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Elevated levels of S100A12 in the seminal plasma of infertile men with varicocele

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Abstract
Purpose Varicocele is among the leading causes of male infertility. Despite decades of research, its pathogenesis is still unclear. S100A12 is a member of the S100 family of calcium-binding proteins with principal extracellular activities. It is secreted by activated neutrophils and interacts with the multiligand receptor for advanced glycation end products (RAGE). Many studies have delineated the patterns of S100A12 expression in a variety of pathologic conditions. These data show that S100A12 could be a potentially useful inflammatory marker. To explore the relationship between S100A12 and infertility, we examined the amount of S100A12 in semen of infertile men who suffered from varicocele.
Methods S100A12 levels in seminal plasma of 68 infertile men with varicocele (age range 30–45 years) and 68 healthy fertile controls were measured using ELISA. Statistical analysis was performed using both parametric and nonparametric tests.
Results Results of this study showed that the seminal levels of S100A12 were significantly increased in infertile men with varicocele when compared to fertile controls ($P < 0.001$).
Conclusions Because of the important role(s) of these molecules in inflammatory response of cell systems, it could be possible that the spermatozoa motility is reduced following increasing neutrophils, S100A12, and reactive oxygen species in semen of infertile patients with varicocele. S100 proteins seem to be potential biomarkers and therapeutic targets for male infertility.

Keywords S100A12 · Male infertility · Semen · Varicocele · ROS

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Introduction

Infertility is a health problem that has increased over the last decades. Varicocele is one of the main causes of male infertility with incidence rate of 35–75 and 10–15 % among infertile men and general population, respectively [1]. Varicocele is an abnormal dilation of the pampiniform venous plexus in the scrotum [2]. The association between varicocele and male infertility is not clear due to the lack of data uniformity and valuable studies. Human semen contains both cellular and chemical compounds, including sperm, white blood cells (WBC), enzymes, hormones, and other unknown biomaterials [3, 4]. An increased number of WBCs are reported in semen samples of men affected by either genital tract infection or infertility [3, 5], which disrupt the fertilizing ability of sperm [6, 7]. Activated WBCs in seminal plasma during genital tract inflammation or cellular immunity against microbial antigens may trigger the release of a range of products, including proteolytic enzymes, reactive oxygen species (ROS), cytokines, and other inflammatory molecules such as S100 proteins [8]. S100A12 (EN-RAGE) is a member of the S100 multigene family

of calcium-binding proteins involved in Ca^{2+}-dependent synchronization of a variety of intracellular activities, including protection from oxidative cell damage, protein phosphorylation, cell proliferation and differentiation, the dynamics of cytoskeletal rearrangement and structural organization of membranes, enzyme activities, intracellular Ca^{2+} homeostasis, and inflammation [9]. S100A12 is principally secreted by activated neutrophils in humans [10]. Therefore, in recent years, increasing attention has been dedicated to the function of this protein and its role as a marker of inflammation. The best-known receptor for S100A12 is the receptor for advanced glycation end products (RAGE) [11, 12]. Furthermore, binding of S100A12 to RAGE lead to activation of intracellular signal cascades such as nuclear factor kappa B (NF-κB) and MAP kinase, which produce a number of pro-inflammatory cytokines, including interleukin-6 (IL-6), tumor necrosis factor alpha (TNF-α), interleukin-1β (IL-1β), and ROS [13, 14]. Several studies on different cell types have shown that ligand–RAGE interaction mediates production of ROS and consequent downstream signal transduction and regulation of gene expression [15]. RAGE produces oxidative stress mainly through activation of NADPH oxidase, while amplification mechanisms in mitochondria can further drive ROS generation [16]. Additionally, in male genital tract, spermatozoa and peroxidase-positive leukocytes (neutrophils and macrophages) are the source of ROS formation. In the ejaculate, leukocytes are the main producers of ROS compared to spermatozoa. However, some studies have shown that ROS levels have beneficial effects on sperm morphology and functions [17, 18]. On the other hand, due to high content of polyunsaturated fatty acids in the membrane, spermatozoa are especially susceptible to ROS attack. It has been demonstrated that increased ROS production by abnormal spermatozoa and leukocytes can seriously damage sperm. Therefore, high levels of ROS may lead to reduced sperm motility, spermatozoa DNA damage, and apoptosis [19]. Extracellular ROS are mainly produced by neutrophils in male infertility caused by various testicular disorders [20, 21]. One of neutrophil antimicrobial strategies against pathogens is ROS production in a process called the respiratory burst. As a result, seminal neutrophils are responsible for oxidative stress. Oxidative stress is now considered as an important factor in varicocele-associated male infertility. There is a high correlation between varicocele-related ROS production and sperm DNA damage [22, 23].

