

Seminal plasma α -glucosidase activity and male infertility

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Measurement of α -glucosidase (α -GLUC) activity by means of a simple colorimetric test using a commercial kit (EpiScreen[®]; FertiPro, Lotenhulle, Belgium) yielded results that were strongly correlated with the values obtained for the neutral iso-enzyme measured by a fluorimetric reference method ($r = 0.85$, $P = 0.003$, $n = 13$). The former method was characterized by a low intra- and inter-coefficient of variation (6.6 and 4.3% respectively). Vasectomized men with azoospermia ($n = 27$) had a significantly lower α -GLUC activity in semen than vasectomized men with residual spermatozoa present ($n = 11$, $P < 0.01$) and men with azoospermia of primary testicular origin ($n = 33$, $P < 0.01$). Receiver operating curve (ROC) analysis showed α -GLUC measurement to be reasonably accurate in differentiation between cases with obstructive versus testicular azoospermia at criterion value 13.5 U/l (sensitivity = 82%, specificity = 70%). In cases with spermatozoa present, α -GLUC activity and output per ejaculate were positively correlated with sperm concentration ($r = 0.53$ and 0.38 , $n = 472$), linear velocity ($r = 0.35$ and 0.30 , $n = 224$), curvilinear velocity ($r = 0.32$ and $r = 0.29$, $n = 224$), semen adenosine triphosphate ($r = 0.35$ and 0.26 , $n = 64$), the concentration of 5α -dihydrotestosterone ($r = 0.31$ and 0.29 , $n = 74$), and γ -glutamyltransferase activity ($r = 0.62$ and 0.32 , $n = 275$) in seminal plasma. The activity of α -GLUC was inversely correlated with ROS generation after 12-myristate, 13-acetate phorbol ester stimulation ($r = -0.27$, $n = 104$), and both α -GLUC activity and total output were inversely correlated with the concentration of peroxidase-positive white blood cells among samples with $\geq 1 \times 10^6$ /ml of these cells ($r = -0.30$ and -0.19 , $n = 165$). It is concluded that simple photometric measurement of α -GLUC activity in seminal plasma reflects the functional state of the epididymis and may be helpful for the differential diagnosis of certain cases with azoospermia.

Key words: epididymis/ α -glucosidase/male infertility/semen analysis

Introduction

The epididymis plays a crucial role in the maturation of spermatozoa and their acquisition of progressive motility and fertilizing capacity (review: Cooper, 1996; Haidl and Schill, 1997). Several markers of epididymal function are available including L-carnitine, glycerylphosphocholine and α -glucosidase (α -GLUC) (Cooper *et al.*, 1988; Garcia Diez *et al.*, 1992), but their use has not been universally implemented. One of the reasons for this is the diversity and relative complexity of some test procedures, and the debatable clinical meaning of the results for patient management. In the era of assisted reproduction, more attention is given to the possible use of epididymal and testicular spermatozoa for intracytoplasmic sperm injection (ICSI), and this has revived the interest in correct classification of cases with azoospermia.

The determination of α -GLUC in semen, particularly of its neutral isoenzyme, has been claimed a rapid, sensitive and non-invasive method to differentiate secretory azoospermia from the excretory type, to localize the site of obstruction in the male genital tract, and to identify partial obstruction at the epididymal level (Guérin *et al.*, 1986; Cooper *et al.*, 1988; Garcia Diez *et al.*, 1992). Low levels of α -GLUC in semen may be related to epididymitis (von der Kammer *et al.*, 1991) and have been associated with defective sperm maturation in the epididymis (Haidl *et al.*, 1993). A high level of α -GLUC was correlated with strong binding capacity of the spermatozoa to the human zona pellucida (Ben Ali *et al.*, 1994), and with a high probability of success following intrauterine insemination (Milingos *et al.*, 1996).

Recently, a simple photometric method for the determination of α -GLUC activity has become commercially available (EpiScreen Kit; FertiPro, Lotenhulle, Belgium). We have compared the results of this method with those of a fluorimetric reference method measuring the neutral iso-enzyme (Warner and O'Brien, 1979; Cabezas *et al.*, 1982). In addition, we have studied the correlation of semen α -GLUC levels with various clinical and sperm parameters, and the concentration of testosterone, 5α -dihydrotestosterone (DHT), adenosine triphosphate (ATP) and reactive oxygen species (ROS) in semen of subfertile patients and vasectomized cases.

