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Male fertility and varicocoele: role of immune factors

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SUMMARY

The role of antisperm antibodies (ASA) in the aetiopathogenesis of varicocoele-related male infertility remains unclear. The objective of this study was to determine whether varicocoele is associated with antisperm immune response and whether this factor provides additional affect on male fertility. We performed a multicentral, prospective study that included the clinical examination of 1639 male subjects from infertile couples and 90 fertile men, the evaluation of the absolute and relative risks of immune infertility associated with varicocoele and the impact of the autoimmune response on the semen quality. The methods used were as follows: standard examination of seminal fluid according to WHO criteria; ASA detection in seminal fluid using mixed antiglobulin reaction (MAR) and direct flow cytometry; measurement of spontaneous and ionophore-induced acrosome reactions; oxidative stress evaluation with luminal-dependent chemiluminescence method and evaluation of DNA fragmentation by sperm chromatin dispersion. The prevalence of varicocoele-related immune infertility is about 15% and does not depend on the grade of vein dilatation both in primary and secondary fertility disorders. Varicocoele is not an immediate cause of autoimmune reactions against spermatozoa, but is a cofactor increasing ASA risk; the OR of immune infertility after a testicular trauma in varicocoele patients increases twofold. In varicocoele patients, the autoimmune antisperm reaction is accompanied by a more significant decrease in the semen quality (concentration and number of progressively motile and morphologically normal spermatozoa in the ejaculate), acrosome reaction disorders (presence of pre-term spontaneous and lack of induced reactions) and an increase in the proportion of spermatozoa with DNA fragmentation. These disorders correlate with the level of sperm oxidative stress; reactive oxygen species (ROS) production in ASA-positive varicocoele patients is 2.8 and 3.5 times higher than in ASA-negative varicocoele patients and fertile men respectively. We did not find correlation between the grade of spermatic cord vein dilatation and ROS production.

INTRODUCTION

Varicocoele is associated with one of four cases of abnormal spermogram parameters and is found in every tenth fertile man (Vital & Health Statistics, 2009; Esteves, 2012; Jungwirth *et al.*, 2013). To date, the pathophysiological mechanisms of male infertility associated with varicocoele remain unclear (Nagler & Grotas, 2009; Weinbauer *et al.*, 2010; Eisenberg & Lipshultz, 2011). Over the recent years, an opinion has emerged that varicocoele is not a single cause, but a cofactor along with other genetic and molecular factors resulting in infertility (Marmar, 2001; Niesclag *et al.*, 2010; Eisenberg & Lipshultz, 2011).

One of the potential pathogenetic factors that adversely affect infertility in varicocoele patients is antisperm immune response (Marmar, 2001; Marconi & Weidner, 2009; Will *et al.*, 2011). The presence of sperm-bound immunoglobulins can be

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associated with a small but significant decrease in both sperm concentration and motility; sperm-bound immunoglobulins are present in a greater percentage of infertile men with varicocoele than infertile men without varicocoele (Gilbert *et al.*, 1989). Many authors highlighted the bilateral character of morphological and functional testicular damage in unilateral varicocoele that suggested the autoimmune mechanism of this disorder (Sizyakin, 1996; Libman *et al.*, 2006; Benoff *et al.*, 2009). Walsh & Turek (2009) consider the hyperthermia a possible cause of antisperm antibodies (ASA) production in varicocoele patients. However, many studies rule out the association between varicocoele- and immune-related infertility (Oshinsky *et al.*, 1993; Heidenreich *et al.*, 1994; Aşci *et al.*, 1998; Gubin *et al.*, 1998; Veräjänkorva *et al.*, 2003; Marconi & Weidner, 2009).

The objective of this study was to determine whether and how varicocoele is associated with antisperm immune response.

MATERIALS AND METHODS

Study population

In this multicentral prospective study, 1639 male patients from infertile couples were examined according to the WHO recommendations (2000), including 599 subjects with ASA. The ASA levels were assessed with a mixed antiglobulin reaction (Sperm MAR test). The study procedures were approved by the Institutional Review Board; the written informed consent was obtained from all subjects.

The inclusion criteria for the study group with ASA were as follows: the duration of involuntary infertility for at least 12 months, a regular sexual life not less than once a week without using a contraception, the age of the female partner under 35 and MAR-IgG > 10% (more than 10% of sperm cells coated with IgG).

