Effect of testis nondescent or orchidopexy on antisperm antibodies and testis histology in rats

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Objective: To examine effects of nondescent of normal testis and of various orchidopexy techniques on antisperm antibody (ASA) production and histologic testicular lesions.

Design: Experimental cohort study.

Setting: Laboratories of surgical research and biology of reproduction, academic medical centers.

Patient(s): Lewis rats, immature and adult.

Intervention(s): Eighteen-day-old rats (6 groups): intra-abdominal stay of testis after closure of inguinal canal, classic dartos pouch orchidopexy, orchidopexy by testis fixation through tunica albuginea, orchidopexy by transparenchymal testicular fixation, sham operation, and bilateral vasectomy. Adult rats (1 group): transparenchymal testicular fixation.

Main Outcome Measure(s): The ASA—antiacrosome and antitail—were measured by indirect immunofluorescence in sera collected preoperatively, on 50th and 120th day in immature rats, and 90 days after surgery in adult rats. Testicular histology was also examined at the end of sera collection.

Result(s): Neither intra-abdominal testicular localization nor orchidopexies induced significant ASA. Testicular nondescent and fixation (transparenchymal or transtunical) caused hypospermatogenesis; dartos pouch was harmless. Bilateral vasectomy produced significantly increased ASA, but no significant testicular lesions. Contralateral testes were unaffected.

Conclusion(s): Intra-abdominal testicular stay and orchidopexy do not elicit autoimmune response to sperm; histologic testicular lesions might not be associated with ASA. In operated cryptorchids, ASA are probably due to other reason than testicular heat or orchidopexy trauma. (Fertil Steril® 2010;94:1504–9. ©2010 by American Society for Reproductive Medicine.)

Key Words: Antisperm antibodies, cryptorchidism, immunofluorescence assay, orchidopexy, testicular histology, testis nondescent
We designed the present prospective study to examine separately the effect of testis nondescent (i.e., exposure to increased temperature) or orchidopexy techniques on serum ASA, as tested by immunofluorescence assay. In separate groups of immature Lewis rats, we inhibited descent of left testes or applied various orchidopexy techniques. We added a group of bilaterally vasectomized rats expected to develop ASA (9) as well as a group with sham operation. Sera were taken before surgery and twice after surgery: in prepuberty, to assure that ASA do not develop against epitopes other than sperm-specific, and in adulthood to examine if ASA were evoked after sperm production. To investigate whether testis fixation could produce ASA in the presence of sperm, we performed it on adult rats and tested for ASA 3 months later, when immune response reaches a maximum (9). To investigate whether ASA were associated with testicular lesions, operated and contralateral testes were histologically examined at the end of follow-up.

MATERIALS AND METHODS
Experimental Groups and Sampling Schedule
This prospective study comprised a total of 84 male Lewis rats, including 74 immature and 10 adult rats. Rats were obtained from the Pasteur Institute, Athens, Greece, and were individually held in cages under a physical light/dark cycle and in a controlled atmosphere with a temperature range of 25 ± 3°C. Rats had access to food and water ad libitum. They were operated under anesthesia (ketamine-chloralhydrate) with a surgical microscope (Carl Zeiss Co., Oberkochen, Germany) under aseptic conditions. All procedures with animals were conducted strictly in accordance with the Guiding Principles in the Care and Use of Animals (DHEW Publication, National Institutes of Health). Before the experiments, ethical approval was acquired from the National Ethical Committee for Animal Experimental Investigations (13/1667/February 4, 2008).

Immature rats were operated on the 18th postnatal day. Testes were still undescended; descent occurs normally at 21–28 days in rats. We established six groups: 1) sham operation (SO; n = 12): negative control; 2) dartos pouch (DP; n = 11): left testis was placed in a pouch between skin and dartos muscle; 3) transtunical fixation (TF; n = 12): left testis was fixed into the scrotum with 9–0 nonabsorbable suture driven through the tunica albuginea without traumatizing testicular parenchyma; 4) fixation (F; n = 11): left testis was fixed into the scrotum (9–0 nonabsorbable suture driven through the parenchyma); 5) mechanically induced “cryptorchidism” (MC; n = 13): unilateral testicular nondescent after closure of the left inguinal ring (8–0 nonabsorbable suture) and anchoring the gubernaculum onto psoas muscle (9–0 nonabsorbable suture); and 6) bilateral vasectomy (BV; n = 15): positive control: the vas deferens was incised between two ligatures (8–0 nonabsorbable sutures). Sera were taken before surgery and twice after surgery: prepubertally (50th day) and in adulthood (120th day).

