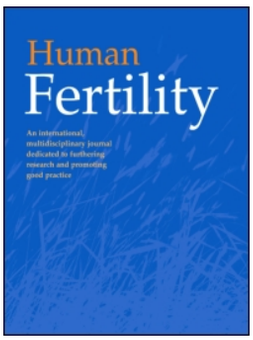

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ORIGINAL ARTICLE

Seminal inflammasome activity in the adult varicocele

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ABSTRACT

Varicocele has been hypothesized to lead to seminal inflammation, which in turn interferes with sperm function. Thus, the aim of this study was to investigate the role of inflammatory cytokines in the pathogenesis of decreased semen quality observed in adult men with varicocele, and to determine if varicocelectomy corrects these potential alterations. A prospective study was carried out including fifteen control men without varicocele and with normal semen quality and 15 men with varicocele with surgical indication. Men with varicocele grades II or III underwent microsurgical subinguinal varicocelectomy. Controls collected one semen sample and men with varicocele collected one before and one 6 months after the surgery. Semen analysis, sperm function, and seminal lipid peroxidation levels were assessed. Seminal plasma inflammasome activity was evaluated by ELISA assays for IL-1β, IL-18 and caspase-1 and by Western blotting for ASC (apoptosis-associated speck-like protein). Groups were compared by an unpaired Student's T test. Varicocelectomy samples were compared using a paired Student's T test ($\alpha = 5\%$). Men with varicocele had decreased semen quality, and increased seminal IL-1β levels, when compared to control men. Varicocelectomy decreased levels of caspase-1, IL-18, and IL1β. Thus, varicocelectomy improves sperm morphology and decreases seminal plasma inflammatory activity, after a six-month post-operative period.

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KEYWORDS
Cytokines; sperm DNA; varicocelectomy

Introduction

Varicocele is defined as an abnormal venous dilation of the pampiniform plexus with blood reflux (Brugh et al., 2003). Due to its prevalence of 15% in the general male adult population, and up to 80% in men with secondary infertility (Gorelick & Goldstein, 1993; Witt & Lipshultz, 1993), varicocele has been suggested to cause a progressive decline of fertility (Evers & Collins, 2003). Despite many hypotheses regarding the detrimental effect of varicocele on testicular function, inflammation is not usually studied in these men. Varicocele is a vascular disease with many hypotheses described in the literature that may explain its pathophysiology, such as blood reflux and the accumulation of adrenal metabolites in the spermatic vein (Masson & Brannigan, 2014). Metabolites present can be toxic to testicular function, leading to an inflammatory state and seminal oxidative stress (Benoff et al., 2009).

Many studies have described an inflammatory state in men with varicocele, by observing the presence of different cytokines in seminal plasma (Belardin et al., 2019; Dousset et al., 1997; Moretti et al., 2014; Nallella et al., 2004; Sahin et al., 2006), and also by unbiased proteomics techniques (Baazm et al., 2020; Camargo et al., 2013, 2019; Del Giudice et al., 2013; Zylbersztejn et al., 2013). In spermatic veins, inflammatory activity was observed due to the presence of high levels of prostaglandins E and F (Ito et al., 1982). In men with spinal cord injury (SCI), the presence of inflammatory cytokines, in seminal plasma, has been related to low semen quality. Furthermore, in these patients, inflammatory activity was associated with the inflammasome complex and with high levels of specific cytokines released in seminal plasma (Zhang et al., 2013).

Inflammasomes are macromolecular complexes that serve as platforms for the innate immune response (Guarda & So, 2010). This complex is formed by a cytosolic NLR-type receptor, ASC protein (caspase domain recruiter) and pro-caspase-1, which is activated by different pathways, leading to the activation of pannexin-1 and P2X7R receptors (Monie, 2013). The activated complex stimulates increased expression of inflammatory cytokines such as caspase-1, IL-1β and

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IL-18, responsible for tissue injury (Guarda & So, 2010; Monie, 2013). The cytokines are released into the extracellular space after their production (Matsushita et al., 2009).