The exclusion criteria were as follows: evident causes of the female infertility (amenorrhoea, anovulation and bilateral tubal occlusion), ejaculation or sexual disorders that interrupt semen penetration into vagina, the infectious inflammatory processes of ancillary genital glands (leucocytes count more than 1 million/mL) in male subjects, the reproductive tract infections and marked oligozoospermia (sperm count less than 5 million/mL). The aim of such a selection was to increase the sensibility of the direct MAR test and to exclude the cases of the genetic hypogonadism.

We identified different groups of the study subjects according to:

- the presence and grade of the spermatic cord vein dilatation in the fertile men (n = 90) and in patients from the infertile couples with primary (n = 958) and secondary (n = 681) infertility, to determine the absolute risk and the degree of antisperm autoimmune response with regards to the varicocoele;
- the diagnosis of immune infertility according to WHO criteria – the subjects with MAR-IgG ≥ 50% (n = 267) and the men with the sperm pathology without ASA (n = 916), to evaluate the relative immune infertility risk associated with the varicocoele.

Ninety fertile and healthy men aged 22–51, who underwent complex clinical laboratory examination, were enroled for the control group. The inclusion criteria for the control group were established as a spontaneous pregnancy at 8–16 weeks of gestation in their spouses.

Methods used

The varicocoele was diagnosed using the standard criteria (Jungwirth *et al.*, 2013). Based on the physical examination, the grade of the spermatic cord vein dilatation was evaluated (0 – no dilation, 1+, 2+ and 3+). The backflow in the veins of spermatic cord and in pampiniform plexus was confirmed by the ultrasound examination. The subclinical forms of the disorder, when vein dilatation was not palpable or visible at rest or during Valsalva manoeuver were diagnosed by a Doppler ultrasound test. The tests were performed using LOGIQ-5 and LOGIQ-9 (GE, Milwaukee, WI, USA) and Flex Focus 1202 (B-K Medical, Herlev, Denmark).

The sperm evaluation was performed according to the WHO requirements (WHO, 2010). The IgG-ASA- and IgA-ASA-coated motile sperm counts were evaluated with Sperm MAR test (Ferti Pro N.V., Beernem, Belgium). The percentage of viable ASA-positive spermatozoa was evaluated with direct flow cytometry assay (FACScan Becton Dickinson Immunocytometry Systems, Mountain View, CA, USA) (Nikolaeva *et al.*, 1993). Immune infertility was diagnosed as MAR-IgG ≥ 50% (WHO, 2010).

The oxidative stress was evaluated by the estimation of free radical processes intensity with the luminol-dependent chemiluminescence method (luminometer; LKB-Wallac 1256, Turku, Finland). The chemiluminescence intensity was estimated according to the light sum and maximal emission amplitude which corresponded to the rate of reactive oxygen species (ROS) generation (Agarwal & Deepinder, 2009).

The spontaneous and ionophore A23187-induced acrosome reaction (AR) was assessed with double fluorescent staining of spermatozoa using fluorescent-labelled lectin from *Pisum sativum* (Sigma, St. Louis, MO, USA) and rhodamine-labelled lectin from *Arachis hypogaea* (Sigma) (Nikolaeva *et al.*, 1998). The reference level was the spontaneous AR in less than 15% of spermatozoa and the induced AR in not less than 15% of spermatozoa (WHO, 2010).

The chromosome damage was determined as the DNA fragmentation level using the method of sperm chromatin dispersion (Halosperm; Halotech DNA, Madrid, Spain) in inert agarose gel with the visual microscopic halation evaluation after the DNA denaturation and the nuclear protein lysis. The percentage of spermatozoa with apoptosis traits and halation defect rate was evaluated. In compliance with the test system manufacturer requirements, the reference level of sperm DNA fragmentation index was defined as less than 20% (Fernández *et al.*, 2005).

Statistical analysis

The data were processed with Statistica software package (StatSoft, Tulsa, OK, USA). Median, mean (M) and standard deviation (SD) were calculated; the differences' significance was assessed according with Student's t-test, Mann–Whitney test, Wilcoxon test, chi-squared test and signed rank test. Also, the correlation analysis was performed (R-Spearman and gamma coefficients were calculated). The partial correlation analysis was carried out using the covariate-adjusted generalized linear models. The effects of between-group factors were evaluated with ANOVA and logistic regression, the step-wise model analysis

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was based on Chi-squared test, Cox and Snell R^2 tests. The results were input into a logit regression equation to calculate the probability of the event. The logit regression equation was constructed using backward variable elimination until the most reliable regression model was found. We used various data imputation techniques, and the model significance was characterized using Wald criterion.

