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ORIGINAL PAPER

Seminal calbindin 2 level in azoospermia and oligoasthenoteratozoospermia and its correlation with seminal and hormonal parameters

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Summary

Objectives: *We aimed to assess seminal calbindin 2 (CALB 2) expression in men with different semen parameters as well as its correlation with reproductive hormones in azoospermic patients and different semen parameters in oligoasthenoteratozoospermic patients. CALB 2 is also known as calretinin and 29 kDa calbindin.*

Materials and methods: *This prospective study was performed on 96 cases from the andrology outpatient clinic divided into 3 groups as follows: group 1 including 32 non obstructive azoospermic (NOA) patients, group 2 including 32 patients with oligoasthenoteratozoospermia (OAT), and Group 3 including normozoospermic individuals as controls. Semen analysis and estimation of seminal CALB 2 concentrations by enzyme linked immunosorbent assay (ELISA) technique were performed for all participants. Reproductive hormones were measured in non-obstructive NOA patients.*

Results: *The mean seminal CALB 2 level was higher in OAT patients compared to NOA patients and controls (7.8 ± 1.30 ng/ml, 7.3 ± 0.80 and 7.4 ± 1.0, respectively). Furthermore, the study had shown strong positive correlations between CALB 2 and sperm normal forms in controls and OAT patients. In contrast, there was no significant correlation between seminal CALB 2 and any of the reproductive hormones measured in NOA patients.*

Conclusions: *Seminal CALB 2 may play a role in increasing the abnormal forms in OAT patients.*

KEY WORDS: *Non obstructive azoospermia; Oligoasthenoteratozoospermia; normozoospermia; Seminal CALB 2 (Calretinin & 29 kDa Calbindin); Reproductive hormones.*

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INTRODUCTION

Abnormalities in the form of oligozoospermia, asthenozoospermia, teratozoospermia and high percentage of sperm DNA fragmentation are associated with decreased probability to conceive (1). Oligozoospermia is classically defined when sperm count is below 10M/mL. 15 M is the 5% percentile of the new 2021 WHO manual for semen analysis (2). Defective sperm motility in the form of decreased progressive motility or absence of motility is defined as asthenozoospermia which is usually accompanied by oligozoospermia or teratozoospermia (3). Harbouring above 85% morphologically abnormal sperms in semen is considered *teratozoospermia* (TS). TS is classified into monomorphic and polymorphic. In monomorphic TS, all sperms have the same morphological abnormality while in polymorphic type, there are different varieties of abnormal sperm morphologies (4). Calbindin 2 (CALB 2; Calretinin; 29 kDa Calbindin) is a calcium binding protein that is mostly secreted in the nervous system as well as the ovary, the adrenal glands, and the testis. The main function of CALB 2 is to buffer intracellular calcium ion to stop Ca2+ overload as well as a Ca2+ receptor (5). Ca2+, which is also a second messenger in the cytoplasm, plays an essential role in different physiological functions such as cell proliferation and apoptosis (6). Additionally, it regulates the synthesis of reproductive hormones (7). The primary studies revealed that the highest CALB 2 was secreted in the cytoplasm of Leydig cells of adult rats in synchrony with androgen level so postulating that CALB 2 might prompt steroidogenesis (8). Increment in viability and proliferation of Leydig cells by CALB 2 was attributed to inhibition of mitochondrial related apoptotic pathway through inducing ERK1/2 and AKT pathways as well as supressing cell apoptosis (9).

Interestingly, CALB 2 can be deployed as a marker of normal and neoplastic Leydig cells of the testis as well as diagnosing atypical Leydig cell tumor (10). *Non obstructive azoospermia* (NOA) and OAT of unknown causes are common and are noticed in a high sector of infertile men. However, the underlying molecular mechanisms of these conditions remain unknown (11). We aimed to assess seminal CALB 2 expression in men with different semen parameters as well as to investigate potential correlations between seminal CALB 2 and different semen parameters in OAT patients. Furthermore, we aimed to find any correlation between seminal CALB 2 and reproductive hormones in NOA patients.

Patients and methods

The current prospective study was conducted in the andrology outpatient clinic during the period from April (2021) till January (2022). Approval by the local research and ethical committee was obtained that conforms to Helsiniki declaration (2013) (FMBUREC/09052021) (12). Written informed consents were obtained from the patients who were involved in the study.

The study was performed on 96 randomized patients using simple numbering method. They were divided into 3 groups as follows: group (1) comprised of 32 NOA patients. Group (2) comprised of 32 patients with OAT. Group (3) comprised of 32 patients with normal semen parameters as controls.

Inclusion criteria of the patients

Any infertile patient with normal testicular volume was included.

Exclusion criteria of the patients

Any patient with varicocele, smoker, leukocytospermia, abnormal karyotyping and finally evidence of severe uncontrolled medical diseases was excluded from the study.

