CatSper gene expression in postnatal development of mouse testis and in subfertile men with deficient sperm motility

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BACKGROUND: The search for Ca²⁺ channels residing in sperm has led to the recent cloning and characterization of a novel gene, named CatSper, which codes for a unique Ca²⁺ channel expressed exclusively in the testis. It plays an essential role in sperm motility, penetration into the oocyte, and ultimately in male fertility. In this study, we assessed the temporal profile of CatSper gene expression during mouse testis development and performed a semi-quantitative evaluation of expression levels in a group of subfertile men which lack sperm motility. METHODS: A small piece of testicular tissue obtained by either multi-site testicular biopsy or orchidectomy was used for semi-quantitative RT-PCR of CatSper and β²-microglobulin (β²m, as an internal control) genes. RESULTS: Our results reveal that: (i) the expression of mouse CatSper is developmentally regulated with a direct correlation between CatSper expression and mouse sexual maturation. CatSper gene expression is first detected at 3 weeks of age and coincides with the appearance of round spermatids in the developing mouse testis. (ii) There is a significant reduction in the level of CatSper gene expression (up to 3.5-fold difference) among patients which lack sperm motility as compared with patients whose infertility cannot be ascribed to a deficiency in motility (used as a control). CONCLUSIONS: The data obtained in this study support a potential role for CatSper in sperm motility and fertility in mouse and human. CatSper is therefore implicated as a potential target to explore the molecular mechanisms of male infertility.

Key words: CatSper/gene regulation/male infertility/sperm motility/testis

Introduction

According to current estimates, almost one out of six couples is unable to achieve pregnancy. Male infertility accounts for ~30–50% of all infertility cases, with unexplained infertility accounting for a further 10–20% of cases. Genetic defects are believed to be the cause of infertility in a significant proportion of infertile men. A chromosomal abnormality or a small Y chromosome deletion is sometimes detectable (Maduro and Lamb, 2002). Nonetheless, little is known about the other possible genetic mechanisms underlying the infertility in the majority of these patients. Identifying the genes associated with these abnormalities would have a great impact on our understanding, diagnosis and treatment of male infertility (Maduro and Lamb, 2002).

Although sperm motility is one of the most important predictors of fertilizing ability, the mechanisms underlying motility abnormalities remain poorly understood (Haidl, 1994). Men with sperm motility <50% or progressive motility <25% are considered to be subfertile (World Health Organization, 1999). A significant proportion of patients presenting to infertility clinics have reduced sperm concentrations and motility, often in combination (Liu and Baker, 1992). These patients are subfertile despite having normal hormonal levels and other semen criteria such as volume, pH, viscosity, and sperm morphology. Their sperm also perform normally during in vitro assessment of the acrosome reaction (Olds-Clarke, 1996). It is hoped that understanding the molecular mechanisms regulating sperm motility will lead to novel strategies aimed at treating infertility in these patients (Katz, 1991; Haidl, 1994).

It is well known that calcium ions and cyclic nucleotides play vital roles during various stages of fertilization, especially in facilitating sperm motility (Darszon, 1999). Accordingly, any increase in the concentration of intracellular calcium ions leads to changes in sperm motility (Ward et al., 1985; Cook et al., 1994). However, the mechanisms controlling swimming behaviour, the entrance site of calcium ions into sperm, and the
molecular identity of the calcium channels themselves all remain poorly understood (Wiesner et al., 1998).

Recently, the search for the calcium channels residing in sperm led to the cloning and characterization of a novel gene, named CatSper, which codes for a unique cation channel (Ren et al., 2001). CatSper is a single-unit channel having a six-transmembrane-spanning repeat within its sequence and a pore region resembling that of the voltage-dependent calcium channels. This channel is exclusively expressed in the testis. Comparison of human and mouse CatSper amino acid sequences reveals a high degree of conservation, suggesting its critical function throughout evolution. Deletion of the CatSper gene in mouse revealed its vital role in sperm motility and male fertility. Although CatSper −/− mice display sexual behaviours that are indistinguishable from wild-type mice, they are infertile. In-depth examination revealed no apparent abnormalities in these mice except for a lack of sperm motility (Ren et al., 2001).

In the present study, we have evaluated the temporal pattern of CatSper gene expression during mouse testis development. Using semi-quantitative RT–PCR, we have also compared the level of CatSper gene expression in two groups of subfertile men to assess any potential abnormality linked to sperm motility. The first group had no observable motile sperm while the control group had motile sperm, as assessed by percutaneous epididymal sperm aspiration (PESA) and testicular sperm extraction (TESE).

