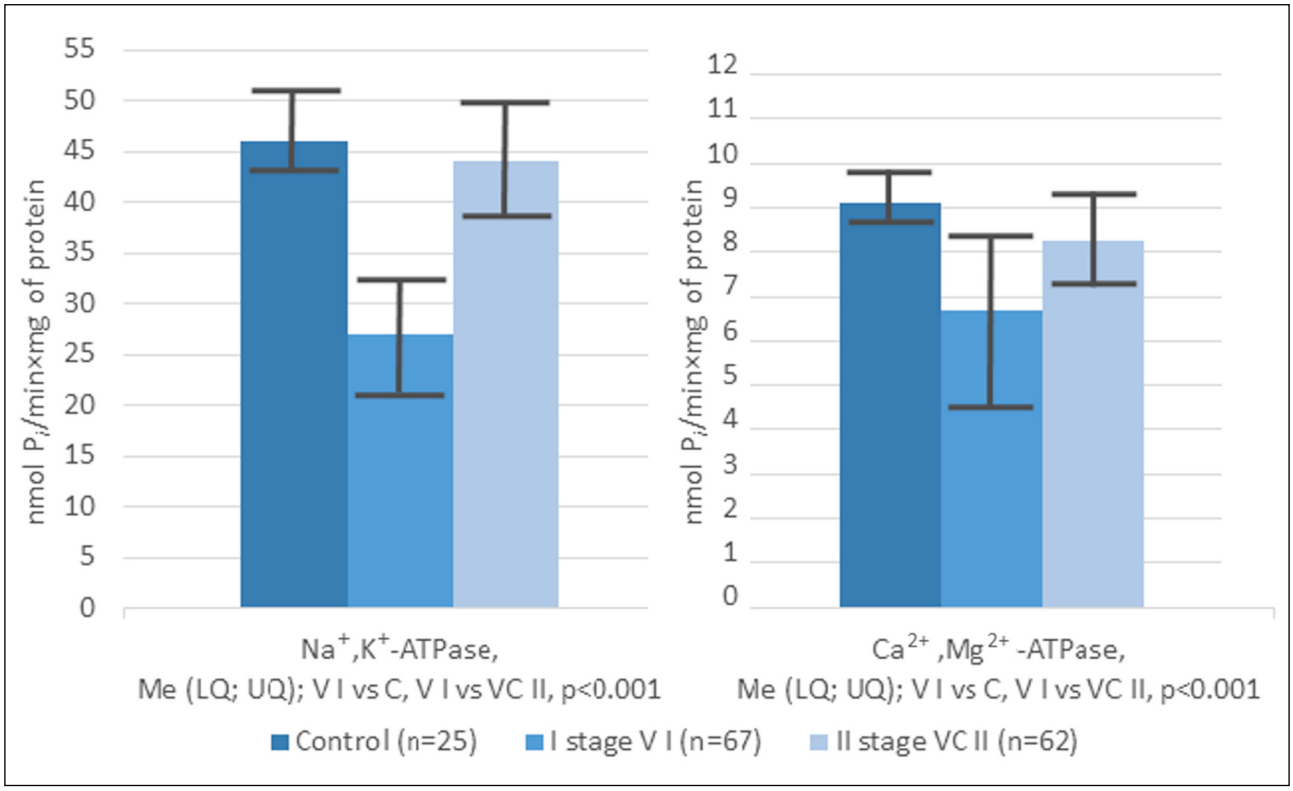

Fig. 3. Ion-transport system of sodium, potassium (Na^+, K^+-ATPase) and calcium, magnesium adenosine triphosphatases (Ca^{2+}, Mg^{2+}-ATPase) in spermatozoa before (V I) and after (VC II) surgery (observation stage I and II) and versus healthy controls.

Table 3. Spearman rank order correlation (r) of adenosine triphosphatase systems in spermatozoa with semen parameters in patients with left-side varicocele grade II–III (V I) before varicocelectomy.

Parameter	Volume	Total sperm count	Motility	Viability	Normal Morphology
Na^+,K^+-ATPase, r	0.05	0.85*	0.28*	0.07	–0.11
Ca^{2+},Mg^{2+}-ATPase, r	0.16	0.82*	0.16	0.05	–0.06

*$p<0.05$; r - Spearman rank order correlation; Na^+,K^+-ATPase, - sodium, potassium adenosine triphosphatase; Ca^{2+},Mg^{2+}-ATPase - calcium, magnesium adenosine triphosphatase.

Table 4. Concentration of selected biochemical substances in seminal plasma of patients with left-side varicocele (grade II–III) at different observations stages (V I vs. VC II) and compared to healthy controls.