RESULTS

In the group of fertile men, the MAR-IgG levels were as follows: median 0%, 25–75% (0; 5); non-outlier range (0–12). In 5% of cases, more than a half of the motile spermatozoa were covered with ASA (MAR-IgG ≥ 50%) and all these cases were 'outliers' (>3S). We found no varicocoele-related differences (Table 1): MAR-IgG ≥ 50% was detected in 4% (3 of 68) of men without varicocoele, in 6% (1 of 16) of men with non-expressed forms (subclinical and 1+) and in 0% (0 of 6) of men with grade 2 varicocoele ($p > 0.05$); mean MAR-IgG% levels were the same ($p > 0.05$).

The men from infertile couples had MAR-IgG ≥ 50% levels three times more frequently than men from fertile couples ($p < 0.05$). No correlation between ASA prevalence and presence of varicocoele was found: MAR-IgG ≥ 50% was detected in 14.4% of patients with varicocoele and 15.8% of patients without varicocoele ($p > 0.05$). Both subgroups had similar mean MAR-IgG% levels (Table 1; $p > 0.05$). We did not find significant correlation between MAR-IgG% and MAR-IgA% and varicocoele grade ($\gamma = -0.15$ and 0.08; $p > 0.05$). However, the direct flow cytometry revealed positive dependence between varicocoele grade (0 through 3+) and the percentage of all viable both motile and non-motile spermatozoa covered with IgA ($\gamma = 0.23$; $p = 0.001$), which was more expressed in primary infertility ($\gamma = 0.31$; $p = 0.0005$).

The ASA-positive varicocoele patients had more expressed sperm functional disorder. The varicocoele patients with autoimmune reactions against spermatozoa demonstrated worse standard spermogram results than the varicocoele patients from the infertile couples without ASA. In the ASA-positive varicocoele patients, we revealed stronger reverse correlation between varicocoele grade and sperm concentration as well as direct correlation between the proportion of abnormal spermatozoa and estimated number of spermatozoa with the motility and normal morphology progressively in the ejaculate (Table 2; $p = 0.0002$ and 0.015 respectively). For example, the ASA-positive patients with 2+ grade varicocoele had 2.25 times less progressively motile spermatozoa (median 15.9 million/ejaculate; 25–75% quartiles = (5.8; 44.9)) than the ASA-negative patients with 2+ grade varicocoele (median 35.7 million/ejaculate; 25–75 quartiles = (8.6; 81.3); $p < 0.01$).

The premature (spontaneous) and lack of induced AR (Fig. 1) were 1.78 times more frequent in the immune infertility patients than in the fertile men ($p < 0.05$). The AR was normal only in 40% of the immune infertility patients.

Antisperm immune response was associated with the increased proportion of gametes with the chromosome structure defects. There was a direct correlation between MAR-IgG% and DNA fragmentation rate: $R = 0.48$ ($p = 0.003$) for the percentage of spermatozoa with DNA fragmentation (Fig. 2A). The DNA

Table 2 The correlation between grade of varicocoele and semen parameters

Parameter	Without antisperm antibodies MAR-IgG = 0% (n = 1130)		With antisperm antibodies MAR-IgG > 10% (n = 599)		p-value Gamma 1–2
	1		2		
	γ	p-value	γ	p-value	
Volume, mL	−0.03	ND	0.06	ND	ND
Concentration, 10^6 per mL	−0.13	0.000005	−0.15	0.0005	ND
Progressive motility (%)	0.04	ND	−0.005	ND	ND
Abnormal forms (%)	0.02	ND	0.20	0.000003	0.0002
Total number of spermatozoa with normal morphology and progressive motility, 10^6 per ejaculate	−0.04	ND	−0.16	0.00009	0.015

ND, no difference.