Inclusion criteria of the controls

Any healthy age matched individual was recruited in group 3.

Sample size was calculated using G power. At least 89 participants should be included in the three groups using F tests - ANCOVA: Fixed effects, main effects and interactions as well as a priori analysis (13). The participants were asked about any relevant medical history and were subjected to clinical examination. Also, 5 cc blood was withdrawn from each participant for hormones investigation [FSH, LH, total testosterone, estradiol, *prolactin* (PRL)] and semen analysis. Furthermore, all participants were asked about a history of hernia repair, scrotal surgery, pelvic surgery, endoscopic urethral instrumentation, or genitourinary infection. Testicular volume of all participants was routinely determined by Prader's orchidometer. Furthermore, scrotal duplex was done for all participants to exclude varicocele.

Semen analysis

Semen samples were collected by masturbation following abstinence for 3-4 days. A special wide-mouth container was used to collect semen and incubated at 37°C until semen was liquefied. Semen analysis was then performed within 1 hour following the WHO manual criteria (5th edition, 2010) (14). Duplicate semen analyses were performed twice at the beginning and 3 months after initiating the study and the average of the two values was used for analysis. The same investigator performed all semen analyses to optimize repeatability (15). Seminal analysis was carried out within 30 minutes after liquefaction. The volume, viscosity, pH and appearance of semen were evaluated together with sperm concentration, progressive and total motility and morphology.

Sperm concentration was evaluated using a Makler counting chamber (*Irvine Scientific, Santa Ana, CA, USA*) under an optical microscope (*Nikon, Nikon Europe B.V., Amsterdam, The Netherlands*) at 200x magnification. Sperm morphology was evaluated by using pre-coloured glasses (Testsimplets) and the eosin Y test was applied to evaluate sperm vitality.

Determination of CALB 2

Human CALB 2 was determined using Calretinin ELISA Kits supplied by *SinoGeneClon Biotech Co., Ltd. Cat. No.: SG-00383*. This kit uses the Sandwich-ELISA principle with sensitivity 0.01 ng/mL and detection range 0.06-4 ng/mL. The microtiter plate of the Calretinin ELISA is coated with a capture antibody. The diluted sample was added and any antigen present bound to capture antibody. After a washing step, the detecting antibody (biotinylated anti-calretinin antibody) was added and bound to antigen. After another washing step the enzyme conjugate streptavidin-peroxidase was added and bound to detect the antibody. The following substrate TMB/peroxidase reaction was monitored at 450 nm (reference wavelength at 620 nm).

Determination of reproductive hormones in NOA patients

Blood samples were obtained from azoospermic patients, and then samples were left to clot for 1 hour at room temperature or overnight at 2-8°C before centrifugation for 20 min at 1000×g at 2-8°C. The supernatant was collected to carry out the assay. *Follicle stimulating hormone* (FSH) was determined using ELISA Kits supplied by *Elabscience Biotechnology, Inc, United States. Cat. No.: E-EL-H1143*. This kit used the sandwich-ELISA principle with sensitivity 0.94 mIU/mL and detection range 1.56-100 mIU/mL. *Luteinizing hormone* (LH) was determined using ELISA Kits supplied by *Elabscience Biotechnology, Inc, United States. Cat. No.: E-EL-H6019*. This kit used the sandwich-ELISA principle with sensitivity: 0.1mIU/mL and detection range 0.16-10mIU/mL. Serum prolactin was measured according to human *prolactin* (PRL) *OneStep ELISA Kit* (*Boster Biological Technology, Pleasanton CA, USA, Catalog # EK7006*) with analytical sensitivity 11.7 pg/mL and assay range 15.6-1000 pg/mL.

Testosterone was determined using ELISA Kits supplied by *MyBioSource, Inc., San Diego, USA*. Cat. No.: MBS580035. Estradiol was determined using ELISA Kits supplied by *Thermo Fisher Scientific, Inc. Third Avenue Waltham, MA USA. Cat. No.: KAQ0621*. This kit used the sandwich-ELISA principle with sensitivity: 5 pg/mL and detection range 13-935 pg/mL.

Statistical analysis

Analysis of data was performed using SPSS v. 23 (*Statistical Package for Social science*) for Windows. Mean, *standard deviation* (SD), minimum and maximum were used to describe quantitative variables whereas number (No.) and percentages (%) were used to describe qualitative variables. Shapiro/Kolomogrov tests of normality were utilized to test for normality. The Chi square test was used to determine the statistical difference of the categorical data between the two groups. Pearson correlation was used to test the correlation between different quantitative variables.

Results

There was no significant difference between the studied participants regarding their baseline characteristics (Table 1).

Furthermore, one case had atrophic right testis and 3 cases presented with small testis in NOA patients. There were three cases with absent right epididymis and 4 cases with absent vas deferens in the same group.