Considering that varicocele occurs in a progressive manner (Witt & Lipshultz, 1993), understanding the role inflammatory cytokines play in determining infertility is important, as well as whether varicocelectomy – the main treatment for varicocele – is able to decrease this inflammatory profile. This is important especially because studies have demonstrated a strong relation between the inflammatory response, oxidative stress and sperm DNA fragmentation (Belardin et al., 2019; Camargo et al., 2019; Nallella et al., 2004; Ni et al., 2016; Sakamoto et al., 2008). Furthermore, there is a significant component of inflammatory pathways (i.e. the inflammasome complex), which is responsible for decreased sperm motility in patients with spinal cord injury (SCI) (Ibrahim et al., 2014; Zhang et al., 2013). Finally, varicocele has been associated with an inflammatory seminal plasma (Camargo et al., 2013; Del Giudice et al., 2016; Zylbersztejn et al., 2013).

Hence, because men with varicocele present with low sperm motility and inflammatory seminal plasma, we wished to investigate the role of inflammatory cytokines in the condition of varicocele, by determining if abnormal cytokine concentrations were corrected following varicocelectomy. Additionally, sperm functional aspects (i.e. sperm mitochondrial activity, DNA fragmentation and acrosome integrity) were determined in these patients.

Materials and methods

Patients and study group

Institutional Review Board approval was obtained from the Universidade Federal de São Paulo Research Ethics Committee (CAAE: 06001412.8.0000.5505). The patients included in the study were recruited from Human Reproduction Section of the Division of Urology of the Universidade Federal de São Paulo (UNIFESP). Thus, a prospective study was carried out with 15 normozoospermic men (according to 2010 WHO guidelines) without varicocele, and any other male factor, serving as controls (control group) and 15 patients with a palpable varicocele as the study group. In the study group, only patients with grade II or III and with a surgical indication were included. These patients were then treated for their varicoceles with subinguinal microsurgical varicocelectomy (Marmar et al., 1985). Both groups included only men between 20 and 50 years old.

Intervention

Physical examination was performed in a room with adequate illumination and controlled temperature (23 °C). Varicocele was diagnosed by scrotal palpation, according to Dubin & Amelar (1977). Men with varicoceles then underwent a microsurgical subinguinal varicocelectomy using a surgical microscope (Zeiss OPMI Vario S8.8).

Men in the control group provided one semen sample and patients from the study group provided two semen samples: one before varicocelectomy (pre-period) and one six months after varicocelectomy (6-months period). After liquefaction, all samples were analysed according to World Health Organization (2010) guidelines and the checklist proposed by Björndahl et al. (2016) was completed. On each sample, sperm functional analysis was performed (see below), and the remaining volume was centrifuged at 800 × G to remove cellular debris. Then, the samples were kept at −20 °C until inflammasome and oxidative stress analysis.

Sperm functional analysis

Sperm mitochondrial activity was evaluated by 3,3′-diaminobenzidine (DAB) sperm staining (Sigma-Aldrich, St. Louis, USA) as previously described (Blumer et al., 2008, 2012; Intasqui et al., 2016). This technique relies on DAB reaction with cytochrome C complex, after which it deposits in the sperm midpiece, thus marking active mitochondria (Hrudka, 1987). Sperm were classified in 4 different classes, according to Hrudka (1987) where Class I is 100% of active mitochondria, and Class IV is 100% of inactive mitochondria. Thus, an index of mitochondrial activity was calculated through the following formula: (2*Class I + Class II)/(Class III + 2* Class IV) (Camargo et al., 2019).

Sperm acrosome integrity analysis was performed with a peanut agglutinin conjugated with FITC, as previously described (Intasqui et al., 2016; Roth et al., 1998). In total, 200 sperm were analyzed and classified according to acrosome integrity.

Sperm nuclear DNA fragmentation was evaluated by Comet assay, as previously proposed by our group (Intasqui et al., 2013, 2016). In total, 100 sperm were analyzed using Komet 6.0.1 software (Andor Technology). The variable Comet Distributed Moment for each cell was used as an index of sperm DNA fragmentation (Kent et al., 1995). This variable was chosen because its distribution was closest to a normal (Gaussian) distribution. This variable calculates comet

length and its fluorescence intensity not differentiating the fluorescence from head to tail (Kent et al., 1995).

Seminal lipid peroxidation evaluation

Seminal plasma lipid peroxidation levels were assessed in each sample by measuring thiobarbituric acid reactive substances (TBARS) levels in seminal plasma, as previously described by (Intasqui et al., 2015). Lipid peroxidation data were presented as nmolTBARS/mL semen.