Table 1 Spermogram of fertile men and the patients from infertile couples with and without varicocoele

Indicator	Fertile men n = 90			Primary infertility patients n = 958			Secondary infertility patients n = 681		
Groups	1	2	3	4	5	6	7	8	9
Left-side varicocoele, grade	0	1+	2+	0	1+	2–3+	0	1+	2–3+
Number of patients, n (%)	68 (75)	16 (18)	6 (7)	659 (69)	233 (24)	66 (7)	468 (69)	159 (23)	54 (8)
MAR-IgG%, M ± S (Min–Max)	8.8 ± 21.2 (0–100)	8.8 ± 15.2 (0–54)	10.7 ± 14.9 (0–36)	19.3 ± 33.2 (0–100)	20.0 ± 34.1 (0–100)	11.8 ± 26.5 (0–100)	17.1 ± 30.8 (0–100)	18.1 ± 29.8 (0–100)	10.6 ± 23.4 (0–100)
MAR-IgG%, Median (25–75%), Non-outlier range	0 (0–5) 0–12	0 (0–15) 0–24	3,5 (0–21) 0–36	2 (0–18) 0–45	1 (0–23) 0–55	0 (0–5) 0–11	1 (0–13) 0–32	2 (0–20) 0–50	0 (0–5) 0–12
MAR-IgG ≥ 50%, n (%)	3 of 68 (4)	1 of 16 (6)	0 of 6 (0)	115 of 659 (17)[1–4]	45 of 233 (19)	6 of 66 (9)	68 of 468 (15)	30 of 159 (19)	3 of 54 (6)[8–9]

The patients with infectious and inflammatory processes of ancillary genital glands and marked oligozoospermia (sperm count less than 5 million/mL) were excluded from the study. [1–4, 8–9 and others] – the differences between the table groups are significant according to Chi-squared test with $p < 0.05$.

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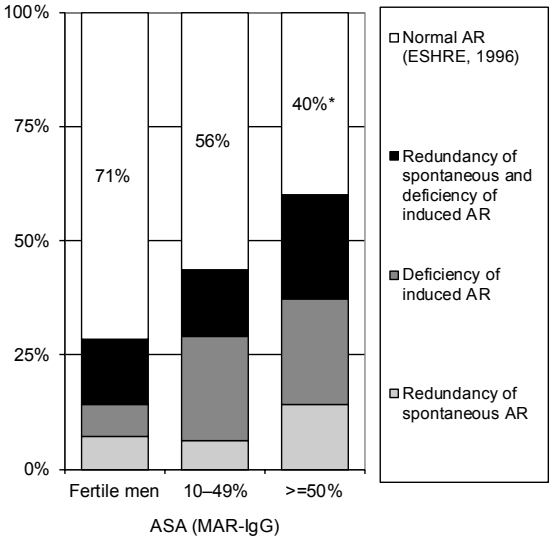
Figure 1 Acrosome reaction (AR) in fertile men (n = 22) and patients from infertile couples with varicocoele and various intensity of autoimmune activity (n = 48 and 35 respectively). *Note.* *The difference from the controls is statistically significant ($p < 0.05$); sperm count is ≥5 million/mL; the patients with infections and inflammation (including pyospermia) were excluded.



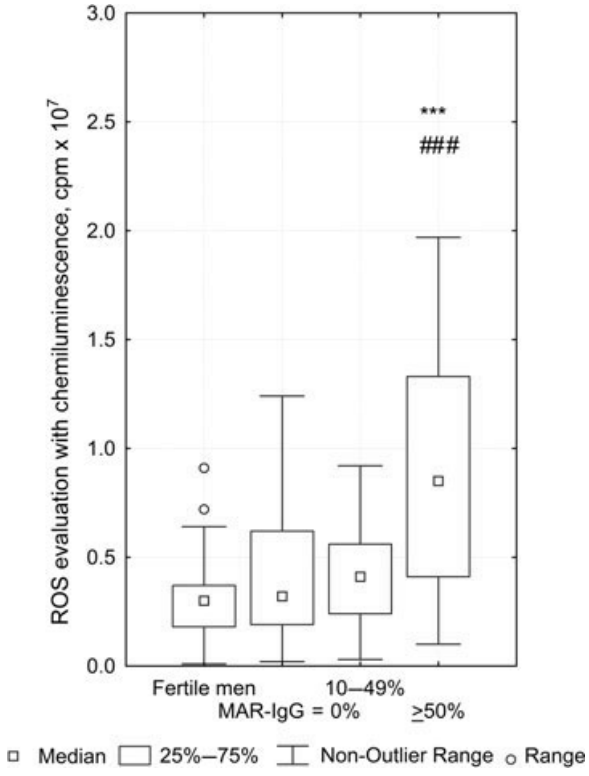
fragmentation analysis in all the varicocoele patients (Fig. 2B) revealed that the elevated sperm ROS production is accompanied by the higher percentage of spermatozoa with the DNA fragmentation (R = 0.38; p = 0.039).