Due to the fact that infertile men may suffer from inflammatory conditions and impaired movement of spermatozoa, the aim of the present study was to assess seminal concentrations of S100A12 in infertile men with varicocele in comparison with fertile men.

Materials and methods

Subjects

In this experimental study, semen samples were collected from 68 consecutive patients suffering from varicocele and 68 matched fertile healthy males. Patients between 30 and 45 years of age who were referred to the urology clinic (Moradi Hospital, Rafsanjan, Iran) from April 2012 to December 2014 were included in the study. The minimum duration of infertility required was defined as a failure to establish a pregnancy within 2 years with unprotected intercourse. All subjects were nonsmokers with a normal body mass index. None of these patients reported exposure to toxic environmental chemicals or radiation.

In order to detect the possible presence of varicocele, physical examination was performed by a well-trained and experienced physician. All patients were also evaluated with color Doppler ultrasonography [24]. However, subclinical varicoceles were not considered. Furthermore, an expert urologist diagnosed and confirmed the occurrence of infertility based on clinical and paraclinical findings. Semen analysis was performed on each patient and control specimen (Table 1). Exclusion criteria included urogenital infection, sexually transmitted diseases, hypogonadism, genetic defects, occupational exposure to spermatogenetic-toxic chemicals, chemotherapy or radiotherapy treatment, vasectomy, and retrograde ejaculation. In this study, ethical approval was obtained from the Rafsanjan University of Medical Sciences local ethics committee. Before sample collection, written informed consent was obtained from each participant.

Semen analysis

Semen samples were collected by masturbation into sterile containers after 2–5 days of sexual abstinence. Samples were allowed to liquefy completely for 30 min at 37 °C. After liquefaction, sperm motility and concentration were manually assessed using a MicroCell counting chamber (Vitrolife, San Diego, CA, USA). Specimens with greater than 1 million leukocytes/ml (or >5/HPF) were considered

Table 1 Semen features of men with varicocele and controls (mean ± SEM)

	Infertile men with varicocele	Fertile men without varicocele
Sperm concentration (10^6/mL)	30.9 ± 28.4	71.6 ± 18.4
Sperm motility (%)	36.2 ± 32.5	59.2 ± 19.42
WBC (HPF)	5.68 ± 0.65	1.8 ± 0.32
Duration of infertility (year)	3.75 ± 2.7	–

as pyospermia [25]. Seminal plasma was obtained by centrifugation of liquefied samples at 10,000×*g* for 30 min at 4 °C. After centrifugation, the seminal plasma was microscopically checked to confirm the absence of spermatozoa. The samples stored at −80 °C until further analysis.

Semen assay of S100A12

We utilized an ELISA Kit (KA0091 Abnova, Taiwan) to detect and quantify S100A12 in aliquots of frozen-thawed seminal plasma. The ELISA was conducted according to the manufacturer's guidelines, with a detection limit of 56 pg/mL.

Statistical analysis

Data were expressed as mean ± SEM. Statistical analysis was conducted using SPSS version 18 (SPSS Inc., Chicago, IL, USA). Differences were evaluated with the parametric *t* test and nonparametric Mann–Whitney *U* test. A *P* value of <0.05 was considered statistically significant.

Results

In this study, we enrolled 68 consecutive patients with varicocele and 68 matched fertile healthy males. Based on physical examination and color Doppler ultrasonography results, all of the infertile males were affected by varicocele. Moreover, our findings demonstrated that the mean semen levels of S100A12 in the patients and controls were 253.2 ± 7 and 94.18 ± 2 ng/ml, respectively. Seminal S100A12 levels were significantly higher in patients with varicocele than in healthy controls ($P < 0.001$) (Fig. 1).