Adult rats, 120 days old, underwent tranparenchymal fixation (AdF; n = 10), as described above. Sera were collected before surgery and 3 months after surgery.

Operated and contralateral testes were removed after last serum collection; then the animals were killed. Sera were stored at −30°C and later tested together.

Antisperm Antibody Assay
Sera were tested by indirect immunofluorescence assay on spermatozoa smears obtained from rat cauda epididymis (9). Smears were prepared with a cytocentrifuge at 800 rpm for 5 minutes, air dried, and fixed in methanol for 10 minutes at room temperature. Smears were incubated for 1 hour at room temperature with rat sera diluted at 1:10, washed with Earle bovine serum albumin (BSA) solution, then incubated for 30 minutes with the fluorescein-conjugated IgG fraction of antisem to rat IgG, diluted 1:40, washed with Earle BSA, and mounted in Citifluor. Stained smears were observed under an epifluorescence microscope (Nikon E 600). Sera reactions were blindly rated by two independent readers according to an ordinal system scale of 5 categories (− or ±, +, ++, ++++, and ++++), and expressed as 0–4 arbitrary units (AU).

Histology
Removed testes were fixed in Bouin solution and embedded in paraffin. Four μm sections were stained with hematoxylin and eosin. Microscopic evaluation was done blindly by a single observer. Seminiferous tubular diameter was measured using a micrometer eyepiece (>10); the mean was calculated by averaging the diameter of 50 randomly selected round seminiferous tubules in each section. Mean tubuli number and Leydig score (10) were also counted. Spermatogenesis was evaluated by the Johnsen scoring method on a scale of 1–10 (10). Morphology of tissue was studied to identify atrophy, vascular injury, calcification, edema or inflammation.

Statistics
The study had a mean sample size of 12 subjects per group; therefore, it had a power of 73% to detect a difference in means of 0.53 AU (SD = 0.93 AU) on Altman nomogram (11). Comparisons between groups within each measurement were performed using one-way analysis of variance (ANOVA) or Kruskal-Wallis test, depending on normal distribution of data (tested by Shapiro-Wilk test) (12); Bonferonni-Dunn tests followed. McNemar test assessed difference in ASA reactivity between consecutive time intervals (childhood–prepuberty, prepuberty–adulthood) in each group (11). Correlation between ASA reactivity and histologic variables was estimated by Spearman rank correlation coefficient (11, 12). Differences in histologic variables between groups were examined by one-way ANOVA or Kruskal-Wallis tests, followed by Bonferonni-Dunn tests. The SPSS program version 15.0 for Microsoft Windows was used for statistics.

RESULTS
Antisperm Antibodies
Antiacrosome and antitail reactivities in each group are depicted in Figure 1. Some reactivity was found preoperatively against sperm acrosome and tail (Fig. 1), i.e., reactivity >0 AU (1 or 2 AU) in 21 out of 74 rats (28%) and in 8 out of 74 (11%), respectively. Evidently, this reactivity in childhood cannot be attributed to sperm antigens but rather to cross-reactions with other epitopes. Similarly, antiacrosome and antitail reactivity was found in the postoperative measurement in prepuberty (Fig. 1). We found reactivity of 1 or 2 AU in 40 out of 74 (54%) against acrosome and in 23 out of 74 (31%) against tail. The increase of these percentages compared with the first measurement may be the effect of aging; sperm had not been produced yet and these reactivities cannot be attributed to sperm epitopes. However, these reactivities were not affected by testicular nondescent, various orchidopexies (F, TF, DP), or vasectomy (no difference from SO group; Shapiro-Wilk tests: P < 0.5; Kruskal-Wallis tests: anticarboxyme: H = 6.245; df = 5; P = .283; antitail: H = 10.903; df = 5; P = .053).

In the postoperative adulthood measurement, i.e., after sperm production, sperm-specific reactivity due to inhibition of testis descent, orchidopexy, or vasectomy may be added to the nonspecific reactivity acquired with aging. Only the effect of aging was present in the SO group; comparison with it allows for separate examination of the effects of testicular nondecent or orchidopexy on ASA reactivity. Neither minimally invasive orchidopexy techniques in childhood (DP, TF) nor testicular fixation in childhood or adulthood (F, AdF) induced a significant antiacrosome or antitail antibody response compared with the SO group (Shapiro-Wilk test: P > 0.05; Kruskal-Wallis tests: antiacrosome: H = 28.277; df = 6; P < 0.001; antitail: H = 39.43; df = 6; P < 0.001; Bonferonni-Dunn tests: P = 1.0). Similarly, testicular nondecent (MC) did not influence antiacrosome or antitail reactivity (Bonferonni-Dunn tests: P = 1.0). Only BV significantly increased antiacrosome...
TABLE 1

Measures of spread of histologic parameters in left (operated) testes in adulthood.