The average ROS production in the immune infertility patients (Fig. 3) was 2.8 times higher than in the ASA-negative patients ($p < 0.001$) and 3.5 times higher than in the fertile men with varicocoele ($p < 0.001$). No correlation was observed between ROS production and the grade of the spermatic cord vein dilatation ($p > 0.05$).

To determine whether the varicocoele is an immediate cause of the immune infertility, we evaluated the prevalence, the absolute risk and the odds ratio (OR) of ASA production associated with varicocoele.

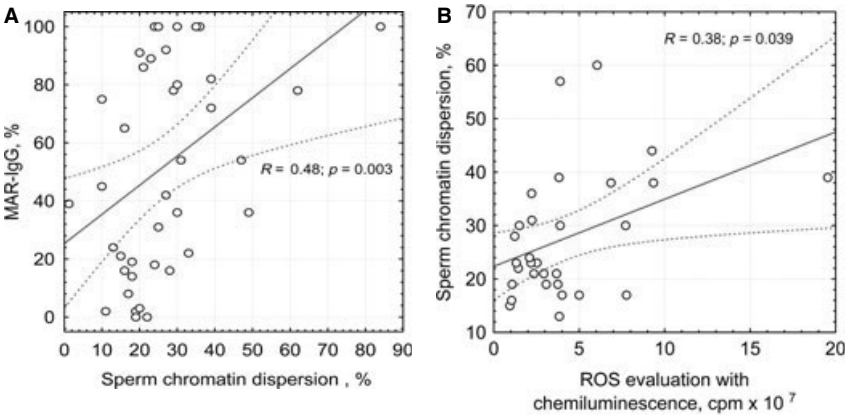
Our findings showed that the varicocoele-related immune infertility was associated with 17% of primary and 15% of

Figure 3 Reactive oxygen species (ROS) production in semen of fertile men and patients from couples with primary and secondary infertility and varicocoele with various ASA sperm counts. *Note.* ROS, reactive oxygen species; sperm count is ≥ 5 million/mL; the patients with infections and inflammation (including pyospermia) were excluded; ROS counts higher ±3S are excluded. The vertical axis: fertile controls (n = 52), infertile men with left-side varicocoele without ASA (n = 145), infertile men with left-side varicocoele with moderate ASA levels (n = 54) and infertile men with varicocoele and immune infertility according to WHO criteria (n = 31). ***The differences from the controls are significant according to the Mann–Whitney U-test ($p < 0.001$); ###The differences between varicocoele groups are significant ($p < 0.001$).



secondary reproductive disorders (Table 1; $p > 0.05$). Moreover, it was often accompanied by other risk factors of antisperm immune response, such as orchitis, epididymis, subclinical

Figure 2 The correlation between the number of motile spermatozoa coated with ASA (MAR-IgG) and (A) the percentage of spermatozoa with DNA fragmentation assessed by sperm chromatin dispersion, (B) the reactive oxygen species (ROS) production in semen. *Note. R*, Spearman rank correlation coefficient; for higher accuracy of MAR% and DNA fragmentation detection, the sperm concentration was not less than 10 million/mL; percentage of progressively motile spermatozoa was more than 10%; dotted line – 95% confidence interval.



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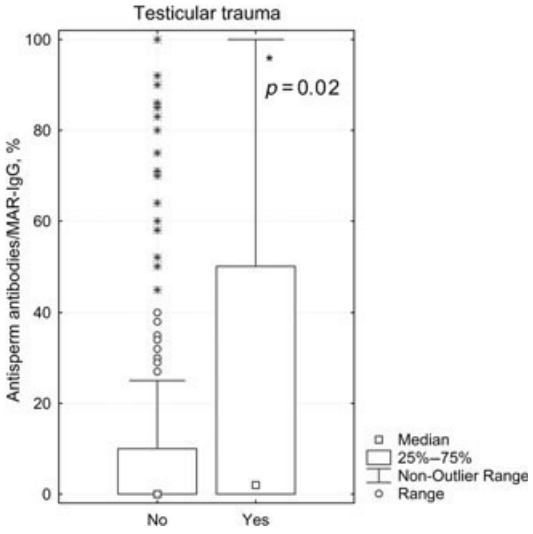
testicular trauma, past medical history of Chlamydia and other reproductive tract infections, unilateral epididymal obstruction (Table 3). After eliminating such cases from the analysis, the absolute risk of varicocoele-related immune infertility reached 9.5%. On the contrary, the varicocoele prevalence was similar in the immune infertility group and the ASA-negative patients (Table 2): 32 and 31% respectively (OR = 1.0, $p > 0.05$).

