Neutralization of ASC improves sperm motility in men with spinal cord injury

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STUDY QUESTION: Does neutralization of apoptosis-associated speck-like protein containing a caspase activation and recruitment domain (ASC) improve sperm motility in men with spinal cord injury (SCI)?

SUMMARY ANSWER: Neutralization of ASC improves sperm motility in men with SCI.

WHAT IS KNOWN ALREADY: Semen of men with SCI contains normal sperm concentrations but abnormally low sperm motility. Inflammatory cytokines, activated via the inflammasome complex, are contributory. A key component of the inflammasome is ASC.

STUDY DESIGN, SIZE, DURATION: This prospective study included semen samples collected from 32 men with SCI.

PARTICIPANTS/MATERIALS, SETTING, METHODS: At a major university medical center, untreated semen was compared with semen treated with anti-ASC polyclonal antibody. Semen treated with IgG was used as a control.

MAIN RESULTS AND THE ROLE OF CHANCE: Addition of anti-ASC polyclonal antibody to semen significantly increased mean sperm motility from 11.5% (95% CI, 6.3–16.7) to 18.3% (95% CI, 11.8–24.8). Improvements were most pronounced in the subgroup whose starting motility ranged between 6 and 40%. In this subgroup, the mean sperm motility improved from 13.3% (95% CI, 9.3–17.3) to 23.9% (95% CI, 14.7–23.0). Sperm motility did not improve after treatment with IgG.

LIMITATIONS, REASONS FOR CAUTION: This study is limited by the small sample size as this is a rare population.

WIDER IMPLICATIONS OF THE FINDINGS: Blockade of the inflammasome via treatment with anti-ASC improved sperm motility in men with SCI. This is the first study to demonstrate that interference with the inflammasome improves sperm motility in men with SCI. This treatment has potential as a therapeutic intervention.

STUDY FUNDING/COMPETING INTEREST(S): This study was funded by the Craig H. Neilsen Foundation, Grant # 224598, the University of Miami Miller School of Medicine and the Miami Project to Cure Paralysis, Miami, FL, USA. R.W.K. and J.P.d.R.V. hold a patent for the treatment of inflammation after central nervous system injury using antibodies against inflammasome proteins. The other authors have no conflicts of interest to declare.

Key words: ASC / infertility / inflammasome / spinal cord injury / sperm motility

Introduction

Spinal cord injury (SCI) has a worldwide incidence of ~20–40 cases per million (Devivo and Chen, 2011). Infertility is ubiquitous in this group owing to a combination of erectile dysfunction, ejaculatory dysfunction and poor semen quality (Brown et al., 2006; DeForge et al., 2006; Brackett et al., 2010b). Phosphodiesterase 5 inhibitors and implantation of a penile prostheses have each been shown to be effective in providing erections satisfactory for intercourse in >80% of men with SCI who have erectile dysfunction (Zermann et al., 2006; Soler et al., 2007). However only 10% of men with SCI can ejaculate with sexual activity (Brackett et al., 2010a). Ejaculation can be induced by either penile vibratory stimulation (PVS) or electroejaculation (EEJ) in 97% of men with SCI whose level of injury is T10 or rostral (Brackett et al., 2010a,b), but these men must rely on some sort of assisted conception to achieve pregnancy (Kafetsoulis et al., 2006).

Despite these advances, poor semen quality, particularly abnormally low motility of spermatozoa, remains. The choice of ART is primarily...
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Materials and Methods

Patients

Semen samples were collected from 32 volunteer men with SCI. All subjects were past the period of spinal shock (>12 months). All subjects were in good general health with no active urinary tract infections. No subject had taken any medication known to affect semen quality within 6 months prior to the study. The cohort demographics are summarized in Table I. The mean age was 38 years, with a mean time of 14 years post-injury. The level of injury ranged from C3 to L1. Semen was obtained using the standard methods of PVS or EEJ (Brackett et al., 2010b).

Semen analysis

Only antegrade semen specimens were used in this study. Following liquefaction at room temperature, an initial semen analysis was performed on each specimen. Semen volume was measured in milliliters (ml) and sperm concentration was measured in millions/ml. In this study, we wished to assess four grades of sperm motility, and we therefore used the four category WHO method: (a) rapid linear motility; (b) sluggish motility; (c) nonprogressive motility and (d) immotility (World Health Organization, 1999). Categories a and b were summed to calculate the percentage of spermatozoa with progressive motility, also referred to as ‘sperm motility’ in this study. Total motile sperm count (TMSC) was calculated by multiplying sperm concentration by semen volume by sperm motility (%) divided by 100. The same technician performed all semen analyses, including assessments of sperm motility.

