ORIGINAL ARTICLE

Seminal plasma granulysin level before and after varicocelectomy

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ABSTRACT

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Background: Varicocele is the most commonly identified correctable cause of male infertility. Varicocelectomy is commonly performed an operation for treatment of male infertility by obstructing the refluxing venous drainage to the testis while maintaining arterial inflow and lymphatic drainage. Granulysin is a catalytic molecule in human Cytotoxic T lymphocytes and natural killer cell granules. **Objective:** Estimate seminal plasma level of granulysin in varicocele patients versus control, its level before and after varicocelectomy and correlate it with semen parameters and varicocele grade**. Methods:** A Case control study conducted on 23 varicocele and 24 non varicocele male as control. All patients were clinically evaluated by history and examination. The assessment of seminal plasma granulysin level, semen analysis and Doppler ultrasonography were done. **Results:** Seminal plasma granulysin level was significantly increased in cases compared to control (P <0.001). SPG was significantly higher among the varicocele cases pre-operation compared to cases after operation (P<0.001). SPG could be an entrant in the physiological apoptosis of spermatogenesis process and its high level was negatively affected of the spermatogenesis in males with varicocele. **Conclusion:** Varicocelectomy led to upgrade semen parameters with a significant effect on increase of concentration, progressive motility, total motility of sperm and sperm morphology and significantly decreases seminal plasma granulysin level.

INTRODUCTION

Varicocele is an abnormal redundancy of testicular veins in the pampiniform plexus [1-2]. It is present in 15% of adult men, in 35% of primary infertility and in 70%-81% of secondary infertility cases [3], and can be diagnosed and graded via regular physical examination, venography, Colour Doppler Ultrasonography (CDU) and thermography [4]. Varicocele normally affects one side, usually the left (90% of the cases) more than the right side but it can be occurred unilaterally or bilaterally in about 10% [5-6]**.** It is presents as heaviness in the scrotum with an asymmetry in the scrotal size, rarely present with testicular pain, it can lead to testicular atrophy, or shrinkage, and the testicle might become smaller and softer [7]. The deleterious effect of varicocele on semen parameters was due to

the stasis of blood in varicocele that led to increasing of the intra scrotal and testicular temperature, decreasing of the spermatogonia number and increasing apoptosis of germinal epithelium cells [8-12].

Granulysin is a cytolytic molecule present in human cytotoxic T lymphocytes and natural killer cell granules [13-14]. Granulysin is a member of the saposin-like protein family and its structure possess lytic properties [15]. Granulysin is related to the mitochondrial pathway of apoptosis, and it can target several human cells that enhance inflammatory and autoimmune processes. The granulysin-mediated increase of intracellular calcium could contribute to the mitochondrial damage and induction of apoptosis which cause the release of cytochrome C and production of reactive oxygen species [16]. Moreover, granulysin participates in activation of caspase 3 and DNA fragmentation that further potentiates mitochondrial damage and provides positive feedback for activation of apoptosis [17]. The previous studies have specified that the incidence of apoptosis was more frequent within the testicular tissue of patients suffering from varicocele than the control individuals, and that the concentration of spermatozoa in semen increased neatly after varicocelectomy [18-19].

Varicocelectomy is the most commonly implemented operation for the treatment of male infertility. The repairing of varicocele aims to obstruct the refluxing venous drainage to the testis while preserving arterial inflow and lymphatic drainage [20]. Varicocelectomy improves sperm parameters (count, total and progressive motility), seminal oxidative stress, sperm ultra-morphology, spontaneous pregnancy rates, reduces sperm DNA damage [21-24].

The aim of the present study was to compare seminal plasma granulysin level before and after three months of varicocele repair and correlate it with semen parameters. Moreover, it was to measure granulysin level in varicocele patients in comparison with free control men and correlate it with varicocele grade and semen parameters including, count, concentration, motility and vitality.

PATIENT AND METHODS:

This is a case-control study included 47 individual males, 23 vricocele patients, and 24 control non varicocele male with unknown reproductive potential., who attend the andrology outpatient clinic and urology outpatient clinic of Aswan University, with full clinical history and full andrological examination, during the period from October 2018 to April 2020.