Additionally, the medians of FSH, LH, Testosterone, E2, and prolactin in NOA patients were 5.40, 6.35, 4.50, 42.00 and 7.50, respectively.

Furthermore, there was no significant difference between NOA, OAT and normozoospermic participants regarding seminal CALB 2 level (7.3 ± 0.80 ng/ml; 7.8 ± 1.40 ng/ml; 7.4 ± 1.0 ng/ml, p = 0.15, respectively). Although seminal CALB 2 levels in NOA and OAT groups were not significantly different to levels of normozoospermic participants (p > 0.05), yet, there was a higher predictive role of seminal CALB 2 levels in predicting OAT cases compared to azoospermic cases (Table 2, Figures 1-2). Moreover, there were significant linear strong positive correlations between sperm normal forms and seminal CALB 2 levels in normozoospermic and OAT cases (Tables 3-4).

Table 1.
Sociodemographic characteristics of the participants.

	Group (1) azoospermic patients (n = 30)		Group (2) oligoasthenoteratozoospermic (OAT) (n = 30)		Group (3) normozoospermia (n = 30)		P value
	Mean	SD	Mean	SD	Mean	SD	
Age (years)	33.3	± 10.4	30.4	± 8.1	29	± 6.7	0.15
Marital status	N	%	N	%	N	%	
Single	5	16.7					-
Married without offspring	16	53.3	30	100			
Married with offspring	9	30.0			30	100	

P value was calculated using Chi-square test.

Figure 1.
Receiver Operating characteristic curve for prediction of non obstructive azoospermia (compared with normozoospermia) using seminal calretinin level.

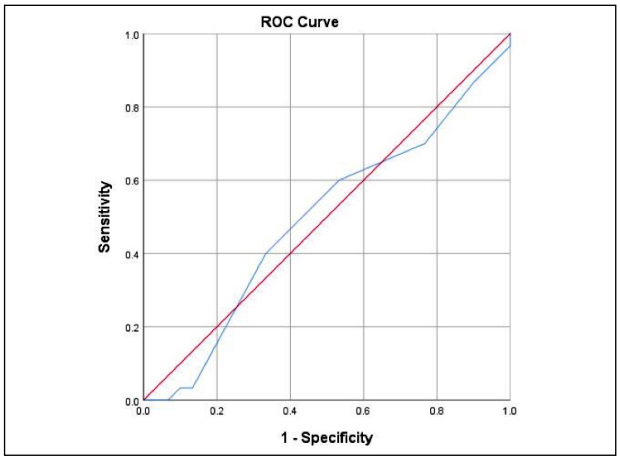

Figure 2.
Receiver Operating characteristic curve for prediction of oligoathenoteratospermia (compared with normozoospermia) using seminal calretinin level.

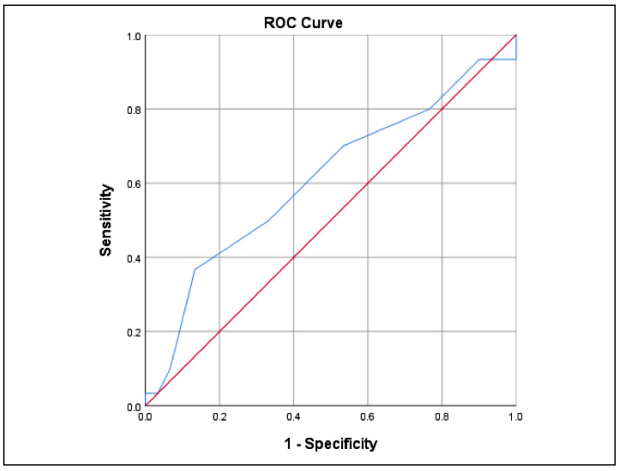

Table 2.
Cutoff values, area under the curve (AUC), sensitivity, specificity, positive predictive values and negative predictive values of seminal CALB 2 in azoospermic and oligoasthenoteratozoospermic (OAT) cases.

	Azoospermic cases	OAT cases
P-value	0.92	0.15
Cut off	7.2500	7.7500
AUC	0.493	0.609
Sensitivity	60 %	50%
Specificity	46%	66%
Positive predictive value	60%	50%
Negative predictive value	45%	65%

Table 3.
Correlation between seminal calbindin 2 (CALB 2) and age and semen parameters in normozoospermic participants.

	Normozoospermic cases	Seminal CALB 2 (ng/ml)
Age	Pearson correlation (r)	0.055
	p-value	0.77
Sperm concentration (10^6/ml)	Pearson correlation (r)	-0.072
	p-value	0.71
Sperm total motility (%)	Pearson correlation (r)	0.285
	p-value	0.13
Sperm normal forms (%)	Pearson correlation (r)	0.709
	p-value	0.00

P value was calculated using Pearson correlation.