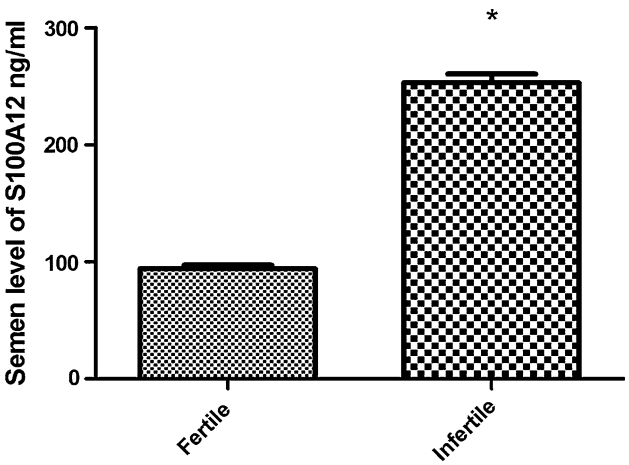

Fig. 1 S100A12 level in seminal plasma of fertile and infertile men. Results are expressed as mean ± SEM of three experiments. *Statistically significant difference between fertile and infertile men ($P < 0.05$)

Discussion

Infertility is defined as incapability of couples to become pregnant after 1 year of unprotected intercourse. Over the last decades, an increasing trend in male infertility has been observed, causing notable psychosocial problems. Among male infertility causes, varicoceles can reduce production of sperm [26, 27]. Neutrophils (polymorphonuclear leukocytes) are involved in host defense against pathogens such as bacteria and fungi using different mechanisms, including production of ROS [28]. Neutrophils and macrophages are the main leukocytes found in semen which can be detrimental to sperm through ROS production and apoptosis induction. Furthermore, it has been shown that immature spermatozoa and varicocele are other major sources of ROS generation in seminal plasma. However, semen of fertile men normally contains neutrophils that do not appear to affect sperm function. It has been suggested that small number of neutrophils may have beneficial effects through removal of abnormal and degenerative spermatozoa [29, 30]. Low levels of ROS have been shown to be necessary for overall sperm fertilizing capacity, including sperm motility, capacitation, acrosome reaction, and oocyte fusion. On the other hand, spermatozoa are especially susceptible to ROS attack because of their stage of differentiation and the composition of their membranes [31]. It could be demonstrated that increased levels of ROS result in oxidative stress, which seriously damages sperm function affecting particularly the motility, the fertilizing potential, and the integrity of sperm genome [32]. The present study was designed to investigate seminal levels of S100A12 in infertile males with varicocele. To the best of our knowledge, this is the first report of an association between seminal S100 protein levels and male infertility. Accordingly, in this case–control study, we demonstrated the elevated seminal S100A12, also known as calgranulin C, level as a member of pro-inflammatory S100 proteins derived from neutrophils. Our findings showed that semen pattern of S100A12 increased significantly in infertile men compared to fertile subjects. Although there were no similar study to be compared with, in a recent in vitro study, Hakimi et al. [33] reported the elevated level of other pro-inflammatory molecules such as CXCL1 and CXCL9 in upper genital tract infections. Increased semen levels of S100A12 in infertile men in the present study may demonstrate that this member of S100 proteins is derived from accumulated neutrophils in the lower part of genital tract, which may be correlated with reduction in the sperm motility in these patients [34]. It is obvious from our findings that the number of WBCs (especially PMNCs) has increased in infertile men compared to fertile men. Therefore, recruited

WBCs may secret a wide range of components such as proteases, proteolytic enzymes, cytokines, ROS, and inflammatory mediators [35]. Moreover, a proportion of S100A12 may possibly be the result of insults and injuries of lower part of genital tract [36, 37]. It has been widely accepted and established that during the inflammatory responses, a broad spectrum of inflammatory mediators, including S100 proteins, are released from inflamed tissues, organs, and migrated immune cells [12]. These factors, in turn, trigger several intracellular signaling pathways as upstream regulatory pathway for stimulation of inflammatory cytokines and ROS production; for instance, ligation of S100A12/RAGE axis could regulate activation of NF-κB, which is responsible for production of inflammatory and pro-inflammatory mediators [38].

According to the findings of current study and studies mentioned above, it could be concluded that several cell types in the male reproductive system, including migrated and resident immune cells, are involved in development of immunological responses in infertility. Activation of S100A12/RAGE axis and ROS production may also play a role in the pathogenesis of infertility. Our results may re-emphasize that these proteins could be considered as potential novel biomarkers and therapeutic targets in male infertility. Furthermore, we suggest evaluation of other members of S100 protein family and their respective receptors (for instance RAGE) on immune cells in varicocele as a major cause of male infertility.

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Compliance with ethical standards

Conflict of interest None of the authors of this study declared conflict of interest.

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