2.1. Ethical approval

An informed consent was taken from all patients. The study was approved from ethics committee, Faculty of Medicine, Aswan University. The study included patients that exhibited clinical varicocele diagnosed by palpation as well as by Doppler ultrasound examination indicated for surgical varicocelectomy operation between the ages of 20–60 years. All selected patients had not been exposed to any environmental stressors, including chemicals or radiation.

2.2. Subjects

All patients were subjected to full clinical history, full andrological examination, semen analysis according to WHO 2010, and seminal plasma granulysin detection using granulysin ELISA Kit before and after three months of varicocele repairing. The correlations between seminal plasma granulysin level and semen parameters before, and after operation were made.

2.2.1. Varicocele examination

All selected patients possessed clinical varicocele that were clinically diagnosed by scrotal palpation in a temperature-controlled room (37°C) with sufficient illumination, as well as using grey scale and duplex colour doppler ultrasonography. The diagnostic criteria were as follows: >2 mm diameter of a vein of the pampiniform plexus and flow reversal duration on valsalva manoeuver of >1 s with an increase in vein diameter of >3 mm. Whereas, the grading of varicocele was achieved according to the following criteria: grade 0: no dilated veins; grade I: dilated veins of <2.5 mm in diameter, with no flow reversal after a Valsalva manoeuver; grade II: dilated tortuous veins of 2.5–3.5 mm in diameter and flow reversal after a Valsalva manoeuver; and grade III: dilated tortuous veins of more than 3.5 mm in diameter and flow reversal after a Valsalva manoeuver.

2.2.2. Semen analysis

Semen analysis was done by using the standard protocol for which the sample was collected following abstinence period from sexual intercourse for 3–5 days. The semen was liquefied then divided into two parts: one for the traditional seminal analysis and the other part was centrifuged at

1,000 *g* for 15 min, then the separated plasma was collected at −80°C until used for granulysin measurement and semen analysis was measured before and after three months varicocelectomy.

2.2.3. Seminal plasma granulysin (GNLY) measurement

Seminal plasma granulysin (GNLY) measurement was carried out by using the Human Granulysin Pico Kine ELISA Kit. The kit is for the quantitative level of GNLY in the sample, adopt purified Human GNLY antibody to coat micro titer plate, make solid-phase antibody, then add GNLY to wells, Combine GNLY antibody with labeled HRP to form antibody-antigen -enzyme-antibody complex, after washing completely, add TMB substrate solution, TMB substrate becomes blue color at HRP enzyme-catalyzed, reaction is terminated by the addition of a stop solution and the color change is measured at a wavelength of 450 nm. The concentration of GNLY in the samples is then determined by comparing the O.D. of the samples to the standard curve

2.2.4. Surgical intervention

Testicular artery and lymphatic sparing inguinal, and sub inguinal varicocelectomy was performed for patients by the urology department at Aswan university under anaesthesia.

2.2.5. Follow-up protocol

Follow-up protocol for all selected patient was done three months' post-operative including physical examination and laboratory investigation in the form of semen analysis with an abstinence period three days which was compared to pre-operative one, seminal plasma granulysin was done and compared to pre-operative values.

3. Statistical analysis

Data were analysed using IBM-SPSS 24.0. Means, standard deviations, medians, ranges, and percentages were calculated. Student t-test analysis was carried out to compare the means of dichotomous data that follow the normal distribution. While Mann Whitney U test was used to compare the medians of dichotomous data that did not follow the normal distribution. For repeated measures (before vs. after varicocelectomy); paired sample t-test analysis was carried out to compare the means of normally distributed data while Related Samples Wilcoxon Signed Rank test was used to compare the medians of dichotomous data that did not follow the normal distribution. ROC curve was depicted to investigate the diagnostic performance of SPG for prediction of varicocele, analysed as area under the curve (AUC), standard error (SE) and 95% CI, (sensitivity, specificity, positive and negative predictive value –PPV & NPV-) were calculated. Spearman Rank Correlation was used to examine the correlation between SPG and associated parameters P value < 0·05 will be significant.