Semen processing

In this within-subjects study design, each subject served as his own control. Each semen sample was divided into aliquots of 50 µl. Aliquots were treated with a vehicle control (i.e. addition of 8 µl sperm wash), or with normal goat IgG-control (5 µg in a volume of 5 µl sperm wash media), or with a polyclonal antibody against ASC (R&D Systems, Minneapolis, MN, USA, Catalog # AF3805) at a concentration of 2–4 µg in a volume of 8 µl sperm wash media (Life Global, Guilford, CT, USA). Sperm motility was assessed 60 min after treatment.

Immunoblotting

To determine if the polyclonal anti-ASC antibody (R&D Systems, Minneapolis, MN, USA, Catalog # AF3805) selectively binds human ASC in spermatozoa, we performed an immunoblot analysis as described in Zhang et al. (2013) by loading ASC positive lysate (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and lysate obtained from samples of spermatozoa. Samples were then blotted for ASC with the antibody of interest (i.e. R&D Systems, Minneapolis, MN, USA, Catalog # AF3805).

Table I Cohort demographics.

<table>
<thead>
<tr>
<th>Number of subjects</th>
<th>32</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>23–52 Years</td>
</tr>
<tr>
<td>Range</td>
<td>23–52</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>38 ± 1.5</td>
</tr>
<tr>
<td>Median</td>
<td>37</td>
</tr>
<tr>
<td>Time since SCI</td>
<td>2–38 Years</td>
</tr>
<tr>
<td>Range</td>
<td>2–38</td>
</tr>
<tr>
<td>Mean ± SE</td>
<td>14 ± 1.5</td>
</tr>
<tr>
<td>Median</td>
<td>14</td>
</tr>
<tr>
<td>Level of injury</td>
<td>n</td>
</tr>
<tr>
<td>C3–C8</td>
<td>15</td>
</tr>
<tr>
<td>T1–T6</td>
<td>12</td>
</tr>
<tr>
<td>T7–L1</td>
<td>5</td>
</tr>
</tbody>
</table>

Statistical analyses

Untreated and treated groups were compared using the student’s t-test and the Mann–Whitney test for non-parametric measures (SPSS Statistics 21, IBM Corporation, Armonk, NY, USA). P-values ≤ 0.05 were considered significant.

The inflammasome is a multiprotein complex thought to be responsible for activation of the innate immune response (Schröder and Tschopp, 2010). The majority of inflammasomes consist of a nucleotide oligomerization domain-like receptor (NLR), caspase-1, and the adaptor protein, apoptosis-associated speck-like protein containing a caspase activation domain and a recruitment domain (ASC). The activation of the inflammasome results in the cleavage of procaspase-1 into active caspase-1, which then cleaves pro–IL-1β and pro–IL-18 into their active forms. IL-1β and IL-18 are potent pro-inflammatory cytokines that play a major role in innate immunity and the body’s response to tissue injury, including lymphocyte activation, recruitment of other inflammatory cells and their products and cytokines, and induction of secondary inflammatory cytokines and other cellular products in T cells and natural killer cells (Guarda and So, 2010; Dunne, 2011).

In a recent report, ASC, caspase-1, IL-1β and IL-18 were found to be elevated in seminal plasma from men with SCI compared with non-SCI control subjects. Immunocytochemistry revealed that ASC was located in the acrosome, equatorial segment and midpiece of the spermatozoa (Zhang et al., 2013). The present study sought to discover whether antibody-mediated blockade of the ASC portion of the inflammasome would result in improved motility of spermatozoa for men with SCI.

The exact mechanism of poor semen quality in men with SCI is currently unknown. It has been shown, however, that seminal plasma from men with SCI is spermatoxic (Brackett et al., 1996, 2010b), and that this toxicity is associated with elevated inflammatory cytokines, the neutralization of which leads to improved motility of spermatozoa (Cohen et al., 2004; Brackett et al., 2007). Recent evidence suggests that the inflammasome may play a role in the pro-inflammatory pathway generating elevated semen cytokines in these men (Zhang et al., 2013).

In vitro fertilization (IVF) and intracytoplasmic sperm injection (ICSI) are the only alternatives for men with severely reduced numbers of motile spermatozoa. In addition to the increased costs associated with IVF/ICSI, there are increased risks of complications to the mothers, multiple births, and possibly mental retardation and autism in the offspring (Blondel and Kaminski, 2002; Sandin et al., 2013). Improvements in motility of spermatozoa may allow the use of less invasive assisted conception procedures such as intravaginal insemination and/or intrauterine insemination (IUI) in couples with SCI male partners.