We hypothesized that the association between varicocoele and antisperm immune response depends on other factors, or covariates. We compared the results of 2 one-way ANOVA tests of the 'left-side varicocoele grade' factor impact on 'MAR-IgG%' and found that one of the covariates significantly affects the dependent variable: the history of testicular trauma accounted for 4.2% of the variance of 'MAR-IgG%' variable ($F = 9.54$; $p = 0.02$). It was confirmed that the 'left-side varicocoele grade' alone did not significantly affect the variance of 'MAR-IgG%' variable ($F = 2.15$; $p = 0.091$) showing decline in the confidence level ($p = 0.088$ and 0.091 respectively), however, the direct correlation between the MAR-IgG test results and the testicular traumas was highly significant ($\gamma = 0.27$; $p < 0.000001$; $n = 1639$). The fertile men had a similar relationship ($\gamma = 0.21$; $p > 0.05$; $n = 80$). 35% of the patients with the primary infertility (MAR-IgG ≥ 50%) had the testicular traumas in the medical history (Table 2) that was twice as frequent compared with the ASA-negative patients ($p < 0.001$) and more than three times higher than in the fertile men ($p < 0.01$). The varicocoele patients with testicular traumas developed the immune infertility 1.9 times more frequently than the patients without varicocoele: in 25.2% (32 of 127) and 13.3% (49 of 367) of cases respectively ($p < 0.01$). The patients with varicocoele and testicular traumas had higher levels of MAR-IgG (Fig. 4): without traumas – mean 15%, median 0%, 25–75% (0; 10); with traumas – mean 24%, median 3%, 25–75% (0; 50), ($p = 0.02$).

The simple ANOVA of all evaluated factors showed that only 'orchitis' factor had a significant effect on the 'MAR-IgG' dependent variable ($F = 3.39$; $p = 0.036$). The medical history of orchitis in the immune infertility patients was 3.7 times more frequent in the primary ($p < 0.001$) and three times higher in the secondary disorders, whereas it was not found in the fertile men (Table 2). There was a significant relationship between the variables 'orchitis' and 'MAR-IgG' in patients both with and without the varicocoele, $\gamma = 0.38$ ($p = 0.0005$; $n = 496$) and 0.33 ($p = 0.000005$; $n = 1109$) respectively. The risk of the orchitis after the testicular trauma is 1.4-fold higher in the varicocoele patients ($p < 0.05$).

Figure 4 The rate of ASA-coated spermatozoa in varicocoele patients from infertile couples with ($n = 127$) and without history of testicular trauma ($n = 370$). *Note.* *The differences from initial parameters are statistically significant according to the Mann–Whitney *U*-test ($p = 0.02$); the patients with azoospermia, infections and inflammation (including pyospermia) were excluded.



DISCUSSION

Given that some of the varicocoele patients remain fertile, whereas the others have a decreased semen quality, the additional genetic or acquired risk factors may contribute to the male infertility related to varicocoele. Many factors are important for understanding the varicocoele pathogenesis: hyperthermia, venous hypertension, hormonal effects (hypoandrogenism), toxic substances exposure (catecholamines, smoking), excessive ROS generation, microdeletion of some mitochondrial genes, the deficiency of heat shock proteins, caspases, poly-ACP-ribose polymerase, metastasis-associated protein 1, Bak, p53 as well as some other oxidative stress

Table 3 Conditions linked with antisperm antibodies (without congenital, inflammation and/or infection of the male reproductive tract, vasectomy and iatrogenic obstruction)

Indicator	Fertile men			Primary infertility patients			Secondary infertility patients		
Groups	1	2	3	4	5	6	7	8	9
	MAR-IgG, %								
	0%	11–49%	≥50%	0%	11–49%	≥50%	0%	11–49%	≥50%
Number of patients, *n*	36	10	4	481	325	158	318	248	95
Patients underwent varicocelectomy, *n* (%)	1 (3)	1 (10)	1 (25)	49 (10)	30 (9)	13 (8)	26 (8)	15 (6)	5 (5)
Clinical varicocoele, *n* (%)									
Left side	8 (22)	4 (40)	1 (25)	150 (31)	95 (29)	51 (32)	98 (31)	77 (31)	31 (33)
Right side	4 (11)	0 (0)	0 (0)	38 (8)	26 (8)	9 (6)	21 (7)	17 (7)	8 (8)
Cryptorchidism, *n* (%)	0 (0)	1 (10)	0 (0)	9 (3)	5 (2)	0 (0)	1 (1)	1 (1)	2 (2)
Testicular trauma, *n* (%)	4 (11)	3 (30)	0 (0)	89 (18)$^{4–6}$	87 (27)$^{5–4,\ 8–5}$	55 (35)$^{6–4}$	47 (15)	48 (19)$^{8–5}$	22 (23)
Orchitis, *n* (%)	0 (0)	0 (0)	0 (0)	14 (3)$^{4–6}$	12 (4)$^{5–6}$	18 (11)$^{6–4}$	10 (3)$^{7–9}$	9 (4)	9 (9)$^{9–7}$
Autoimmune diseases, *n* (%)	1 (3)	0 (0)	0 (0)	37 (8)	16 (5)	15 (10)	17 (5)	15 (6)	9 (10)