Materials and methods

Animals
A total of 24 Balb/C male mice was obtained from the Razi institute (Tehran, Iran) and divided into eight different age groups (n = 3 in each group: 1 day of age; 1, 2 and 3 weeks of age; 1, 2, 3 and 4 months of age). Animals were housed in small groups under standard lighting conditions with free access to water and food. They were rapidly killed by cervical dislocation and both testes were removed. One testis was fixed in Bouin’s fixative and processed for histological analysis. The other one was snap-frozen in liquid nitrogen and stored at −80°C for subsequent RT–PCR analysis.

Human testis biopsies
Testicular tissue was obtained from unused portions of multi-site testicular biopsies (TESE) from patients undergoing ICSI treatment as well as from patients undergoing orchidectomy. The tissues were immediately snap-frozen in nitrogen vapor and kept at −80°C until RNA extraction. The experimental group included men in whom no motile sperm were visible in either semen samples or in epididymal and testicular tissues retrieved by either PESA or TESE respectively. The control group included subfertile patients in whom motile sperm were evident in either semen samples or in epididymal and testicular tissues. The experimental design was approved by the Ethics Committee of Tabriz Modares University. All patients were informed about the study and gave written consent prior to participation.

RNA extraction and RT
Total RNA was isolated from frozen tissues using the RNX plus solution (Cinnagen, Iran) according to the manufacturer’s instructions. Briefly, after homogenizing the tissue, 1 ml of RNX solution was added to the tube and incubated at room temperature for 5 min. Chloroform was added to the solution and centrifuged for 15 min at 12,000 g. The upper phase was then transferred to another tube and an equal volume of isopropanol was added. The mixture was centrifuged for 15 min at 12,000 g and the resulting pellet was then washed in 70% ethanol and dissolved in DEPC-treated water. The purity and integrity of the extracted RNA was evaluated by optical density measurements (260/280 nm ratios) and by visual observation of samples electrophoresed on agarose gels. Both methods indicated integrity of the extracted RNA with little or no protein contamination.

Complementary DNA synthesis reactions were performed using 1 μg RNA and MMLV reverse transcriptase (Gibco BRL, Germany) with oligo(dT)18 priming in a 20 μl reaction as described elsewhere (Sambrook and Russel, 2001).

PCR
PCR primers for human samples were designed using previously described human CatSper (hCatSper) and beta-2microglobulin (hβ2m) sequences (GenBank accession numbers: AF407333 and NM-004048 respectively). Primers were designed using Genrunner software (version 3.02; Hastings Software Inc.) and were as follows: hCatSper forward, 5′-TTTCTGATCATCTACGTTGGT-3′; hCatSper reverse, 5′-CTTTCTCCAGCTCAAACT-3′; hβ2m forward, 5′-TGGCTACTCTCTCCTCTCT-3′; hβ2m reverse, 5′-GGTTACATGTCTCGATCCAC-3′.

PCR primers amplified 868 and 334 bp segments from hCatSper and hβ2m cDNA respectively. PCR was performed using 5 μl of synthesized cDNA with 1.25 U of Taq polymerase (Roche, Germany), as described elsewhere (Sambrook and Russel, 2001). The PCR amplification was performed for either 35 (hCatSper) or 30 (hβ2m) cycles. The cycling conditions were as follows: 94°C for 30 s, 58.5°C for 30 s, 72°C for 1 min with a final extension at 72°C for 5 min.

Primers for mouse CatSper (mCatSper) and mouse β2m (mβ2m) sequences (GenBank accession numbers: AF407332 and NM-009735 respectively) were designed as follows: mCatSper forward, 5′-CACCACCCGGAAATATCT-3′; mCatSper reverse, 5′-TGGGAGAACACACAGAGAAG-3′; mβ2m forward, 5′-TGACGCCGTGTGATGCTTAC-3′; mβ2m reverse, 5′-CACATGTCCTGATCCAGTAG-3′. These primers amplified a 566 bp segment from mCatSper cDNA and a 316 bp segment from mβ2m cDNA with the amplification performed for either 30 or 25 cycles respectively.

Quantification of PCR products
PCR products were separated on a 1.5% agarose gel and visualized by ethidium bromide staining. The amount of DNA was quantified by measuring the intensity of light emitted from corresponding bands under UV light using Labimage software (version 2.6; Kapelan GmbH Co., Germany). The results were expressed as the ratio of the intensity of the CatSper gene band to that of β2m to account for any differences in the starting amounts of RNA.

Statistical analysis
All experiments were replicated two or three times and the results were analysed by performing t-tests with P < 0.05 considered as statistically significant. The SEM was also calculated for each value.

Results

RT–PCR optimization
The RT–PCR reaction was optimized by varying the number of amplification cycles, in increments of five, from 20 to 45 cycles, in order to select the lowest cycle number where...
transcripts could be quantified without reaching stationary phase. Band intensity increased almost linearly as the reaction approached the stationary phase (data not shown). While 30 and 25 cycles were used to amplify mCatSper and mβ2m respectively, 35 and 30 cycles were required to amplify hCatSper and hβ2m respectively.