Materials and methods

Clinical evaluation and semen analysis

Semen samples of 474 men attending the andrology outpatient clinic of the Ghent University Hospital were included in the study. Clinical evaluation of the patients, semen analysis, and semen classification followed the recommendations of the World Health Organization (WHO, 1987; Rowe *et al.*, 1993). Sperm concentration and motility

characteristics, however, were evaluated using a computerized system (Autosperm; Fertipro) (Hinting *et al.*, 1988). γ -Glutamyltransferase (GGT) was measured in seminal plasma using a kinetic enzymatic kit (Boehringer Mannheim, Germany) (Comhaire *et al.*, 1989), and the categorization of round cells as leukocytes was based on their capacity to react with a peroxidase stain (Leucoscreen; Fertipro) (Endtz, 1972).

The patients were divided into the following groups: normozoospermic ($n = 36$), asthenozoospermic ($n = 112$), teratozoospermic ($n = 40$), oligozoospermic ($n = 33$), oligoasthenoteratozoospermic (OAT, $n = 172$), 38 azoospermic ($n = 38$) (five with proven obstruction at the head of the epididymis and 33 with idiopathic azoospermia of primary testicular origin), and successfully vasectomized men ($n = 38$) of whom 11 had residual spermatozoa present in their ejaculate, and 27 were azoospermic. In addition, 11 semen samples were collected in two split fractions and these fractions were analysed separately.

Measurements of α -GLUC

Seminal plasma α -GLUC was measured in all samples according to the photometric method described by Guérin *et al.* (1990) using a commercially available kit (Episcreen; Fertipro). In this method, the substrate (paranitrophenyl α -D-glucopyranoside) is hydrolysed specifically by α -GLUC into paranitrophenol, during 4 h of incubation at 37°C, pH 6.8. The reaction is stopped by adding NaOH (0.02 N). The quantity of paranitrophenol is measured by spectrophotometer (Carl Zeiss PM QII) at a wavelength of 407 nm. One unit of activity was defined as that producing 1 μ mol of D-glucose/min at 37°C, pH 6.8. The intra- and inter-assay coefficients of variation were calculated by measuring α -GLUC in a frozen pool of seminal plasma eight times on one day, and by performing the test 19 times 1 week apart respectively. In addition, the activity of the neutral and acidic isoenzymes of α -GLUC were measured by a fluorimetric method in 13 randomly selected samples (Warner and O'Brien, 1979; Cabezas *et al.*, 1982) and results of the two methods were compared.

Determination of ROS

In 104 samples with spermatozoa present, ROS were monitored by chemiluminescence (Aitken and Clarkson, 1987) using 5-amino-2,3-dehydro-1,4-phthalazinedione (luminol), stored as a 25 mM solution in dimethyl sulphoxide. Four microlitres of this probe were added to 400 μ l of unwashed cell suspension at concentration 10×10^6 spermatozoa/ml in Biggers/Whitten/Whittingham (BWW) medium, and the chemiluminescent signal was measured on a Berthold LB 9501 luminometer (Benelux Analytical Instruments, Vilvoorde, Belgium) in the integration mode. The cell suspension was supplemented with 3 purpurogallin Units of horseradish peroxidase (Type VI, 318 U/mg, Sigma, St Louis, MO, USA) to sensitize the assay for the generation of extracellular hydrogen peroxide (Aitken and West, 1990), and the signal obtained at this moment was considered the basal state. Once a basal signal had been established, the cells were stimulated by adding 100 nM of 12-myristate, 13-acetate phorbol ester (PMA) which has a predominant effect on the peroxidase-positive white blood cells (Aitken *et al.*, 1992). The ROS result is the expression of the oxidative effect of mainly white blood cells and the antioxidant effect of seminal plasma. It is the excess of ROS produced by the peroxidase-positive white blood cells and by poorly matured spermatozoa over the latter that is relevant in causing damage to the membrane and DNA of spermatozoa (Aitken *et al.*, 1992; Zalata *et al.*, 1997).

Measurement of ATP in semen

ATP was extracted and measured by a bioluminescence assay after the addition of luciferin-luciferase reagent and using an internal standard (Comhaire *et al.*, 1983).

Hormone determinations

Serum concentrations of luteinizing hormone (LH), follicle stimulating hormone (FSH), testosterone (Medgenix, Fleurus, Belgium), and prolactin (Behring, Marburg, Germany) were measured by radioimmunoassay using appropriate kits.