IL-1β, IL-18 and Caspase-1 analysis

Seminal levels of Caspase-1, IL-1β, and IL-18 were analyzed by ELISA assay according to the manufacturer's protocol (R&D Systems, Inc – USA) (Zhang et al., 2013). Seminal plasma samples were thawed at room temperature and then placed in microplates pre-coated with a monoclonal antibody specific for caspase-1, IL-1β, or IL-18. Each sample was analyzed in duplicate. Absorbance was measured at 492 nm in a Stacker II microplate spectrophotometer (BMG LabTech, Germany). Caspase-1, IL-1β, and IL-18 concentrations were calculated from a standard curve using linear regression analysis. Constructed standard curves ranged from 0 to 400 pg/mL for caspase-1, 0 to 125 pg/mL for IL-1β, and 0 to 1000 pg/mL for IL-18. Data were described as pg/mL semen.

ASC measure

Initially, total protein concentration from the samples was evaluated individually using the bicinchoninic acid (BCA) protein assay (Sigma, St Louis, MO, USA) (Smith et al., 1985). Each sample was measured in triplicate, and a standard curve was measured in duplicate. Then, a volume corresponding to 50 μg of seminal plasma proteins was used to verify ASC levels by Western blotting according to previously described (ASC antibody from Santa Cruz Biotechonology sc-271054, Texas, USA) (Belardin et al., 2019). Signals were detected using an ImageQuant LAS 4000 system (GE Healthcare, Amersham, UK). Images were processed using ImageQuant TL 7.0 software (GE Healthcare, Amersham, UK).

Statistical analysis

All statistical analyses were performed in SPSS 18.0. Initially, the data were submitted to a Kolmogorov–Smirnov normality test. Then, in order to verify the effect of varicocele, samples were compared using an unpaired Student's T-test; otherwise, samples were compared using a Mann–Whitney test. To verify the effect of varicocelectomy, samples were compared using a paired Student's T-test, or a Wilcoxon test, when appropriate.

For ASC protein analysis, the band volume was normalised for DJ-1 constitutive protein (Sigma Aldrich – SAB2500750) through the formula ASC/DJ-1 (Belardin et al., 2019). Also, two bands were observed – one at 31 and one at 24 kDa – so that a 31/24 kDa variable was created to study possible protein isoform turnover.

Results

Of the 15 men with a varicocele included in the study, 2 had bilateral grade III varicocele, 10 had bilateral grade II varicocele, 2 had left grade II, right grade I varicocele, and 1 had unilateral left grade II varicocele. Clinical data of the control and varicocele groups are presented in Table 1. No statistical difference was observed between the groups ($p > 0.05$).

Seminal and sperm functional analysis are presented in Table 2. Men with varicocele showed lower ejaculate volume ($p = 0.011$), progressive motility ($p = 0.010$), sperm concentration ($p = 0.00004$), sperm morphology ($p = 0.00003$). In addition, men with varicocele sherif lower sperm mitochondrial activity ($p = 0.002$) when compared to controls. Acrosome integrity, DNA fragmentation and lipid peroxidation levels did not differ between the groups ($p > 0.05$). Inflammatory data demonstrated higher IL-1β levels in men with a varicocele ($p = 0.006$), however, IL-18, caspase-1 and ASC levels were not significantly different (Figure 1), including ASC of 31/24 kDa, with a mean ± SD (arbitrary units, a.u.) of 0.6 ± 0.45 (control group), and 0.6 ± 0.44 in the varicocele group ($p = 0.819$).

Data regarding the effect of varicocelectomy are presented in Table 3. Varicocelectomy led to increased semen volume ($p = 0.027$) and improved sperm morphology ($p = 0.005$). Progressive motility and sperm concentration were not altered after the surgery ($p > 0.05$). Sperm functional analysis did not

Table 1. Clinical data of patients from control group (without varicocele) and varicocele group.

	Control group	Varicocele group	p
Age (years)	34.8 ± 3.10	33.6 ± 8.43	0.648
BMI (kg/m^2)	25.8 ± 3.01	23.9 ± 2.98	0.228
Left testicular volume (cm^3)	19.4 ± 3.90	17.4 ± 3.80	0.358
Right testicular volume (cm^3)	21.1 ± 3.77	18.0 ± 4.41	0.057

Data were presented as mean ± standard deviation.

demonstrate any difference following varicocelectomy, however, inflammasome cytokines caspase-1, IL-1β and IL-18 decreased after 6 months (Figure 1). ASC a.u. was not different before and after the surgery, including ASC a.u. of 31/24 kDa, which was 0.6 ± 0.44 before surgery and 0.5 ± 0.43 after surgery (mean ± SD). These results demonstrated a treatable inflammatory status in men with varicocele.