Parameter	**α-glucosidase (mIU/semen)**	**Fructose** (mg/semen)	**Citric acid** (mg/semen)
	Me (LQ; MQ)	Me (LQ; UQ)	Me (LQ; NQ)
I stage (n=27)	90.09 (59.74; 130.06)	8.45 (5.42; 11.41)	14.995 (11.19; 24.83)
II stage (n=15)	26.66 (17.38; 71.92)	4.5 (4.49; 8.53)	5.84 (5.47; 7.66)
Control (n=12)	34.4 (29.5; 39.85)	9.6 (7.1; 10.15)	13.8 (12.55; 15.1)
$U_{[27;\ 15]}$	25	56	3
V I vs. VC II	p<0.013	–	p<0.001
$U_{[27;\ 12]}$	14	140	119
V I vs. C	p<0.001	–	–
$U_{[15;\ 12]}$	28	19	0
VC II vs. C	–	–	p<0.001

V I - patients with varicocele before operation; VC II - patients after varicocelectomy; C - controls; U - Mann-Whitney U test - statistically significant differences between values obtained for (V I) patients before (I) and (VC II) after varicocelectomy, and vs. healthy controls (C); Me - median; LQ - lower quartile; UQ - upper quartile; p - statistical significance of changes in parameters between two observation stages and versus healthy controls.

healthy males. Similarly, the level of non-enzymatic antioxidant CA after surgical intervention was lowered (2.0-fold) in statistically significantly manner (p<0.001), however, still remained twice reduced as compared to those obtained for healthy males. Fr levels did not demonstrate statistically significant change after varicocelectomy and remained at 2.0-fold below the values obtained for healthy males.

ROC-analysis have shown statistically significant values for positive (98.5%) and negative (91.7%) predictive values concerning α-Glu levels in seminal plasma (SP) delineating patients with varicocele and their subsequent successful treatment by varicocelectomy

DISCUSSION

According to several studies, approximately from 25% to 40% of men with semen pathological spermiogram reveals varicocele, but the exact mechanism through which this condition impairs sperm quality remains unclear (37). Varicocele has been correlated with a high percentage of sperm with inactive mitochondria, and the level of oxidative stress found in these patients might lead to decreased sperm motility (21, 38). According to our data, the main sperm parameters observed in patients with varicocele were decreased, *i.e.* concentration of spermatozoa, motility, viability and normal morphology in respect to values found in healthy volunteers (*Table 1*). All out of these parameters statistically significantly increased after surgical treatment, however, remained lower than in the healthy control group. Among them sperm motility and sperm with normal morphology remained lower after varicocelectomy, but not significantly lower than in normal subjects (*Table 1*).

In patients with varicocele the main source of excessive ROS production in semen seems to be derived from pathological forms of spermatozoa (3). It is confirmed that in patients with varicocele oxidative stress has been maintained by sperm with remaining cytoplasmic droplets (12), and concomitantly with deficient antioxidant system in spermatozoa (39). ROS levels have been usually reduced at 3 months after varicocelectomy while spermatogenesis lasts around 74 days (40). It is known that ROS exert harmful effects on sperm function, including critical pathophysiological environments in both testes and epididymis of men with varicocele (3, 8, 12, 41). Also, MDA levels reflect extracellular ROS action (42). ROS may cause damage to cellular membranes as well as DNA integrity in spermatozoa (43).

According to recent data, low TAC levels were observed in patients with high grades varicocele and long infertility period (39, 41), so they have been unable to protect lipid peroxidation in the cell membranes (44). In general, TAC includes two types of major antioxidants: enzymatic and non-enzymatic ones (45). They should protect the semen redox status and the quality of spermatozoa, since the balance between pro- and antioxidants preserves the optimal sperm function (42, 45). In patients with varicocele, some authors observed significantly changed SOD activity, and others did not indicate its response to high ROS levels (38). In general, SOD should protect sperm from superoxide anions by catalyzing conversion of superoxide into atomic oxygen and H_2O_2 (45) and although ROS and antioxidants balance seems to be universal preserving fertility in the whole mammalian family it should be particularly difficult to study this complex system based on enzymatic activities of its particular components (46).

It was, however, reported, that varicocelectomy increases the antioxidant capacity of seminal plasma (44, 47). In our group of patients, SOD activity decreased (statistically insignificant) after varicocelectomy. In turn TAC level in our study increased after surgery in seminal plasma in statistically significant way, so we can assume that in patients with varicocele, surgical intervention may be efficient to reduce ROS release (*Table 2*).