3.1 Sample size calculation

Sample size calculation was performed by using G*Power 3 software. A calculated minimum sample of patients will be needed. The sample were divided into two groups: 23 varicocele patients undergoing varicocelectomy and 24 control free non varicocele group that were needed to detect an effect size of 0.2 in the mean seminal plasma level of granulysin with an error probability of 0.05 and 90% power on a two-tailed test.

RESULTS

Regarding to the distribution of semen parameters in varicocele cases vs .control that was illustrated in table 2**.** The mean sperm count (p = 0.027), concentration (p=0.032), vitality (P<0.001), motility(total(P=0.008), progressive (P=0.037)) among varicocele cases were lower in comparison with control and this was statistically significant. While the mean age (p=0.340) (table1) and mean semen volume (p=0.412)**,** among varicocele cases was higher in comparison with control and this was statistically insignificant.

Also there was significant elevation of the mean seminal granulysin level in cases with varicocele compared with the control men (P<0.001) table 2. In addition, the mean seminal granulysin level was insignificantly higher in grade II varicocele cases compared with grade III varicocele cases (P=0.413) (Table 5).

In addition, the diagnostic performance criteria of seminal plasma granulysin level illustrated in tables 3, 4 showed that the AUC was excellent (0.938; 95% CI: 0.849-1.000, p<0.001). The

accuracy was 96%, sensitivity was 92%, specificity was 100%. Also, PPV was 100% and NPV was 92.5%. As well, False Detection Rate was 0% and False Omission Rate was 7%.

Regarding semen parameters levels in varicocele cases pre-, post- varicocelectomy (table 6) the mean semen volume ($p>0.05$) and sperm morphology ($p=0.218$) among varicocele cases pre operation were insignificantly lower in comparison with the cases after operation .While the mean sperm count ($p=0.019$), concentration ($p=0.004$), vitality ($p<0.001$), and progressive motility ($p=0.149$) among varicocele cases preoperative were lower in comparison with cases after operation and this was statistically significant .

Seminal plasma granulysin level among varicocele cases pre operation was significantly higher ($p<0.001$) in comparison with cases after operation (table7).

For the control group ($r = -0.386$ and -0.378, $p = 0.031$ and 0.034, respectively), and the group after operation ($r = -0.366$ and -0.310, $p = 0.043$ and 0.048, respectively)**,** there was significant moderated negative correlation between SPG and both total and progressive motility. In other words, with increase in SPG level there was decrease in the total and progressive motility. The other parameters showed minimal to mild ($r = 0.054$ and -0.288), ($r = 0.218$ and -0.230) negative insignificant ($p<0.05$) correlation with SPG for both groups. While in the group before operation the parameters showed minimal to mild negative insignificant ($p<0.05$) correlation with SPG (table 7).

Table 1: Age distribution of the studied cohort

Parameter	Varicocele cases (n=23)	Control (n=24)	P-value
Age/years			
• **Mean ± SD**	**31.83 ± 10.6**	**29.25 ± 7.5**	**= 0.340**
• **Median (Range)**	**29 (22 - 59)**	**29.5 (19 - 45)**	

***Independent t-test test was used to compare the mean difference between groups.**

Table 2: Semen parameters and seminal plasma granulysin in cases vs. controls

Parameter	Varicocele cases (n=23)	Control (n=24)	P-value
Volume of semen (ml)			
• **Mean ± SD**	**2.51 ± 1.0**	**2.38 ± 0.9**	**= 0.412***
• **Median (Range)**	**2.5 (0.5 - 5)**	**2 (1.5 - 5)**	
Sperm count (million/ejaculate)			
• **Mean ± SD**	**81.18 ± 61.5**	**148.91 ± 99.1**	**= 0.027***
• **Median (Range)**	**86 (2 - 225)**	**138 (11.5 - 270)**	
Sperm concentration (million/ml)			
• **Mean ± SD**	**38.52 ± 32.9**	**65.36 ± 44.8**	**= 0.032***
• **Median (Range)**	**32 (1 - 100)**	**60 (15 - 146)**	
Vitality %			
• **Mean ± SD**	**48.89 ± 10.3**	**67.89 ± 8.3**	**< 0.001****
• **Median (Range)**	**50.5 (19.5 - 70)**	**68 (57 - 82)**	
Total motility (%)			
• **Mean ± SD**	**51.56 ± 14.2**	**63.13 ± 13.1**	**= 0.008***
• **Median (Range)**	**55 (16 - 80)**	**65 (41 - 82)**	
Progressive motility (%)			
• **Mean ± SD**	**30.82 ± 13.9**	**38.05 ± 12.1**	**= 0.037***
• **Median (Range)**	**30 (10 - 60)**	**35.5 (20 - 60)**	
Seminal plasma granulysin (ng/ml)			
• **Mean ± SD**	**118.78 ± 50.2**	**44.05 ± 11.5**	**< 0.001***
• **Median (Range)**	**108 (8 - 225)**	**60 (26 - 60)**	