In addition, seminal plasma testosterone and 5 α -dihydrotestosterone (DHT) were measured in 74 samples by radioimmunoassay after extraction with diethyl ether and paper chromatography (Vermeulen, 1973; Zalata *et al.*, 1995).

Statistics

Statistical analysis was performed using the MedCalc program for medical statistics (MedCalc Software, Mariakerke, Belgium) (Schoonjans *et al.*, 1995). The significance of differences was assessed by one-way analysis of variance, correlations were calculated using the Spearman rank test, and ROC curves (Griner *et al.*, 1981) were used to determine the discriminative power between two groups and to identify criterion values. Not all samples were subjected to every analysis, and the number of samples is given for each test.

Results

There was a significant positive correlation between the results of α -GLUC determination by the photometric method and the neutral iso-enzyme as assessed by the fluorimetric method ($r = 0.85$, $P = 0.003$). The working range for the photometric method was 11–100 U/l, while that of the fluorimetric method was 3–20 U/l. In the latter method dilutions were used to assess values of >20 U/l. The detection limit of the two methods was 11 and 0.001 U/l respectively. For the photometric method, the intra- and inter-assay coefficients of variation were 6.6 and 4.3% respectively. The median value of α -GLUC activity in the seminal plasma pool assayed was 23 U/l.

Higher levels of α -GLUC were detected in men with normal sperm concentration than in cases with oligozoospermia, azoospermia of primary testicular origin, or after vasectomy (Table I). Significant differences in the α -GLUC levels were registered between azoospermic cases of primary testicular origin and vasectomized men with residual spermatozoa, and between the former and those men successfully vasectomized with azoospermia (Figure 1). All five patients with proven obstruction of the caput epididymidis had α -GLUC values >22 U/l, whereas those with azoospermia of testicular origin or azoospermia following vasectomy had α -GLUC values <20 U/l.

ROC curve analysis indicated that α -GLUC was reasonably accurate in differentiating between azoospermia due to vasectomy or to primary testicular causes [criterion value ≤ 13.5 U/l, area under ROC curve = 0.76, 95% confidence interval (CI) = 0.62–0.87, sensitivity = 82%, specificity = 70%, positive likelihood ratio = 2.72, negative likelihood ratio = 0.26, positive predictive value = 78.6%, negative predictive value = 73.7%], and in differentiating between vasectomized men with or without residual spermatozoa (criterion value >13.4 U/l, area under ROC curve = 0.83, 95% CI = 0.76–0.93, sensitivity = 73%, specificity = 82%, positive likelihood ratio = 3.93, negative likelihood ratio = 0.33, positive predictive value = 61.5%, negative predictive value = 88.0%).

Table II shows that both the α -GLUC activity and the total content in the ejaculate (total α -GLUC) presented a significant

positive correlation with sperm concentration, seminal ATP concentration and GGT activity in seminal plasma. The correlation with total progressive sperm motility and with sperm morphology, though statistically significant, is not considered clinically meaningful since it is not identified as a dependent variable in multiple regression analysis. In addition, α-GLUC levels correlated negatively with ROS and the concentration of peroxidase positive white blood cells in semen among cases with >1×10⁶/ml round cells. The total α-GLUC activity correlated positively with serum testosterone. Both α-GLUC activity and total content were strongly correlated with the concentration of DHT in seminal plasma. Multiple regression analysis showed that 67% of variability (*R*-adjusted) of α-GLUC activity could be explained by variability of semen GGT, sperm concentration and progressive motility, and the concentration of white blood cells. For total α-GLUC per ejaculate, 70% of variability could be explained by the variabil-

ity of semen GGT, progressive sperm motility, concentration of white blood cells, and testosterone concentration in serum.

No correlation was found between α-GLUC levels and sperm concentration among cases with oligozoospermia, whether or not associated with asthenoteratozoospermia. The concentration of α-GLUC in the first fraction of the ejaculate (median = 37 U/l, range = 16–78) was significantly higher (*P* < 0.01) than that in the second fraction (median = 12 U/l, range = 8.4–21).

When the results from non-azoospermic non-vasectomized patients with and without palpable abnormalities of the epididymides were compared, no differences were detected in either α-GLUC levels or sperm parameters (data not shown).