<table>
<thead>
<tr>
<th>Group</th>
<th>Mean Johnsen score</th>
<th>Mean tubular diameter (μm)</th>
<th>Tubuli number</th>
<th>Leydig score</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median  CI</td>
<td>Median  CI</td>
<td>Median  CI</td>
<td>Median  CI</td>
</tr>
<tr>
<td>SO (n = 12)</td>
<td>9.5   9.5, 9.5</td>
<td>223.0 208.8, 227.8</td>
<td>439 372, 442</td>
<td>3.0 3.0, 3.0</td>
</tr>
<tr>
<td>DP (n = 11)</td>
<td>7.4    5.1, 8.2</td>
<td>198.5 183.3, 205.4</td>
<td>397 386, 427</td>
<td>2.0 2.3, 2.3</td>
</tr>
<tr>
<td>TF (n = 12)</td>
<td>6.1*   3.9, 7.4</td>
<td>181.5* 165.3, 202.9</td>
<td>372 197, 383</td>
<td>2.0 1.9, 1.9</td>
</tr>
<tr>
<td>F (n = 11)</td>
<td>4.4*   3.0, 6.6</td>
<td>201.0* 135.7, 195.4</td>
<td>393 187, 413</td>
<td>2.0* 2.0, 2.0</td>
</tr>
<tr>
<td>AdF (n = 10)</td>
<td>7.9*   3.8, 8.8</td>
<td>190.0 118.3, 206.5</td>
<td>352 167, 396</td>
<td>2.5* 2.2, 2.2</td>
</tr>
<tr>
<td>MC (n = 13)</td>
<td>4.6*   3.6, 6.2</td>
<td>180.0 181.6, 196.4</td>
<td>371 305, 391</td>
<td>2.0 2.2, 2.2</td>
</tr>
<tr>
<td>BV (n = 15)</td>
<td>8.9    6.6, 9.2</td>
<td>211.5 194.4, 220.1</td>
<td>419 393, 439</td>
<td>3.0 2.5, 2.5</td>
</tr>
</tbody>
</table>

Note: AdF = adult rats undergoing fixation (orchidopexy); BV = bilateral vasectomy; CI = 95% confidence interval; DP = dartos pouch (orchidopexy); F = fixation (transparenchymal) (orchidopexy); MC = mechanically induced “cryptorchidism” (intrabdominal testis); SO = sham operation; TF = transtunical fixation (orchidopexy).

*Significant difference from left testes of SO group (P < .0001).

reactivity compared with the SO group and the other groups (Shapiro-Wilk test: $P < .05$; Kruskal-Wallis test: $H = 28.277$; $df = 6$; $P < .0001$; Bonferroni-Dunn tests: $P < .0001$; Fig. 1A). The same result was obtained for antitail reactivity after BV (Shapiro-Wilk test: $P < .05$; Kruskal-Wallis test: $H = 39.43$; $df = 6$; $P < .0001$; Bonferroni-Dunn tests: $P < .0001$; Fig. 1B).

As an additional measure for comparison of the evolution of ASA reactivity, we applied the criterion that an increase of $\geq 2$ AU between two consecutive measurements signifies “positive” ASA (i.e., presence of sperm-specific antibodies) during this time interval. For all orchidopexy techniques and testicular non-descent, no change over time in “positive” ASA between first (childhood–prepuberty) and second (prepuberty–adulthood) postoperative intervals was found (McNemar tests: $P > .06$). Only rats undergoing BV had a significant increase in “positive” antitail antibodies during the second interval compared with the first interval (McNemar test: $P = .03$).

**DISCUSSION**

The present results demonstrate that inhibition of descent of normal testis did not provoke significant ASA production. Therefore, hyperthermia or other factors related to high localization of testis may not be responsible for a defect leading to immunization against sperm, as has been suggested to explain serum ASA in patients with operated cryptorchidism (1, 2). In other studies, testicular hyperthermia also did not harm blood-testis barrier and inter-Sertoli junctions (4, 5).