Table 3: Diagnostic performance of seminal plasma granulysin for prediction of varicocele, analysed as area under the curve (95% CI)

	AUC*	95% CI+	SE**	P-value***
Seminal plasma granulysin	**0.938**	**0.849 - 1.000**	**0.046**	**< 0.001**

***AUC = Area under the Curve**

****SE = Standard Error+CI = Confidence Interval**

*****Null hypothesis: true area = 0.**

Table 4: Diagnostic criteria of seminal plasma granulysin for varicocele prediction

Diagnostic criteria of SPG	
• **AUC**	**0.938 (0.849 – 1.000) (p<0.001)**
• **Cut-off**	**68 ng/ml**
• **Accuracy**	**96%**
• **Sensitivity %**	**92%**
• **Specificity %**	**100%**
• **PPV %**	**100%**
• **NPV %**	**92.5%**
• **FDR (False Detection Rate)**	**0%**
• **FOR (False Omission Rate)**	**7%**

Table 5: Comparison of seminal plasma granulysin levels according to grades of varicocele in the studied patients

Variable (n=23)	**n (%)**	**Mean ± SD**	**Median (Range)**
Seminal plasma granulysin (ng/ml)			
• **II**	**12 (52.2%)**	**120.81 ± 50.4**	**120 (8 – 202.5)**
• **III**	**13 (47.8%)**	**116.57 ± 52.4**	**97.5 (46.5 – 225)**
P-value*	**= 0.413**		

Table 6: Semen parameters in varicocele cases (pre- vs. post- varicocelectomy

Parameter	Varicocelectomy (n=23)		P-value
	Before	After	
Volume of semen (ml)			
• Mean ± SD	2.51 ± 1.0	2.61 ± 0.6	= 0.733*
• Median (Range)	2.5 (0.5 - 5)	2 (1.2 – 7.5)	
Sperm Count (million/ejaculate)			
• Mean ± SD	81.18 ± 61.5	113.44 ± 82.6	= 0.019**
• Median (Range)	86 (2 - 225)	94 (5 - 300)	
Sperm concentration (million/ml)			
• Mean ± SD	38.52 ± 32.9	50.22 ± 36.4	= 0.004**
• Median (Range)	32 (1 - 100)	45 (2 - 120)	
Vitality %			
• Mean ± SD	48.89 ± 10.3	58.51 ± 9.6	< 0.001**
• Median (Range)	50.5 (19.5 - 70)	60 (37 - 57)	
Morphology			
• Mean ± SD	23.07 ± 20.8	27.91 ± 23.4	= 0.218**
• Median (Range)	15 (0 - 71)	22 (3 - 75)	
Total motility (%)			
• Mean ± SD	51.56 ± 14.2	55.22 ± 17.7	= 0.235**
• Median (Range)	55 (16 - 80)	55 (15 - 90)	
Progressive motility (%)			
• Mean ± SD	30.82 ± 13.9	34.53 ± 15.2	= 0.149**
• Median (Range)	30 (10 - 60)	35.5 (5 - 75)	
Seminal plasma granulysin (ng/ml)			
• Mean ± SD	118.78 ± 50.2	72.64 ± 31.8	< 0.001**
• Median (Range)	108 (8 - 225)	60 (20 - 132)	

