Auto-antibodies against disulphide isomerase ER-60 as a possible diagnostic marker in male immunological infertility

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Introduction

Inflammation and infection of the male genital tract have been reported in up to 15% of cases as main aetiologies of male fertility disturbances. In the majority of these patients, however, diagnosis is hampered by an asymptomatic course of the disease, especially subacute or chronic inflammatory conditions in the testis and/or epididymis remain obscure. Definitive diagnosis of suspected chronic testicular inflammation is based only on invasive testicular biopsy (1). Chronic inflammatory conditions of the male genital tract do not only impair basic sperm parameters, but also negatively influence sperm functions and, subsequently, the outcome of IVF/ICSI treatment.

Objective

To establish an enzyme immunoassay for the diagnosis of silent subacute or chronic inflammation in the testis.

Materials and Methods

High resolution 2D-SDS-PAGE was used to separate human testicular protein extracts. Subsequently auto-antigens were identified by Western blotting using a set of serum samples from patients with chronic testicular inflammation confirmed by testicular biopsy. Candidate protein spots were excised and characterized by MALDI-ToF. Highly purified recombinant human (rh) ER-60 (recognized by 92% of sera) was selected and used for an ELISA development. The DRG ER 60 Autoantibody ELISA Kit is a solid phase ELISA based on the direct principle. The 96-well microtiterplate with breakable strips is coated with rhER-60 protein. Diluted patient sample containing endogenous ER-60 auto-antibodies is incubated in the well with additional assay buffer. After incubation the unbound material is washed off. In the second incubation step a complex is formed between ER-60 autoantibody and polyclonal anti-human IgG peroxidase conjugate. The intensity of colour development after addition of the TMB/H_2O_2 substrate is proportional to the concentration of anti-ER-60 in the patient sample. The principal of the assay and assay protocol are shown in **Figure 1 and 2**.

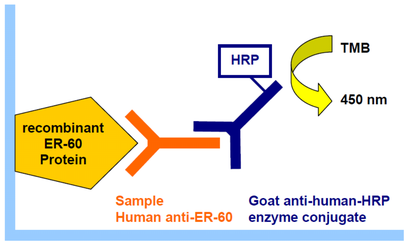

Figure 1. Assay design of the DRG ER 60 Autoantibody ELISA.

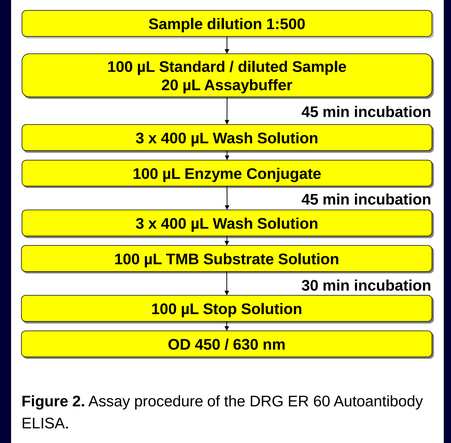

Figure 2. Assay procedure of the DRG ER 60 Autoantibody ELISA.

Results

By proteomic approach using 2D-SDSPAGE and immunoblotting followed by mass spectrometry, we have identified three proteins (ER-60, transferrin, TCP-1) as immunodominant antigens recognized by auto-antibodies in sera from patients with testicular inflammation (**Figure 3, Table I**). Of note, ER-60 was also identified as an auto-antigen in a rodent model of experimental testicular inflammation (2). In the testis ER-60 is localized in the developing germ cells, spermatozoa as well as in Sertoli and Leydig cells (3).

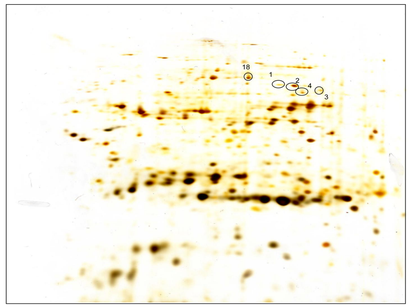

Figure 3. Identification of auto-antigens in male testicular chronic inflammation by 2D gel electrophoresis; Silver-stained 2D gel of human testicular proteins; numbered protein spots were recognized by auto-antibodies from patient sera and identified by mass spectrometry (**Table I**).

protein spot	reactive sera/total number of tested sera (% reactivity)	identified protein (names, synonyms)	Protein ID (PubMed)
1, 2	12/13 (92%)	**Disulfide isomerase ER-60 (Syn. Erp60, ERp57)**	P30101
3, 4	8/13 (61%)	Transferrin (Siderophilin)	P02787
18	6/13 (46%)	T-complex Protein 1 subunit epsilon (TCP-1-epsilon)	P48643

Table I. Human testicular auto-antigens identified by MALDI-ToF mass spectrometry.