The patients with infectious and inflammatory processes of ancillary genital glands and marked oligozoospermia (sperm count less than 5 million/mL) were excluded from the study. $^{4–6,\ 7–9\ \text{and others}}$ – the differences between the table groups are significant according to Chi-squared test with $p < 0.05$.

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antagonists and factors that impact the proliferation–apoptosis balance (Marmar, 2001; Benoff *et al.*, 2009; Chang *et al.*, 2010; Eisenberg & Lipshultz, 2011; Esteves, 2012; Guo *et al.*, 2012; Gashti *et al.*, 2013 and others).

Our research goal was to answer the question whether the elevated ASA production leads to infertility in varicocoele patients or, vice versa, whether varicocoele causes male immune infertility. The current available literature data are contradictory (Veräjänkorva *et al.*, 2003; Benoff *et al.*, 2009; Marconi & Weidner, 2009; Walsh & Turek, 2009; Al-Daghistani *et al.*, 2010; Restrepo & Cardona Maya, 2013).

Naturally produced ASA can affect male fertility by various mechanisms. Some of them are mainly related to the extent of the sperm autoimmunization (e.g. sperm agglutination and impaired cervical mucus penetration); others are also related to immunoglobulin isotype (e.g. complement-mediated sperm injury through the female genital tract), or to antigenic specificity of ASA (e.g. interference with gametes interaction) (Bohring & Krause, 2003; Chiu & Chamley, 2004; Francavilla & Barbonetti, 2009; Restrepo & Cardona Maya, 2013).

We performed a multivariate analysis of the clinical and laboratory data of 1729 men of reproductive age and found that the varicocoele was not an immediate cause of antisperm immune response.

Our findings suggest that the antisperm immune response in varicocoele patients is associated with the decrease in quantitative parameters of a standard spermogram and the deterioration of the sperm functional properties such as AR disorder (both spontaneous and induced) and DNA fragmentation. The primary effect of autoimmune reactions on the sperm quality is a decrease in motility, agglutination and AR disorders (Bohring & Krause, 2003; Francavilla & Barbonetti, 2009; Krause, 2009). We found that ASA-positive varicocoele patients demonstrated a more significant decrease in the semen quality (concentration, total number of progressively motile spermatozoa) which correlates with the grade of spermatic cord veins dilatation. The essential finding is the correlation between ASA and DNA fragmentation in spermatozoa. We agree with Zini *et al.* (2010) that ASA is not the immediate cause of DNA fragmentation. We think that this correlation reflects not the chromosome damage by the antibodies, but the interaction between ASA and already damaged spermatozoa with DNA fragmentation. Some of these spermatozoa with DNA damage may have undergone 'abortive apoptosis' in which they started but subsequently escaped the apoptotic pathway (El-Fakahany & Sakkas, 2011). The presence of DNA damage and apoptotic proteins in ejaculated spermatozoa may be linked to defects in cytoplasmic remodelling during the later stages of spermatogenesis. ASA binding to the inactive form of caspase 3 as a cognate antigen was demonstrated (Bohring *et al.*, 2001). The pathophysiologic significance of these ASA is still unclear (Bohring & Krause, 2003). In this situation, the antibodies may be produced against spermatozoa antigens following the decrease in tolerance resulting from their modification after the damage. The pathogenetic mechanism of such damage may involve ROS, the production of which was increased in fertile and infertile men with ASA. We described ROS production increase in ASA-positive patients several years ago (Korotkova *et al.*, 2001; Bozhedomov *et al.*, 2009). The DNA fragmentation analysis revealed that the elevated spermatozoa ROS production was accompanied by the higher percentage of spermatozoa with the DNA fragmentation.