Table 7: Correlation between seminal plasma granulysin and related parameters

Parameter	Seminal plasma granulysin		
	Control (n=24)	Before (n=23)	After (n=23)
	rho* (P-value)		
Age/years	**-0.288 (=0.088)**	**0.152 (=0.244)**	**0.230 (=0.145)**
Semen parameter			
• **Volume (ml)**	**0.054 (=0.400)**	**0.067 (=0.381)**	**0.218 (=0.159)**
• **Concentration**	**-0.112 (=0.301)**	**-0.062 (=0.390)**	**-0.242 (=0.133)**
• **Total sperm count**	**-0.172 (=0.211)**	**-0.039 (=0.430)**	**-0.088 (=0.345)**
• **Progressive motility %**	**-0.386 (=0.031)**	**-0.060 (=0.393)**	**-0.366 (=0.043)**
• **Total motility %**	**-0.378 (=0.034)**	**-0.043 (=0.439)**	**-0.310 (<0.048)**
• **Vitality %**	**-0.189 (=0.184)**	**0.287 (=0.092)**	**0.050 (=0.410)**
• **Morphology %**	**------**	**0.070 (=0.375)**	**-0.194 (=0.186)**

***Spearman's ranked correlation coefficient**

DISCUSSION

Varicocele is an abnormal dilated tortuous testicular veins in the pampiniform plexus of the spermatic cord, and is graded according to the findings on palpation [25]. The condition is correlated with infertility (20–40%) representing about 15–20% of asymptomatic healthy men [26].

Granulysin is a cationic molecule located in the granules of cytotoxic T lymphocytes and natural killer cells and can regulates apoptosis in several cell types [27-28].

The excessive apoptotic spermatozoon death is correlated with male infertility and there has been increasing interest in oxidative stress as one of the underlying reasons for deterioration of semen parameters in infertile men with varicocele, particularly sperm DNA damage. The most important effects of oxidative stress on spermatozoa were mitochondrial anomalies, sperm lipid peroxidation, apoptosis, sperm ultrastructural and DNA fragmentation [29].

Our present study found that there is statistically significance increase in the mean seminal plasma granulysin level in patient group with varicocele (118.78 ± 50.2 ng/ml) compared with the control free men (44.05 ± 11.5ng/ml group, $p<0.001$). The previous study showed that seminal plasma granulysin level exhibited a significant increase in infertile men compared with fertile men and, infertile men with varicocele displayed significantly higher seminal granulysin compared with infertile men without varicocele, in the cases of bilateral varicocele and in grade III varicocele [30].

Regarding the validity of seminal plasma granulysin for prediction of varicocele, in the present study the diagnostic performance criteria of seminal plasma granulysin level for diagnosis of varicocele showed that the AUC (area under the curve) was excellent, using a cut-off of 68 ng/ml of seminal granulysin at which it was 96% accurate, 92% sensitive, 100% specific, 100% PPV and 92.5% NPV.

Also in agreement with our results, Kamal *et al.*, [31] found in their study as a receiver operating characteristic (ROC) curve of seminal granulysin and MDA (malondialdehyde) levels were conducted for differntiation between infertility cases and control patiant groups. The excellent AUC was 0.971 for the seminal granulysin and 0.991 for the MDA level. The best cut-off value of seminal

granulysin was (=2.47) at which it was 92% accurate, 88.2% sensitive, 100% specific, 100% PPV, and 80% NPV, whereas, the best cut-off value of seminal MDA was (=1.38) at 96% accurate, 94.1% sensitive, 100% specific, 100% PPV, and 88.9% NPV.

As regard the relation between SPG level and degree of varicocele our study showed statistically insignificant results between grade II and grade III varicocele (P=0.413) while Mikhael *et al.,* [30] showed that the mean seminal granulysin level was significantly higher in grade III compared with grade I and II varicocele cases**.** In addition, Kamal, *et al.,* [31] has evaluated the relation between malondialdehyde and seminal granulysin in infertile men with varicocele and the potential effect of varicocelectomy it showed that basal seminal granulysin and MDA levels increased gradually with increased grades of varicocele on left and right sides, in all studied patients while grade III varicocele cases exhibited significantly mean seminal granulysin and MDA levels higher than in cases of grade I and grade II**.**

In our study regarding seminal plasma granulysin there was statistically significant decrease in its value after operation compared with the high result before varicocelectomy (P<0.001).