The last stage of capacitation phase, followed by an acrosomal reaction as well as hyperactive sperm motility requires the presence of certain ions (Ca^{2+}, $K^{+,}$ Mg^{2+}, Zn^{2+}). At normal situation, during capacitation, Ca^{2+}-ATPase cooperates with calmodulin and seems to be quite active. The viability of the sperm requires high concentration of K^+ and low levels of Na^+, Ca^{2+} inside the cell. This status is achieved by membrane-connected Na^+-K^+-ATPase and Ca^{2+}-ATPase. K^+-ATPase promotes the excretion of Na^+ and penetration into the spermatozoa of K^+, while Ca^{2+}-ATPase promotes the release of Ca^{2+} out of the cell (36). In hyperthermic conditions, the epithelium of the caudal epididymis demonstrates impaired ion and water transport, resulting in influx of water, higher concentrations of Na^+ and Cl^-, and decreased concentrations of K^+ within the lumen. Subsequently, the sperm from these conditions maintained decreased viability during storage (48). According to *Fig. 3*, we have determined that the Na^+, K^+-ATPase activity in patients with varicocele was reduced, associating with poor semen motility, however after

varicocelectomy, this activity was elevated in statistically significant fashion recovering to the normal values. The level of Ca^{2+}, Mg^{2+}-ATPase activity in patients with varicocele was also lower in comparison to healthy controls, but after varicocelectomy, there has been noted statistically significant recovery. This indicates, that the capacitation process may normalize upon varicocelectomy.

Sperm motility is an ATP-dependent process. *ATP5D*, another gene that encodes a segment of the mitochondrial adenosine triphosphate synthase (ATPase) which provides energy necessary for sperm flagellar motion was found to be down-regulated in men with varicocele (48).

Sperm motility and ATP production are supported by glucose, therefore, ATP production due to glycolysis is important for maintaining sperm motility. When glycolysis is depressed, sperm motility decreases (44). In patients with varicocele of grade II–III, α-Glu activity was found to be statistically significantly higher than in healthy controls while after varicocelectomy, it dropped to a level significantly lower than observed in normal healthy individuals. This may indicate an effort for glycolysis normalization and, in consequence, increased sperm motility after surgery can be expected.

Sperm DNA damage can be another cause of male infertility, and also a reason of miscarriages, offspring abnormalities, and failures in conception even by assisted reproduction technologies (ART) (20). Scrotal hyperthermia and generation of ROS could be potential mechanisms of varicocele-related sperm dysfunction and DNA damage. Presence of spermatozoa with damaged DNA may indicate apoptotic process, which is an ongoing phenomenon, that starts during oxygen stress and redox deregulated conditions, such it is observed in varicocele. In patients with varicocele, sperm DNA damage has been reported to correlate with abnormal chromatin packaging and mitochondrial membrane potential (MMP) as well as apoptosis (49, 50). Induced apoptosis is one of the main pathophysiological mechanisms of testicular dysfunction in men with varicocele. There are three possible causes for apoptosis: elevated testicular temperature, reduced testosterone levels and toxic metabolites accumulation (18, 21, 31). In addition to early apoptosis, increased necrosis and sperm degeneration are also observed in patients with varicocele (3, 21). DNA integrity with the presence of single- and double-strand DNA breaks was found in infertile men sperm as a consequence of oxidative stress. Sperm DNA integrity is one of the key elements for normal fertilization and embryogenesis in natural conception as well as in cases when ART is used (41, 44, 45). In our study, we have been shown that increased DNA fragmentation in varicocele patients returned to normal values in result of varicocelectomy (*Fig. 2*).

Proper function and protection of sperm DNA depend on the compact chromatin structure. Replacement of histones by protamines in the nuclei of spermatids at the late stage of spermatogenesis is an important step for male fertility maintenance. A defect in this process makes the spermatozoa more susceptible to DNA damage, decreasing their fertility potential (21). Clinical evidence shows that abnormal sperm chromatin packaging is correlated with a reduced spermatozoa ability for oocytes fertilization in normal conception or in ART cases. In accordance with the other authors that have used cytochemical tests (CMA3) to evaluate the DNA chromatin status in patients with varicocele (37), we have indirectly shown a tendency to abnormal chromatin packaging in semen in patients with varicocele (*Fig. 2*) as it was indicated in the other reports (3, 46). Oxidative stress, evidenced by a low TAC, high ROS levels and high DNA fragmentation (DFI) (*Table 2*, *Fig. 2*), was found to be associated with high grades of varicocele and long infertility duration (18, 48).

There is no conclusive evidence that varicocelectomy improves spontaneous pregnancy rates. However, its possible influence on sperm DNA integrity and testicular microcirculation, have been evaluated (41, 43, 51). As we have established, varicocelectomy alleviates oxidative stress in seminal plasma (5) and causes a decrease of DFI, that could serve as a possible indicator of successful treatment (*Fig. 2*) and what can be recommended during the postoperative follow-up (29, 41). Preoperative DFI was found to be the only significant predictor so far that reflects a negative impact of varicocele on sperm quality and may also indicate not well responding patients. This can be explained by the assumption, that in the presence of oxidative stress sperm DNA may denature and spermatogenesis may be impaired (41).