**Confirming the identity of the amplified DNA**

The sizes of the amplified DNA fragments for hCatSper (868 bp) and hβ2m (334 bp) were as expected for both genes. To further confirm the accuracy of the PCR products, the amplified hCatSper and hβ2m products were digested with BamHI and EcoRI respectively. Digestion generated three smaller fragments for hCatSper and two smaller fragments for hβ2m, all with sizes as expected from the primary sequences (data not shown).

**Expression of CatSper in mouse testis is developmentally regulated**

To determine the temporal profile of CatSper gene expression during postnatal mouse testis development, different ages of developing and adult mice were examined. Both testes were excised from the killed animals; one testis was fixed for histological examination while the other was immediately frozen in liquid nitrogen for gene expression experiments. The quality and purity of total RNA obtained from each sample was evaluated by UV spectrophotometry and agarose gel electrophoresis (data not shown). The data show that the expression of the gene is developmentally controlled. Expression was first detected postnatally at 3 weeks of age (Figures 1 and 2). There was no expression of CatSper prior to this age even after increasing the PCR cycle number to 45 (Figure 1, last three lanes).

**Evaluation of CatSper gene expression in subfertile patients**

To compare the profile of CatSper gene expression in subfertile patients with deficient sperm motility to that of subfertile or fertile patients with motile sperm (controls), we obtained unused portions of testicular biopsies from the Koasar Infertility Center and Labbafi-Nejad Medical Center. A total of 18 samples was obtained and divided by an expert urologist into two groups (7 cases and 11 controls) based on clinical examination including direct microscopic visualization of sperm motility. Except for a single 76 year old control patient who underwent orchidectomy for advanced prostate cancer, the range of patient ages was 29–39 years (average 34 ± 3.7) for the case group, and 22–53 years (average 36.6 ± 8.5) for the control group (Table I).

Gene expression levels were examined by semi-quantitative RT–PCR. To ensure that equal amounts of RNA were used for each reaction and that potential differences in signal intensity were not due to differences in the amounts of starting RNA, hβ2m was used as an internal control for each reaction. RT–PCR was performed in separate tubes under similar conditions (except for the cycle number) for both hCatSper and hβ2m with results expressed as an hCatSper:hβ2m expression ratio. Amplified products from all sample sets were loaded onto a single agarose gel and electrophoresed. Pictures were then captured under identical brightness/contrast conditions, thus maximizing the accuracy of quantification. Electrophoresis of the hCatSper and hβ2m PCR products showed two bands with sizes as expected (Figure 3). The experiment revealed an overall reduction in the signal intensities for the cases as compared with the controls. Except for case numbers 6 and 7 (Figure 4A), CatSper signals are barely detectable while control CatSper signal intensities appear strong.

The signal intensities for both hCatSper and hβ2m were quantified using Labimage software. In Figure 4A, the relative expression of hCatSper for each patient is presented separately, while in Figure 4B, the pooled expression profile of the two groups is presented. We observed considerable variability in hCatSper gene expression among individuals within each
group. However, statistical analysis comparing the means between the two groups (Student’s t-test) revealed a significant difference in the level of hCatSper gene expression ($P = 0.009$).

Discussion

In mammals, sperm must travel long distances along the female reproductive tract prior to reaching and fertilizing the ovum. Presented with several biological barriers, only a few sperm achieve proximity to the oocyte from the millions initially deposited. Thus, adequate sperm motility is vital for success of the fertilization process (Olds-Clarke, 1996).

A prolonged search to identify the key molecular mediators of sperm motility led to the recent cloning and characterization of a novel gene, named CatSper (Ren et al., 2001). The gene encodes for a unique calcium channel (exclusively expressed in the testis) that controls sperm calcium influx. Deletion of the gene from the mouse germline diminishes sperm motility and the ability to penetrate the oocyte's outer layer. Except for their sterility, the appearance and sexual behaviour of CatSper ±/± mice are indistinguishable from their wild-type littermates (Ren et al., 2001).

To further explore the precise role of CatSper in male fertility, the issues that need to be addressed include: (i) the description of the temporal expression profile of CatSper during pre- and postnatal development and (ii) the identification of the potential alterations in gene expression levels or sequence in subfertile men. The ability to inhibit the expression and/or function of CatSper itself could lead to the design of a non-hormonal contraceptive taken by either men or women in a non-continuous manner.