Table I. α-Glucosidase activity (in U/l) in the groups studied and differences between groups (*P* values calculated using one-way analysis of variance)

	Median (range)	Different from group number ^a
Sperm concentration >20×10 ⁶ /ml		
Group 1: Normozoospermia	29.0 (14–86)	4, 5, 6, 7
Group 2: Asthenozoospermia	26.4 (14–87)	4, 6, 7
Group 3: Teratozoospermia	28.2 (14–76)	4, 6, 7
Sperm concentration >0 to <20×10 ⁶ /ml		
Group 4: Oligozoospermia	17.2 (12–59)	1, 2, 3, 7
Group 5:	20.0 (12 to >100)	1, 7
Oligoasthenoteratozoospermia		
Azoospermia/vasectomy		
Group 6: Azoospermia of primary testicular origin	16.7 (12–30)	1, 2, 3, 7
Group 7: Vasectomized patients	12.8 (<11–26)	1, 2, 3, 4, 5, 6

^a*P* < 0.05.

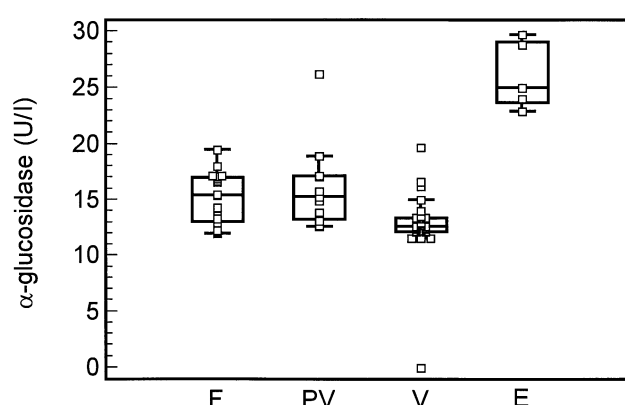


Figure 1. Box-and-whisker plots in cases with azoospermia due to primary testicular failure (F, *n* = 33), in vasectomized men with residual spermatozoa (PV, *n* = 11), vasectomized men with azoospermia (V, *n* = 27) and cases with azoospermia due to obstruction of the caput epididymis (E, *n* = 5). The central box in the box-and-whisker plot represents the lower and upper quartile, and the middle line in the box represents the median. From each end of the box a line is drawn to the most remote data point that is not an outlier. Outliers are displayed as separate points.

Table II. Rank correlations between seminal α-glucosidase (α-GLUC) activity (U/l) and total content (Total α-GLUC) (U/l multiplied by ejaculate volume) with the semen parameters studied and serum testosterone (*n* = number of samples)

	α-GLUC		Total α-GLUC		<i>n</i>
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	
Sperm concentration (×10 ⁶ /ml)	0.53	< 0.001	0.38	< 0.001	472
Sperm motility (total progressive %)	0.13	0.01	0.15	0.002	401
Sperm velocity (μm/s)	0.32	< 0.001	0.29	< 0.001	224
Sperm linear velocity (μm/s)	0.35	< 0.001	0.30	< 0.001	224
ATP (μg/ml)	0.35	0.005	0.26	0.04	64
Sperm morphology (% normal)	0.18	0.001	0.19	< 0.001	384
GGT (U/l)	0.62	< 0.001	0.32	< 0.001	275
ROS (PMA)	-0.27	< 0.001	-0.09	0.34	104
Serum testosterone (ng/ml)	0.07	0.38	0.19	0.01	169
Seminal testosterone (nmol/l)	0.19	0.098	0.14	0.24	74
Seminal DHT (nmol/l)	0.31	0.009	0.29	0.02	74
WBC (×10 ⁶ /ml) ^a	-0.30	0.0001	-0.19	0.02	165

ATP = adenosine triphosphate, GGT = γ-glutamyltransferase, ROS = reactive oxygen species, PMA = 12-myristate, 13-acetate phorbol ester, DHT = 5α-dihydrotestosterone, WBC = peroxidase-positive white blood cells.

^aAmong cases with >1×10⁶/ml round cells.

Discussion

The bulk activity of α -GLUC, and particularly of its neutral isoenzyme, is secreted by the epididymis, mostly in the corpus and cauda (Yeung *et al.*, 1990). It appears to be a sensitive indicator of epididymal function (Paquin *et al.*, 1984; Cooper *et al.*, 1990). We found a significant positive correlation between α -GLUC levels measured by the photometric method and the activity of its neutral iso-enzyme measured by the reference fluorimetric method. The intra- and inter-assay coefficients of variation of the photometric method are acceptable, and they correspond with those reported by others (Spiessens *et al.*, 1996) so that this method can confidently be used for routine semen analysis.