Table 2. Semen and functional analysis between control group and varicocele group.

	Control group	Varicocele group	*p*
Volume (mL)			
Mean ± SD	3.9 ± 1.45	2.5 ± 1.25	0.011*
CI 95%	3.1 – 4.7	1.8 – 3.2	
Progressive motility (%)			
Mean ± SD	54.3 ± 7.6	37.1 ± 22.54	0.010*
CI 95%	50.1 – 58.5	23.4 – 50.7	
Concentration (million/mL)			
Median; Interquartile	62.8; 35.75	13.1; 21.30	0.00004*¥
25-75	36.7 – 72.5	1.9 – 23.2	
Morphology (%)			
Mean ± SD	6.6 ± 2.44	2.5 ± 1.51	0.00003*
CI 95%	5.2 – 7.9	1.5 – 3.4	
Acrosome integrity (%)			
Mean ± SD	78.6 ± 6.61	76.1 ± 9.34	0.443
CI 95%	74.4 – 82.8	70.1 – 82.0	
Mitochondrial activity (a.u.)			
Mean ± SD	14.4 ± 8.69	4.9 ± 3.08	0.002*
CI 95%	8.5 – 20.2	2.9 – 6.8	
DNA fragmentation (a.u.)			
Mean ± SD	39.3 ± 11.69	43.6 ± 11.90	0.425
CI 95%	31.4 – 47.1	34.4 – 52.7	
Lipid peroxidation (nmol/mL)			
Mean ± SD	20.1 ± 4.59	21.6 ± 8.97	0.710
CI 95%	15.3 – 24.9	15.6 – 27.6	

Key: a.u.: arbitrary units; SD: standard deviation; CI: confidence interval; *significant difference; ¥non-parametric test.

Table 3. Seminal and functional analysis between pre- and post-varicocelectomy periods.

	Pre-period	6-Months period	*p*
Volume (mL)			
Mean ± SD	2.5 ± 1.25	3.3 ± 1.53	0.027*
CI 95%	1.8 – 3.2	2.4 – 4.1	
Progressive motility (%)			
Mean ± SD	37.1 ± 22.54	36.4 ± 20.85	0.854
CI 95%	23.4 – 50.7	23.8 – 49.0	
Concentration (million/mL)			
Mean ± SD	17.0 ± 20.62	20.3 ± 24.14	0.507
CI 95%	51.–29.8	5.7 – 34.9	
Morphology (%)			
Mean ± SD	2.5 ± 1.51	3.8 ± 1.94	0.005*
CI 95%	1.5 – 3.5	2.5 – 5.1	
Acrosome integrity (%)			
Mean ± SD	76.0 ± 9.34	70.6 ± 10.83	0.446
CI 95%	70.1 – 82.0	62.8 – 78.3	
Mitochondrial activity (a.u)			
Mean ± SD	4.9 ± 3.08	7.4 ± 4.19	0.099
CI 95%	2.9 – 6.8	4.6 – 10.2	
DNA fragmentation (a.u.)			
Mean ± SD	43.6 ± 11.90	31.3 ± 6.37	0.106
CI 95%	34.4 – 52.7	26.0 – 36.7	
Lipid peroxidation (ng/mL)			
Mean ± SD	21.6 ± 8.97	23.1 ± 7.90	0.355
CI 95%	15.6 – 27.6	16.4 – 29.6	

Key: a.u.: arbitrary units; SD: standard deviation; CI: confidence interval; *significant difference.