Table 4.
Correlation between seminal calbindin 2 (CALB 2) and age and semen parameters in oligoasthenoteratozoospermic (OAT) cases.

	OAT cases	Seminal CALB 2 (ng/ml)
Age	Pearson correlation (r)	-0.596
	p-value	0.00
Sperm concentration (10^6/ml)	Pearson correlation (r)	-0.088
	p-value	0.64
Sperm total motility (%)	Pearson correlation (r)	0.347
	p-value	0.06
Sperm normal forms (%)	Pearson correlation (r)	0.763
	p-value	0.00

P value was calculated using Pearson correlation.

Table 5.
Correlations between seminal calbindin 2 (CALB 2) and age and reproductive hormones in azoospermic patients.

	Azoospermic cases	Seminal CALB 2 (ng/ml)
Age	Pearson correlation (r)	0.032
	p-value	0.87
FSH	Pearson correlation (r)	-0.329
	p-value	0.17
LH	Pearson correlation (r)	-0.307
	p-value	0.22
Total testosterone	Pearson correlation (r)	-0.282
	p-value	0.29
Estradiol	Pearson correlation (r)	-0.173
	p-value	0.46
Prolactin	Pearson correlation (r)	-0.167
	p-value	0.59

P value was calculated using Pearson correlation.

However, there was a significant linear strong negative correlation between age and seminal CALB 2 levels in OAT cases (Table 4). Finally, there was no significant linear correlation between seminal CALB 2 levels and any of the reproductive hormones measured in NOA cases (Table 5).

Discussion

The present study is a case-control study conducted comparing seminal CALB 2 expression in men with normal semen parameters, OAT and NOA. The mean seminal CALB 2 level was higher in OAT cases compared to azoospermic cases and controls. Although, the values of seminal CALB 2 levels in OAT and azoospermic cases were not significantly different compared to normozoospermic participants, there was a higher predictive role of seminal CALB 2 levels in predicting OAT cases compared to NOA cases. Moreover, there was no significant linear correlation between seminal CALB 2 level and any of the reproductive hormones measured in NOA cases. On the other hand, there were significant linear strong positive correlations between sperm normal forms and seminal CALB 2 levels in normozoospermic and OAT cases. There was a high significant linear negative correlation between age and seminal CALB 2 level in OAT cases.

Bar-Shira Maymon et al. (16) demonstrated that the expression of CALB 2 in abnormal Sertoli cells in non-obstructive azoospermia contributes to the multifactorial etiology of spermatogenic failure. In the same context, *GamalEl Din et al.* (17) demonstrated a negative impact of seminal CALB 2 on sperm normal forms in patients with varicocele (17). Furthermore, OAT patients showed the highest median seminal CALB 2 compared to the patients of other 2 groups in the current study. This can be explained by the fact that OAT patients would have more immature Sertoli cells compared to azoospermic patients and controls. Thus, they have increased levels of CALB 2 expression by the seminiferous epithelium and consequently have increased levels of seminal CALB 2. This agrees with *GamalEl Din et al.* (2023) who demonstrated that patients with bilateral varicocele had higher seminal CALB 2 compared to unilateral varicocele (17). Interestingly, the current study did not show any correlation between CALB 2 and any of the reproductive hormones measured in NOA cases. On the contrary, several studies have demonstrated a potential link between CALB 2 and steroidogenesis and reproductive hormones. Firstly, steroidogenesis in Leydig cells can be enhanced by increased free Ca2+ (18). In the same context, *Altobelli et al.* (2017) had detected CALB 2 immunoreactivity in human fetal testis, testis Leydig cells, seminiferous epithelium and epididymal epithelial cells (13). They assumed that CALB 2 is involved in the processes of production and/or secretion of hormones as well as in all calcium dependent differentiation processes that occur during gonadal development suggesting its involvement in steroidogenesis and spermatogenesis (13). However, the role played by CALB 2 cannot be precisely determined similarly to other calcium-binding proteins that behave differently in the presence of this ion to the extent that they are classified as "*Ca-buffer*" or "*Ca sensor*" proteins (19).

This uncertainty of their exact role is due to the possible exposure of their hydrophobic residues after binding with calcium (19).

Admittedly, our study is no free from limitations. Firstly, the small sample size can be considered as a major limitation. Moreover, the inability to use immunohistochemistry in the azoospermic cases to properly localize the site of production of CALB 2 can be regarded as another limitation. Also, not including cases of obstructive azoospermia could be seen as an additional limitation. Besides, another limitation was including one case with atrophic right testis and 3 cases with small testis in NOA patients. Finally, we were unable to evaluate reproductive hormones in all participants.

Conclusions

In brief, seminal CALB 2 may play a role in increasing the abnormal forms in OAT cases.

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Conflict of interest: The authors declare no potential conflict of interest.