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Results

The mean semen volume was 2.0 ml and the mean sperm concentration was 68 million/ml in the initial semen analysis. The mean percentage of spermatozoa with progressive motility was 11.5% (Table II). Following treatment with anti-ASC, this motility increased significantly to 18.3% ($P \leq 0.005$), and from 40.0 to 43.3% in Subgroup C (not statistically significant). Only three subjects fell into this group.

Table II  Semen parameters: all subjects.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean ± SE</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Semen volume</td>
<td>2.0 ± 0.2</td>
<td>1.5</td>
</tr>
<tr>
<td>Sperm concentration</td>
<td>68 ± 12</td>
<td>50</td>
</tr>
<tr>
<td>Progressive motility</td>
<td>11.5 ± 2.6</td>
<td>6.0</td>
</tr>
<tr>
<td>Rapid linear motility</td>
<td>2.0</td>
<td>4.5</td>
</tr>
<tr>
<td>Total motile sperm count</td>
<td>22.6 ± 8.5</td>
<td>4.5</td>
</tr>
</tbody>
</table>

Summary of semen parameters prior to treatment with anti-ASC. SE, standard error of the mean.

Sperm motility

A sub-analysis (Table III) revealed that the degree of improvement in sperm motility related to the subjects’ starting sperm motility. For example, the largest increase in absolute percentage occurred in the sub-group of subjects whose starting sperm motility ranged between 6 and 40% (Subgroup B). In this subgroup, the mean sperm motility increased from 13.3 to 23.9% after treatment with anti-ASC ($P \leq 0.005$). The subgroup of subjects whose starting sperm motility ranged between 0 and 5% (Subgroup A), a small but significant increase occurred (2.2 to 6.0%, $P \leq 0.001$). In contrast, the subgroup of subjects whose starting sperm motility ranged between 41 and 60% (Subgroup C), sperm motility increased only slightly from 49.7 to 54.0% (not statistically significant). Only three subjects fell into this group.

Additional semen parameters

A similar outcome occurred in the fraction of spermatozoa with rapid linear motility (Table III). In all subjects ($n = 32$), the mean percentage of spermatozoa with rapid linear motility increased from 6.7 to 10.6% ($P \leq 0.001$). A sub-analysis revealed rapid linear motility increased from 0.7 to 2.3% in Subgroup A ($P \leq 0.01$), from 5.9 to 12.4% in Subgroup B ($P \leq 0.005$), and from 40.0 to 43.3% in Subgroup C (not statistically significant).
Neutralization of ASC improves sperm motility in SCI

Table III  
Semen treated with anti-ASC.

<table>
<thead>
<tr>
<th>Group</th>
<th>% of sperm with progressive motility</th>
<th>% of sperm with rapid linear motility</th>
<th>Total motile sperm count in millions</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean (95% CI)</td>
<td>Mean (95% CI)</td>
<td>Mean (95% CI)</td>
</tr>
<tr>
<td>Untreated</td>
<td>10.6 (5.8 to 15.3)</td>
<td>2.3 (1.1 to 3.4)</td>
<td>2.7 (1.0 to 5.2)</td>
</tr>
<tr>
<td>Treated with anti-ASC</td>
<td>6.7 (1.1 to 24.8)</td>
<td>0.7 (0.3 to 1.0)</td>
<td>0.7 (0.3 to 1.0)</td>
</tr>
<tr>
<td></td>
<td><strong>Comparison of semen untreated (vehicle control) and semen treated with anti-ASC.</strong> Mean % of sperm with progressive motility, mean % of sperm with rapid linear motility and mean total motile sperm count increased significantly in all groups except Subgroup C (Table III).</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Discussion**

Most men with SCI have normal sperm concentrations, but abnormally low sperm motility. Despite the improvements in treatment modalities for erectile dysfunction (Zermann et al., 2006; Soler et al., 2007) and ejaculatory dysfunction (Brackett et al., 2010a,b), impaired semen quality remains problematic. IVF or ICSI may be the only alternatives available to those men with the poorest of the semen parameters. Assisted reproductive technologies such as IVF and ICSI can be expensive, and may carry an increased risk to the unborn child as well as to the mother (Blondel and Kaminski, 2002; Sandin et al., 2013). Less expensive and less risky methods of conception are thus an objective for this population (Kafetsoulis et al., 2006; Kathiresan et al., 2011a,b).

There is evidence that the inflammasome complex is involved in impairing sperm motility in men with SCI (Zhang et al., 2013). The present study aimed to improve sperm motility by interfering with the inflammasome complex. Specifically, activation of the inflammasome complex was blocked by addition of anti-ASC to semen of men with SCI.