Altogether, this aligns with common concepts of immune reactions' role in infertility development. Many investigators reported the correlation between spermatozoa functional deterioration and ASA production: the ASA-positive patients demonstrated higher occurrence of spermatozoa agglutination and motility reduction (Bohring & Krause, 2003; Francavilla & Barbonetti, 2009) and a premature AR (Bozhedomov *et al.*, 2001; Bohring & Krause, 2003); Bohring *et al.* (2001) reported ASA binding with the functional proteins involved in apoptosis (caspase 3, HSP70).

The ASA were found with the similar frequency in infertile patients both with and without varicocoele, and varicocoele was not associated with significant differences in absolute and relative risks of immune infertility. These data confirm the existing opinion of doubtful aetiological role of the varicocoele in the immune infertility (Heidenreich *et al.*, 1994; Aşci *et al.*, 1998; Gubin *et al.*, 1998; Veräjänkorva *et al.*, 2003; Marconi & Weidner, 2009). At the same time, the varicocoele might be an important cofactor contributing to the risk of ASA production in presence of other damaging impacts. In particular, mechanical testicular traumas in past medical history of varicocoele patients were associated with significantly more frequent antisperm immune response (twofold) and orchitis (1.4-fold) compared with those in patients without varicocoele who experienced the testicular trauma. The direct association between ASA-positive sperm counts and testicular traumas was highly significant. Apparently, the antisperm immunity in such cases is triggered by the damage of the blood–testis barrier resulting in clinically manifested or subclinical orchitis (Bozhedomov & Teodorovich, 2005; Marconi & Weidner, 2009; Walsh & Turek, 2009). Our findings suggest that varicocoele is not an immediate cause of antisperm immune response, but it increases the probability of the immune infertility following the damaging impact on the testicles (e.g. testicular trauma).

We found correlation between varicocoele grade and ASA levels revealed by direct flow cytometry. Hjort (1999) stated that direct flow cytometry seems to be a promising technique which may be able to determine the exact amount of IgA and IgG on individual spermatozoa. However, this correlation is significant for IgA, but not for IgG. This may result from immediate ASA production by the testicles when dilatation of spermatic cord veins is expressed. ASA-IgA and ASA-IgG are considered to be of testicular and post-testicular origin respectively (Krause, 2009). It may result from testicular traumas as we mentioned earlier in the study.

The characteristics of ASA target antigens have been actively discussed recently (Bohring & Krause, 2003; Krause, 2009; Nagler & Grotas, 2009; Walsh & Turek, 2009); in particular, in the aspect of contraceptive vaccine development (Naz, 2011). Nevertheless, the causes of immune responses against the host sperm antigens remain unclear. Neither does the pathogenesis of the immune infertility: whether a fertility decrease is caused by the antisperm immune response, or, on the contrary, whether ASA production is triggered by the structural alterations in sperm functional proteins and by morphological and antigen pathozoospermia resulting in the immunological tolerance mechanism failure? This is the issue to be addressed in further investigations.

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CONCLUSIONS

The prevalence of varicocoele-related immune infertility is about 15% and does not depend on the grade of vein dilatation both in primary and secondary fertility disorders. In varicocoele patients, the autoimmune antisperm reaction is accompanied by a more significant decrease in the semen quality (concentration and number of progressively motile and morphologically normal spermatozoa in the ejaculate), AR disorders (presence of pre-term spontaneous and lack of induced reactions) and an increase in the proportion of spermatozoa with DNA fragmentation.

These disorders correlate with the level of sperm oxidative stress; ROS production in ASA-positive varicocoele patients is 2.8 and 3.5 times higher than in ASA-negative varicocoele patients and fertile men respectively. We did not find correlation between the grade of spermatic cord vein dilatation and ROS production.

Varicocoele is not an immediate cause of autoimmune reactions against spermatozoa, but is a cofactor increasing ASA risk; the OR of immune infertility after a testicular trauma in varicocoele patients increases twofold.

CONFLICT OF INTEREST

None declared.

AUTHORS' CONTRIBUTIONS

V.A.B. – conception and design of the study, acquisition of data, analysis and interpretation of data, critical review of article for important intellectual content and editing the article. N.A.L. – acquisition of data/laboratory tests. I.M.R. – acquisition and analysis of data. R.A.A. – acquisition and analysis of data. I.V.U. – acquisition of data/laboratory tests. G.T.S. – critical review of article and final approval of draft.

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