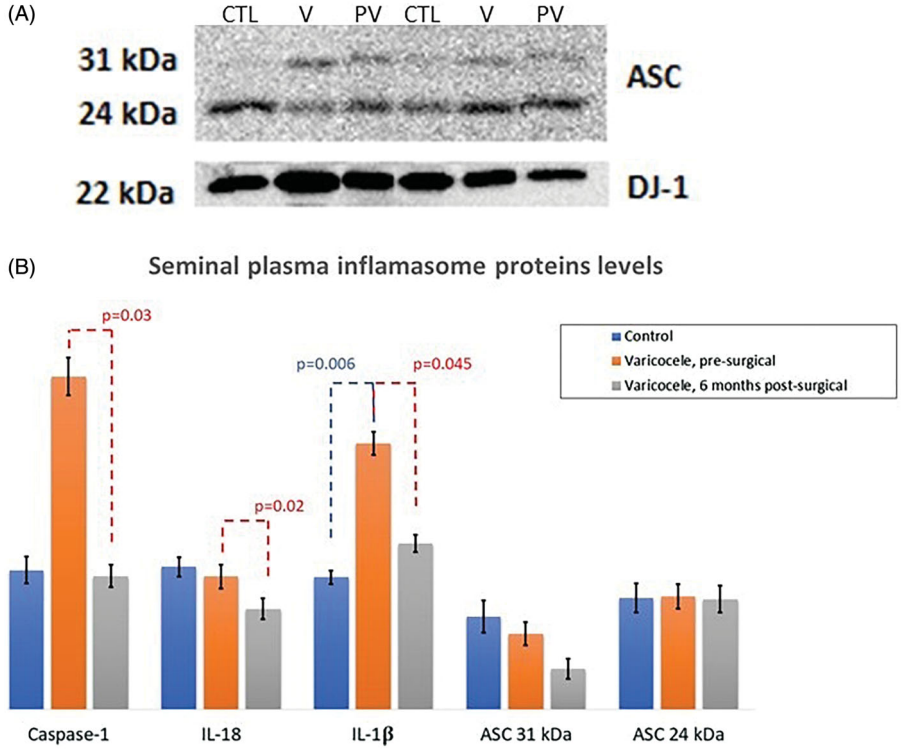

Figure 1. (A) Image representative of a typical Western blot for ASC and DJ-1 proteins, indicating two different bands for ASC: 24 and 31kDa. (B) Bar graphs for seminal plasma inflammasome cytokines and for ASC 24 and 31kDa bands (compared by a Mann–Whitney test between the control and varicocele groups, and by a Wilcoxon test between varicocele and post-varicocelectomy periods). CTL: control; V: varicocele; PV: post-varicocelectomy.

Discussion

A relationship between the inflammasome complex and male infertility was first observed by Zhang et al. (2013). The authors verified the presence of this complex in men with SCI by evaluating seminal plasma levels of IL-1β, IL-18, and caspase-1, and ASC protein in sperm by immunohistochemistry. SCI led to increased seminal levels of these cytokines, compared to controls. Later, Ibrahim et al. (2014) verified that *in vitro* blocking of the inflammasome complex led to an increase in sperm motility in these patients. Because low sperm motility is not pathognomonic to SCI, and because asthenozoospermia is detected in about 18% of men with varicocele (Al-Ali et al., 2010), we hypothesised that men with varicocele could potentially present with higher seminal levels of proteins from this complex (Baazeem et al., 2011). This hypothesis is also supported by another study that confirmed the association between inflammatory cytokines and asthenozoospermia (Ghandehari-Alavijeh, Zohrabi, et al., 2019). In addition, Baazm et al. (2020) confirmed the presence of inflammasome proteins in seminal plasma of men with varicocele. In order to establish causality, we then aimed to verify if surgical treatment of varicocele could decrease seminal levels of these proteins.

While 84% of men with varicocele will not present difficulties in achieving pregnancy at any time (Pinto et al., 1994), 35% of men with primary infertility and up to 80% of men with secondary infertility present with a clinical varicocele (Gorelick & Goldstein, 1993; Witt & Lipshultz, 1993). Thus, it has long been suggested that varicocele produces progressive alterations to male fertility. Varicocele, then, clearly associates with, but does not determine, male infertility. However, although in our study patients with varicocele had lower semen quality, the aim of this study was not to further the knowledge on conventional semen analysis in men with varicocele. Rather, these results are presented so as to demonstrate to which population of men with varicocele our results apply specifically – in the case of this study, to men with varicocele and with a surgical indication, as per ASRM guidelines (American Urological Association and the Practice Committee of the American Society for Reproductive Medicine, 2001).

In our study, men with varicocele presented lower sperm mitochondrial activity and higher seminal plasma levels of IL-1β when compared to men without varicocele, corroborating the study by Baazm et al., (2020). Sahin et al. (2006) observed that IL-1β levels increase in a time-dependent manner in the testis of varicocele-induced rats. In seminal plasma, studies have observed different interleukins in infertile patients (Buch et al., 1994; Dousset et al., 1997; Gérard et al., 1991), demonstrating their importance to male infertility. Any other difference in cytokines was observed between the groups. However, normally semen samples from healthy men may contain immune cells that do not affect sperm function (Zeinali et al., 2017).