For the group after operation and control group, there was significant moderated negative correlation between SPG and both total and progressive motility but the other parameters showed minimal to mild (r = 0.054 and -0.288) negative insignificant (p<0.05) correlation with SPG.

This finding was in agreement with Mikhael *et al.,* [30]**.** As their study suggest that granulysin plays an important role in male infertility by decreasing sperm production, sperm motility and sperm normal forms that could be attributed to exaggerated apoptosis method in these cases.

Also this study is in agreement with Kamal *et al.,* [31] he found that seminal granulysin could be a participant in the physiological apoptosis of the spermatogenesis process, and its' high level negatively impact spermatogenesis in infertile males with varicocele. Seminal granulysin was significantly negatively associated with sperm count, concentration, progressive motility and positively correlated with abnormal sperm forms. Varicocelectomy leads to ameliorate semen parameters and significantly decreases seminal plasma granulysin and MDA levels**.**

In our study in comparison between varicocele and control group as regard semen parameters there was statistically significant relationship as regard (conc.ml, total sperm count, total motility, progressive motility, vitality) as theses parameters was lower in varicocele cases in comparison with control group.

In agreement with our study Agarwal and his colleagues in a systematic review and meta-analysis concluded that varicocele has a significant negative effect on semen quality [32-33]**.**

Regarding semen parameters in cases before and after varicocelectomy there is statistically significant increase and improvement in semen parameters including count, concentration, vitality and, progressive motility in all cases after operation.

In agreement with this Mohamed *et al.,* [34] made a study to compare semen parameters and pregnancy rates after varicocele repair in two age groups: group1 (patients aged 30 years or younger) and group 2 (those older than 30 years)**.** The mean sperm concentration increased significantly in the both groups. The percentage) The mean sperm concentration increased significantly in both groups. The percentage of motile sperm increased from 48.2% to 56.6% in group 1 and from 47.2% to 53.2% in group 2 one year after varicocele repair. Thiago *et al.,* [35] in his study displayed that there were improvement of semen parameters following varicocele repair especially in concentration and motility**.**

In patients with varicocele high levels of oxidative stress results from an unbalance between the reduced total antioxidant capacity and the over-production of reactive oxygen species, which led to alterations of sperm DNA integrity and semen parameters [36]. Apoptosis of testicular tissue triggered via multiple pathways; and hence, it was balanced by the plasma membrane externalization of negatively charged membrane phosphateidyl serine, mitochondrial dysfunction, DNA fragmentation and chromatin condensation [37]. The positively charged granulysin molecule attracted to the negatively charged cell membrane which perhaps illustrate its mechanism of action in this

process [38]. Some previous studies showed that granulysin compound triggers cellular death including induction of apoptosis under the effect of activation of the mitochondrial apoptotic route especially the apoptosis-inducing factor [39] or via oxidative stress that is participated by the decreased K^+ and the increased Ca^{2+} inside the cell and this led to improvement of the mitochondrial reactive oxygen production and hence, seminal plasma granulysin can target several human cells, enhancing inflammatory and autoimmune processes [17]. Also, there is important role of apoptosis during spermatogenesis specially at the phase of the development of stem spermatogonia into mature spermatozoa, and so apoptosis regulators may play an important role in pathologies associated with qualitative and quantitative deficits of spermatozoa [27]. Varicocelectomy leads to improve semen parameters and significantly decreases seminal plasma granulysin. More studies and longer duration of follow-up is recommended to give more definite results and illustrate the exact role of granulysin and oxidative stress in testicular dysfunction in varicocele patients and to confirm the benefits values of varicocelectomy.

CONCLUSION

In conclusion, seminal plasma granulysin could be a participant in the physiological apoptosis of the spermatogenesis procedure, and its high level negatively impacts spermatogenesis in males with varicocele.

Surgical treatment of patients with clinically and ultrasound proved varicocele (varicocelectomy) leads to improve semen parameters, and had significant effect on increase of concentration per ml, progressive motility, total motility of sperm and sperm morphology and significantly decreases seminal plasma granulysin level**.**

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