Our data on α -GLUC determination in split ejaculates and samples with obstructive azoospermia due to vasectomy confirm the mainly epididymal origin of this enzyme (Yeung *et al.*, 1990). The persistence of α -GLUC, though at a low level, in semen of successfully vasectomized men with azoospermia suggests that a proportion of this enzyme is secreted by the seminal vesicles, whereas the contribution of the prostate is probably negligible (Tremblay *et al.*, 1979; Guérin *et al.*, 1986; Cooper *et al.*, 1990).

In cases with azoospermia, an α -GLUC concentration of <13.5 U/l indicates bilateral obstruction of the vas deferens, values between 13.5 and 20 U/l are found in cases with primary testicular azoospermia, and values >20 U/l may suggest bilateral obstruction at the caput epididymis. The latter suggestion is, however, based on a small number of cases due to the rarity of this condition among our patients. However, a low α -GLUC activity may occur in patients with azoospermia of primary testicular origin, possibly due to a direct effect of the testis on the epididymis. Impaired Sertoli cell function may result in both decreased secretion of binding protein for testosterone and maturation arrest in spermatogenesis. Hence, our data confirm the observation that assessment of α -GLUC activity in seminal plasma of azoospermic men, together with measurement of testicular volume, serum FSH and testosterone, can help in differentiating between the major causes of this condition (Guérin *et al.*, 1986; Cooper *et al.*, 1988). The commercial test is simple to perform in any andrological laboratory, and adds value to semen analysis in so far that it can help the clinician to direct his azoospermic patient either to reconstructive surgery, or to assisted reproduction.

Vasectomized men with residual spermatozoa seem to maintain some epididymal fluid in their ejaculate. It is well-known that a relatively large number of ejaculations may be needed to completely empty the ductal reservoir of spermatozoa and — apparently—of epididymal fluid.

The biological significance of α -GLUC is reflected by our finding that its activity correlates positively with conventional sperm characteristics (Cooper *et al.*, 1988) and with seminal ATP, which factors are indicative of the fertilizing capacity of spermatozoa (Comhaire *et al.*, 1983; Hinting *et al.*, 1988). Also, α -GLUC is negatively correlated with both ROS and the concentration of peroxidase-positive white blood cells, which are known to both adversely affect fertilization *in vivo* (Milingos *et al.*, 1996) and *in vitro* (Aitken *et al.*, 1991). The

strong correlation between the activity of α -GLUC and seminal 5 α -DHT suggests that measurement of the former may be informative about the effect of androgens on the function of the epididymis. Indeed, it has been reported that epididymal function depends especially on the concentration of 5 α -reduced testosterone at the level of the epithelium (Zalata *et al.*, 1995).

We found low α -GLUC levels in seminal plasma of patients with oligozoospermia and this finding may reflect either varying degrees of epididymal obstruction, or epididymal hypofunction due to the influence of the testis on the epididymis (Cooper *et al.*, 1988). The positive correlation between serum testosterone concentration, a marker of testicular endocrine function, and the total α -GLUC content per ejaculate supports the latter hypothesis.

Our data indicating similar values of α -GLUC and sperm parameters in patients with and without palpable abnormalities of the epididymis, none of them with acute epididymitis, are in agreement with the findings of Cooper *et al.* (1988).

In contrast to our data regarding the negative correlation between α -GLUC and inflammatory parameters, Wolff *et al.* (1991) did not find a significant decrease of α -GLUC in semen with high levels of elastase produced by polymorphonuclear granulocytes among patients with clinically silent infection of the accessory sex glands. There is no evident explanation for this discrepancy, except that elastase may not adequately reflect the inflammatory status of the epididymis.

The relatively strong correlation between GGT and α -GLUC, and the fact that GGT is selected as the most important dependent variable, suggests simultaneous changes in the function of the epididymis and the prostate. Several explanations can be found for this observation, namely the functional dependence of both glands on testosterone supply (Zalata *et al.*, 1995), or the parallel and simultaneous effect of infection or inflammation on both accessory sex glands (Comhaire *et al.*, 1989).

It appears that the photometric method for the assessment of α -GLUC activity in semen using a commercial kit is a simple, reproducible and specific test. The activity of α -GLUC in semen measured by this method is a marker of the contribution of epididymal secretion to the ejaculate. In conjunction with other variables, it can be helpful in differentiating between different causes in a number of cases of azoospermia. In addition, it may help to localize and assess the effect of inflammation on the function of the epididymis.

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