As a first step, we have focused on CatSper’s gene expression profile during mouse testis development. Our data

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>Age (years)</th>
<th>SCI</th>
<th>Motile sperm</th>
<th>Cause of surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Co1</td>
<td>53</td>
<td>Nil</td>
<td>+</td>
<td>Vasectomized 2 years ago (ICSI)</td>
</tr>
<tr>
<td>Co2</td>
<td>34</td>
<td>Nil</td>
<td>+</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co3</td>
<td>35</td>
<td>Yes</td>
<td>Nil</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co4</td>
<td>36</td>
<td>Nil</td>
<td>+</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co5</td>
<td>22</td>
<td>+</td>
<td>NA</td>
<td>Trans-sex surgery (orchidectomy)</td>
</tr>
<tr>
<td>Co6</td>
<td>33</td>
<td>Nil</td>
<td>+</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co7</td>
<td>35</td>
<td>Nil</td>
<td>+</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co8</td>
<td>48</td>
<td>Nil</td>
<td>+</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co9</td>
<td>37</td>
<td>Yes</td>
<td>NA</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co10</td>
<td>33</td>
<td>+</td>
<td>NA</td>
<td>Obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>Co11</td>
<td>76</td>
<td>Nil</td>
<td>+</td>
<td>Prostatic cancer (orchidectomy)</td>
</tr>
<tr>
<td>C1</td>
<td>39</td>
<td>Yes</td>
<td>Nil</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C2</td>
<td>33</td>
<td>Nil</td>
<td>–</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C3</td>
<td>39</td>
<td>Nil</td>
<td>–</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C4</td>
<td>36</td>
<td>Yes</td>
<td>Nil</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C5</td>
<td>38</td>
<td>Nil</td>
<td>–</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C6</td>
<td>32</td>
<td>–</td>
<td>–</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
<tr>
<td>C7</td>
<td>31</td>
<td>Nil</td>
<td>–</td>
<td>Non-obstructive azoospermia (ICSI)</td>
</tr>
</tbody>
</table>

SCI = spinal cord injury; Co = control sample; C = case (experimental sample); NA = not available; + = any history of observed motile sperm; – = no history of observed motile sperm.
revealed that the expression of the gene is developmentally controlled. Expression begins postnatally at the age of 3 weeks, coinciding with the appearance of lumen within seminiferous tubules as well as with the appearance of particular germ cell types, e.g. round spermatids (Vergouwen et al., 1993). Identification of potential regulatory factors with similar expression profiles could eventually result in novel ways of manipulating CatSper gene expression.

We have also investigated CatSper gene expression levels in subfertile men that lack sperm motility. Our results showed that CatSper gene expression was significantly reduced in these patients as compared with controls. This finding provides clinical confirmation of conclusions drawn in the original report by Ren et al. (2001), linking CatSper gene expression to sperm motility and male fertility.

A potential weakness of the present study is the relative lack of data on males with normal fertility patterns, primarily due to the ethical considerations of requesting testicular biopsies from healthy subjects. For this reason, controls were primarily selected from patients requesting assisted reproductive technology whose subfertility was not primarily due to a lack of sperm motility. In addition, orchidectomy control numbers 5 and 11, one performed for sex reassignment surgery and the other for locally advanced prostatic cancer, were also included. As is shown in Figures 3 and 4A, the level of CatSper gene expression in these two control orchidectomy cases, who had normal sperm counts and motility (according to parameters determined by the World Health Organization, 1999), was significantly higher than the average level for the other controls. This was true even for control 11, who was 76 years old, despite several reports suggesting a correlation between ageing and reduced sperm motility (Nankin, 1985; Plas et al., 2000; Kidd et al., 2001). Unfortunately, the subfertile control samples had often undergone TESE procedures for reasons such as obstructive azoosperma, making it impossible to identify the exact proportion of motile sperm in their semen.

While our findings suggest a possible correlation between lowered CatSper gene expression levels and deficient sperm motility in a proportion of subfertile patients, the involvement of other factors cannot be discounted. The recent discovery of another voltage-gated channel (CatSper2) with several shared features suggests the possibility of the involvement of other factors in sperm motility (Quill et al., 2001). The relatively high level of CatSper expression in case numbers 6 and 7 can be explained by either of two possibilities. First, these patients might not have any abnormality of CatSper gene expression or function and thus their deficiency might be due to an abnormality(ies) in other sperm motility-related factors. Second, although these cases reveal high CatSper gene expression levels, coding abnormalities at either transcriptional or translational levels may result in either poorly functioning or non-functional CatSper channels.

The findings presented in this study could potentially contribute to the diagnosis and treatment of male infertility cases attributable to deficient sperm motility. Further study on the molecular aspects of CatSper gene function could also result in the use of novel non-hormonal contraceptive, which could be used by either men or women. Among potential candidates are factors capable of binding and blocking the CatSper calcium channel itself. Considering the exclusive localization of CatSper channels in sperm, such a specific blocker would likely have fewer side-effects as compared with hormonal contraceptives.

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