The results of our study showed that this treatment significantly increased sperm motility in men with SCI. The amount of increase was related to the subjects’ starting sperm motility. For example, subjects who had very low starting sperm motilities (0–5%), or normal starting sperm motilities (41–60%) had only modest increases after treatment with anti-ASC. In contrast, subjects with starting sperm motilities between 6 and 40% showed the greatest increase in sperm motility. These increases in sperm motility translated to significant increases in TMSC, even in the group with the lowest starting sperm motilities (Subgroup A). In this subgroup, the mean TMSC increased from 1.7 million to 4.1 million after treatment with anti-ASC. This increase is clinically significant. For example, a study by Dong et al. showed that a TMSC > 2 million led to a higher pregnancy rate for IUI compared with a TMSC < 2 million (Dong et al., 2011a). Similarly, a study by Cao et al. showed that IUI pregnancy rates increased from 4 to 14% if the TMSC was > 2 million (Cao et al., 2014).

The presence of sperm with rapid linear motility has long been linked to increased chances of pregnancy. Belker et al. showed a 2.7-fold increase in the odds of achieving pregnancy through IUI if spermatozoa with rapid linear motility were present, especially in subfertile men whose TMSC was < 10 million (Belker et al., 1994). In our study, the percentage of spermatozoa with rapid linear motility improved significantly in all groups except Subgroup C, i.e. men whose starting sperm motility was essentially normal.

The reason for a differential effect on improvement in sperm motility is unknown. Mechanisms other than inflammasome activation, such as elevated concentrations of reactive oxygen species, may contribute to low sperm motility in men with SCI (Sharma and Agarwal, 1996; Barbonetti et al., 2013). Additionally, men with very low sperm motility may have a high percentage of sperm that are dead, rather than living but immotile (Brackett et al., 1998), and these dead sperm cannot be improved by treatment. In contrast, men whose sperm motility is normal or near-normal may not have any significant pathology and therefore treatment would have minimal effect.

In this study we used a polyclonal antibody. It is possible that this antibody may have cross reacted with other antigens. To examine the statistically significant. Similarly, the total motile sperm count (TMSC) increased significantly in all groups except Subgroup C (Table III).
specificity of our antibody, we performed an immunoblot experiment, which showed that the anti-ASC antibody used in this study recognized ASC in human sperm (Fig. 1). Based on these promising results with our polyclonal antibody, future studies will focus on using a humanized monoclonal antibody against human ASC to target more specific epitopes. Our approach of using anti-ASC to inhibit an intracellular complex such as the inflammasome, is consistent with previous reports showing that this antibody can be used to inhibit inflammasome activation (Rivero Vaccari et al., 2008, 2009; Abulafia et al., 2009).

The current investigation shows the potential benefits of blockade of the inflammasome on semen quality in men with SCI. Although this treatment is still in the investigatory stage, our results indicate that blockade of the inflammasome will likely benefit men with SCI who have low sperm motility, potentially increasing their TMSC to ranges that allow alternatives to IVF and ICSI. There does not appear to be a benefit in the group which has essentially normal sperm motility. This study is limited by the small sample size in this rare population of severely affected patients, even more so in Group C, i.e. SCI patients with essentially normal sperm motility.

Blockade of the inflammasome via treatment with anti-ASC resulted in improved sperm motility in men with SCI. In doing so, this treatment significantly increased TMSC in these men. It is expected that additional refinements in our methodology will lead to even greater improvements in sperm motility, and thus increase the number of candidate couples for IUI or even intravaginal insemination (Kathiresan et al., 2011a), instead of the more invasive and expensive options of IVF or IVF/ICSI (Kathiresan et al., 2011b).

This is the first study to demonstrate that interference with the inflammasome improves sperm motility in men with SCI. This treatment has potential as a therapeutic intervention.

**Authors’ roles**

E.I.: Conception and design, acquisition of data, interpretation of data, revising article critically for important intellectual content and final approval of the manuscript. S.M.C.: Analysis and interpretation of data, drafting the article or revising it critically for important intellectual content and final approval of the manuscript. T.C.A.: Acquisition of data, revising article critically for important intellectual content and final approval of the manuscript. R.W.K.: Conception and design, interpretation of data, revising article critically for important intellectual content and final approval of the manuscript. J.P.d.R.V.: Conception and design, interpretation of data, revising article critically for important intellectual content and final approval of the manuscript. C.M.L.: Conception and design, interpretation of data, revising article critically for important intellectual content and final approval of the manuscript. N.L.B.: Conception and design, interpretation of data, revising article critically for important intellectual content and final approval of the manuscript.

**Funding**

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**Conflict of interest**

R.W.K. and J.P.d.R.V. hold a patent for the treatment of inflammation after central nervous system injury using antibodies against inflammasome proteins. The other authors have no conflicts of interest to declare.
References