Neither minimally invasive orchidopexy techniques (DP, TF) nor testis transparenchymal fixation (F) performed in childhood induced significant ASA in adulthood. Apparently, orchidopexy resulted in minor or transient trauma which healed quickly, before postmeiotic spermatozoa antigens appeared. Similarly, fixation in adulthood seemed to cause minor trauma, which, despite sperm presence, did not provoke ASA production. Correspondingly, ASA were absent from adult humans with childhood orchidopexy and subsequent bilateral testicular biopsy (13) or those with adult testis biopsy (14).

In previous experiments with the same design and methods using Sprague-Dawley rats, we found that neither testis non-descent nor orchidopexy techniques induced antiacrosome or antitail antibodies in prepuberty and adulthood; only BV elicited significant antiacrosome antibody production in adulthood, without affecting antitail antibodies. These results (unpublished data) could be attributed to the poor immune response of Sprague-Dawley rats to sperm antigens (9). Therefore, to avoid false negative results, we chose Lewis rats, known to be “good responders” to sperm (9).

Among orchidopexies investigated, DP seems to be the technique of choice, as previously suggested (15), producing neither histologic...
harm to testes nor immunization against sperm. In contrast, F and TF resulted in hypospermatogenesis and decreased tubular diameter.

The present results do not support an etiologic relationship between serum ASA and histologic lesions in operated or contralateral testes, as suggested by others (8). For example, BV induced increased ASA but no histologic alterations; in contrast, testis nondescent or fixation techniques provoked histologic lesions without increasing ASA. In addition, with nondescent or fixation of left testes, no histologic lesions were found in contralateral testes, although operated testes had lesions. It follows that humoral response via ASA is rather improbable as the mechanism of testicular lesions. Others have proposed alternative mechanisms for testicular lesions: Tung and Alexander (16) suggested that there was a T-lymphocyte response to sperm antigens in a delayed hypersensitivity reaction; Bigazzi et al. (9) proposed the existence of a cytotoxic lymphokines–mediated path.

In some animals, either control or experimental, we observed a weak ASA reactivity in childhood, when sperm was absent and rats had not yet undergone surgery. When using methanol-fixed spermatozoa, antibody reactivity could be attributed to antibodies directed against internal antigens cross-reacting with bacterial epitopes (17, 18). These spontaneously occurring antibodies tended to increase with increasing antigenic experience during growth (Fig. 1). This increase is in accord with the findings of Flickinger et al. (19). To exclude this “natural” (nonspecific) reactivity from the overall ASA measured in the experimental groups, we always compared each group with the time-paired measurement of the SO group. The postoperative measurements of the SO group were not affected by the surgical stress of operation: We have previously shown that natural reactivity increases after injury, but normalizes within a time period much shorter than the intervals between measurements in the present study (20).

Immunofluorescence assay is the most specific and frequently used method for ASA detection among available assays in rats. However, cross-reactivity, with consequent false positive results in immunofluorescence assay, is a possible limitation of this study. We aimed explicitly to avoid this bias by comparing experimental groups with both the SO group, expected to have only false positive ASA (negative control), and the BV group, expected to have true positive and false positive ASA (positive control).

The “clinically relevant” serum ASA reported for infertile males with a history of operated cryptorchidism (1, 2, 6) cannot be attributed to mere intrabdominal location of testis or orchidopexy. In cryptorchid testes, ASA may be due to an ongoing developmental anomaly probably unrelated to testis position or orchidopexy. Focal cryptic obstruction of testicular seminiferous tubules (17) or other obstructive anomalies of sperm pathway associated with cryptorchidism could be responsible for serum ASA in operated cryptorchids.

Because we investigated noncryptorchid testes, our study excluded the inherent pathology of cryptorchidism. We documented the effects of testicular nondescent and orchidopexy trauma on ASA production and testis histology. This is the benefit of our animal experimental model, because in humans these effects cannot be investigated separately from cryptorchidism.

**Acknowledgments:** The authors are grateful to Phyllis Bazinet and Carol Foman for skilled editorial assistance.

**REFERENCES**


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**TABLE 1**

<table>
<thead>
<tr>
<th>Group</th>
<th>Vasculitis</th>
<th>Atrophy</th>
<th>Calcifications/microcystosis</th>
<th>Interstitial edema</th>
<th>Granulomas</th>
<th>Polymorphonuclear cells inside seminiferous tubules</th>
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<tr>
<td>SO (n = 12)</td>
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</tr>
<tr>
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<td>0</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TF (n = 12)</td>
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<td>1</td>
<td>2</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>F (n = 11)</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>AdF (n = 10)</td>
<td>0</td>
<td>2</td>
<td>4</td>
<td>0</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>MC (n = 13)</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>BV (n = 15)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

**Note:** Abbreviations as in Table 1.