The epididymis which plays a central role in sperm maturation and transport has several epididymal tubule principal cell linings that are capable of generating ROS. In addition to the metabolically active principal cells, other sources of epididymal ROS include the luminal fluid spermiated from the testis and the endothelial cells (50). The seminal vesicles and ductus deferens are main producers of Fr to seminal fluid. Fr is a source of energy for spermatozoa and their deficiency is associated with asthenozoospermia (25).

The redox balance within the epididymis is maintained by the counteracting enzymatic and nonenzymatic antioxidants to action of pro-oxidants. In varicocele stressful conditions such as hypoxia and heat stress can trigger overproduction of ROS by the principal cells along with compromised antioxidant production in the epididymal tubules, resulting in creation of oxidative stress environment within the epididymis. An experimental animal model, in which varicocele was induced, it resulted in increased apoptosis of principal epididymal cells and decreased levels of carnitine (antioxidant), and α-Glu activity. Epididymal carnitine and α-Glu activity reflect the functional status of the epididymis highlighting its involvement in sperm maturation often seen as failing in infertile men with varicocele (50). The seminal fluid is rich in α-Glu, while its low level is associated with defects of maturation of spermatozoa in epididymis and in capacitation (25, 31).

The data from *Table 4* demonstrated, however a statistically significant decreased concentration of α-Glu after varicocelectomy (α-Glu is the biochemical parameter of epididymis activity) (52). In our view, the increased activity of α-Glu in patients with varicocele may be reflected by intense work of the epididymis, which apparently indicate its compensatory action. Three months after varicocelectomy Fr and CA remained in our studies below the levels observed in control group. In our opinion, this indicates the lack of action of non-enzymatic antioxidants and shortage of supply of energy shortly after surgical treatment. Although α-Glu behavior in epididymis should be treated as controversial sperm maturation both in testis and epididymis could be confirmed in the semen by looking for example to the aquaporin system usually downreguled (AQP1 and AQP9) in varicocele individuals (53).

The level of non-enzymatic antioxidant CA was lowered after varicocelectomy in statistically significantly fashion (2.0-fold). The low concentration of CA constitutes a risk factor for: 1) the spermatozoa viability; 2) elevated ROS secretion in seminal plasma (54, 55). Previous meta-analysis showed the influence of the three surgical approaches (high ligation, inguinal, and subinguinal) on sperm count and motility (29). The results showed that all three surgical approaches led to significant postoperative improvement of these parameters. There was observed significant decrease of ROS as well as a significant increase in SOD, CAT, glutathione peroxidase, and vitamin C levels ($p<0.001$) at 6 months after surgery. However,

the random model analysis did not show significant differences in semen concentration, total motility and spermatozoa with normal morphology between the two investigating groups (pre- and postoperative) (1). Almost 50 % of female patterns of individuals undergoing varicocelectomy got pregnant by 6 and 12 month after varicocelectomy.

On the basis of our data, however, we tend to conclude that varicocelectomy in the adult population may likely improve main semen parameters namely sperm total count, motility, morphology and also sperm DNA integrity. Additional investigations of total antioxidant levels (TAC levels) and enzymatic antioxidants mainly SOD and CAT will be continued in near future in a bigger group of patients.

In conclusion: 1) In semen of patients with varicocele of grade II–III (V I group) we have identified statistically significant decreased values of main spermiogram parameters (semen volume, sperm number, motility, viability and morphology) which were then reversed by varicocelectomy particularly in respect to sperm motility and morphology in a group of VC II (after varicocelectomy) patients. 2) In patients with varicocele of grade II–III we have identified a dysfunction of the pro-antioxidants systems associated with significant reduced levels of TAC, compared to healthy controls which then markedly improved after varicocelectomy (VC II group) with SOD and CAT levels remaining unchanged. 3) Varicocelectomy exerted clear effect on sperm DNA integrity, lowering DNA fragmentation in spermatozoa of patients with varicocele of grade II–III (VC II group) which reached after operation the levels close to observed in healthy controls. 4) Varicocelectomy exerted a positive effect (statistically significant) increasing activity of Na^+, K^+-ATPase while markedly decreasing α-Glu activity in a group of VC II patients. 5) Total sperm count has been delineated as statistically significant for estimation of both positive and negative predictive values to discriminate between VC patients at observation groups I (V I group) and II (VC II group) and versus controls (C) while all the remaining semen factors (volume, sperm motility and morphology) expressed significant positive predictive values for analyzed populations before and after operation. 6) Novel biomarkers presented statistically significant predictive positive or negative predictive values discriminating between varicocele patients and normal controls which was demonstrated by levels of TAC, sperm DNA fragmentation measured by TUNEL assay, and Na^+, K^+-ATPase activity or α-Glu levels in seminal plasma.

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