The new developed DRG ER 60 Autoantibody ELISA is processed in 3 incubation steps resulting in a total incubation time of 2 hours. The exact assay procedure and design of the assay is shown in **Figure 1 and 2**. The assay characteristics are summarized in **Table II** and the typical standard curve is shown in **Figure 4**. High-Dose-Hook Effect was not observed in this test up to 320 U/mL. Hemoglobin (up to 4 mg/mL), Bilirubin (up to 0.5 mg/mL) and Triglyceride (up to 30 mg/mL) had no influence on the assay results.

Sample Material	1:500 dilution of Serum or Plasma, 100 µL diluted sample per well	
Incubation Times	45 min + 45 min + 30 min	
Total Assay Time	approx. 2.5 hours	
Standard Range	0 – 5 U/mL	
Evaluation	**Sample**	**Result**
Analytical Sensitivity	20 x Standard 0	0.02 U/mL
Recovery	1.0 U/mL	102.4 %
	2.0 U/mL	102.0 %
	3.0 U/mL	101.5 %
Linearity of Dilution	1.1 U/mL	105.7 %
	2.2 U/mL	95.5 %
	2.3 U/mL	99.5 %
Intra Assay	0.8 U/mL	5.6 %
	1.8 U/mL	4.1 %
	3.2 U/mL	4.0 %

Table II. Assay characteristics of the DRG ER 60 Autoantibody ELISA.

The dynamic range of the assay is between 0.02 – 5.0 U/mL. The analytical sensitivity was calculated from the mean plus two standard deviations of twenty replicate analyses of the Zero Standard (S0) and was found to be < 0.02 U/mL. The intra-assay (within-run) variation of the ELISA was determined by 20 measurements of 3 control samples and was found to be 5.6 % for a serum with 0.75 U/mL, 4.1 % for a serum with 1.76 U/mL and 4.0 % for a serum with 3.24 U/mL. Recovery of spiked samples was determined by adding increasing amounts of the ER-60 autoantibody to three different sera containing different amounts of endogenous analyte. The percentage recoveries were determined by comparing expected and measured values of the samples. The recovery of dilution was evaluated by serial dilution of serum samples containing different amounts of ER-60 autoantibody with zero standard. The percentage recovery was calculated by comparing the expected and measured values for the analyte.

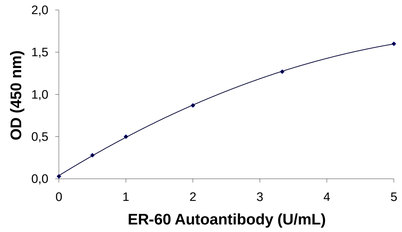

Figure 4. Standard Curve of the DRG ER 60 Autoantibody ELISA. The Standard concentrations are 0, 0.5, 1.0, 2.0, 3.3 and 5.0 U/mL.

Group	Description
1	healthy normozoospermic men (n=22)
2	male blood donors (n=14)
3	patients with impaired semen quality according to WHO reference values, without symptoms of genital tract infection/inflammation (n=16)
4	patients similar to group 3, but with symptoms of genital tract infection/inflammation (n=22)
5	patients with chronic testicular inflammation confirmed by testicular biopsy (n=20)
6	patients after pharmacotherapy of genital tract infection/inflammation (n=15)
7	patients with acute epididymo-orchitis (n=31)

Table III. Classification of patient population used for the development of DRG ER 60 Autoantibody ELISA.

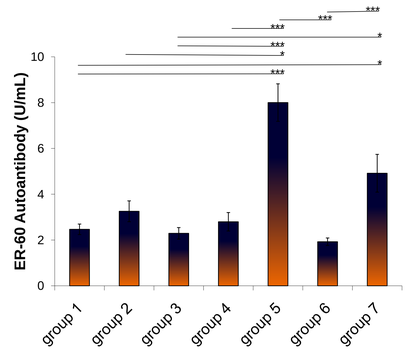

Figure 5. ELISA immunoreactivities of control and patient sera (**Table III**) with ER-60 antigen. Each serum was tested in duplicate; (*p<0.05, ***p<0.001).

Significantly elevated titers of auto-antibodies against ER-60 were found in the sera from infertile men with chronic testicular inflammation confirmed by testicular biopsies (group 5; p<0.001) and patients suffering acute epididymo-orchitis (group 7; p<0.05) as compared to healthy normozoospermic man (group 1) and male blood donors with unknown fertility status (group 2). Compared to group 5 and 7 significantly lower levels of anti-ER-60 antibodies were measured in the sera from patients after the use of anti-inflammatory pharmacotherapy (group 6, p<0.001). Important to note that sera from other investigated groups such as patients with impaired spermatogenesis (group 3) or patients with symptoms of genital tract infection/inflammations (group 4) did not significantly differ from those of the control groups (**Figure 5**).

Conclusion

Our results show that the determination of ER-60 auto-antibodies in male serum is a promising marker for the diagnosis of asymptomatic inflammatory processes in the testis causing male fertility disturbances. The determination of ER-60 antibodies in the serum by ELISA might offer new non-invasive possibilities in the classification of silent testicular inflammation. A multi-center clinical trial will be performed for validation.

References

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