ASC, the main effector of the inflammasome complex, can be found in 4 different isoforms (Monie, 2013). These isoforms are produced during the inflammatory response (Franklin et al., 2014) and can perform different functions (Bryan et al., 2010): two isoforms promote inflammasome activation (24 and 19 kDa), one inhibits inflammasome activation (15 kDa) and one has not yet been elucidated (13 kDa) (Bryan et al., 2010). In the present study, two bands were observed: one with 24 kDa – the usual ASC present in the inflammasome complex (Adamczak et al., 2014; Monie, 2013), and the other with 31 kDa. It is possible that this 31 kDa band corresponds to a homodimer of the 15 kDa isoform, as has been previously reported (Adamczak et al., 2014; Cheng et al., 2010; Ibrahim et al., 2014). The 15 kDa isoform inhibits inflammasome activation by blocking the NLRP3-ASC-caspase-1 complex (Bryan et al., 2010). This inhibition occurs because 15 kDa ASC does not present the PYD domain, so that NLRP3 cannot link to ASC to form the initial inflammasome complex (Bryan et al., 2010). Therefore, the 31/24 kDa variable, which we calculated in this study, would indicate whether the effect was more of an inhibitory nature (when the ratio was higher than 1), or of a stimulatory nature (when the ratio was lower than 1), which was true in our case.

The formation and activation of the inflammasome leads to inflammatory cell death (Cookson & Brennan, 2001), and consequently the release of its contents to the extracellular space (Franklin et al., 2014), in an event called pyroptosis (Fernandes-Alnemri et al., 2007). This is the reason why ASC – an intracellular protein – is detected in the extracellular space (seminal plasma). However, no difference was observed in ASC levels between men without varicocele and men with varicocele.

In this study, an increase in semen volume and sperm morphology was observed six months after varicocelectomy, with a concomitant decrease in seminal levels of caspase-1, IL-18 and IL-1β. This indicates that treating varicoceles reduces the inflammatory seminal profile observed in varicocele, shifting the seminal profile back to its original functional status (Belardin

et al., 2016; Del Giudice et al., 2016). Also, sperm morphology is a qualitative indicator of spermatogenesis (O'Donnell, 2014) and therefore its increase indicates an improvement in the testicular environment, which has also been observed by other authors (Camargo et al., 2019; Schatte et al., 1998; Zini et al., 2005).

In the present study, the observed decrease in inflammatory cytokine levels was not associated with differences in ASC levels. This was an interesting finding because there are alternative pathways for caspase-1 activation (Dunne, 2011), and some of them do not require ASC activation (Bednash & Mallampalli, 2016). As an example, caspase-1 is regulated by NEDD8 protein, a ubiquitin that links to the CARD domain of caspase-1, activating it in an auto-catalytic manner (Segovia et al., 2015). This mechanism would explain why varicocelectomy decreased cytokine levels produced by the inflammasome complex, but did not alter ASC levels. Another hypothesis to explain the observed results would be a hypoxia pathway present in men with varicocele, which has already been demonstrated to be related to inflammation and the presence of sperm DNA fragmentation in men with varicocele (Ghandehari-Alavijeh, Tavalaee, et al., 2019).

Other studies have suggested that both the adolescent and adult varicoceles lead to an inflammatory seminal state, and that varicocelectomy partially corrects this alteration (Camargo et al., 2013, 2019; Del Giudice et al., 2013). However, the specific pathways, and thus the therapeutic targets, have yet to be fully determined. This study demonstrated an evident role for inflammasome proteins in varicocele. Nevertheless, the number of cases studied is very low, which is the main limitation of our findings, which may explain the insignificant results. On the other hand, Baazm et al. (2020) observed similar results, with similar number of patients. However, the lack of pregnancy prevents us from extending our results to a clinical benefit. In any case, understanding that inflammation is detected in semen of men with varicocele is, by itself, important information, with a potential clinical impact, especially if we think that inflammation and varicocele are treatable conditions. Understanding how these inflammation affects the semen, treatments with anti-inflammatory can be applied in some cases.

In conclusion, varicocele leads to decreased semen and sperm functional quality, and to increased cytokine activity. Varicocelectomy decreases seminal plasma inflammatory activity, observed at six-month post-operative period.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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