Gonadal protection from radiation by GnRH antagonist or recombinant human FSH: a controlled trial in a male nonhuman primate (Macaca fascicularis)

A Kamischke, M Kuhlmann, G F Weinbauer, M Luetjens, C H Yeung, H L Kronholz1 and E Nieschlag

Institute of Reproductive Medicine of the University, Domagkstr. 11, D-48129 Münster, Germany

1Department of Radiation Oncology of the University, D-48129 Münster, Germany

(Requests for offprints should be addressed to E Nieschlag; Email: Nieschl@uni-muenster.de)

(G F Weinbauer is now at Covance Laboratories GmbH, D-48163 Münster, Germany)

Abstract

Chemotherapy and radiation often damage spermatogenesis irreversibly in oncological patients and various approaches to gonadal protection have been tested with equivocal results. In rats, hormonal protection of spermatogenesis can be achieved by blocking gonadotropin secretion. However, whether the same mechanisms can effect gonadal protection in primates remains questionable. To clarify this issue we conducted a placebo-controlled trial in a preclinical animal model using macaques (Macaca fascicularis). Twenty adult male monkeys (five in each group) were randomized to receive either recombinant human FSH, GnRH antagonist or saline injections (two groups) for 36 days. On day 29 all groups except one saline-treated control group were exposed to a single testicular irradiation of 4 Gy. Every 2 weeks before, during and after the treatment, ejaculates, body weight, testicular volume and hormones were analyzed until day 539. In addition, repeated testicular biopsies were performed. Testicular volume and inhibin B decreased significantly in all irradiated groups compared with baseline and with the non-irradiated control group, followed by a gradual recovery of these parameters, which was, especially at the earlier time points, significantly better in the FSH-treated group compared with both other irradiated groups. Irradiation caused a drastic decrease of sperm parameters in all groups, followed by a partial recovery of sperm parameters, which was significantly slower in the early phases of recovery in the GnRH antagonist group compared with the vehicle group. Testicular histology showed a significant depletion on study day 261 in all irradiated animals. In conclusion, in clear contrast to rodent studies, GnRH antagonist treatment did not provide gonadal protection in this primate model. FSH treatment resulted in slightly better recovery of spermatogenesis, which appears to be of no or only little clinical relevance.

Introduction

Today some of the common malignancies in children and adults such as testicular tumors, acute leukemias and lymphomas can be cured by surgery, chemoradiotherapy with high success rates. Therefore the long-term toxicity following therapeutic intervention and the resulting quality of life become increasingly important (Kliesch et al. 2000). Chemo- and/or radiotherapy almost always damages spermatogenesis in oncological patients. However, depending on substance and dosage, the degree and rate of spermatogenic damage vary from nearly full recovery (e.g. cycles involving mainly mitoxantrone, doxorubicin and thioguanine) to irreversible (e.g. if multiple cycles of alkylating substances are involved) Sertoli-cell-only syndrome (Meirow & Schenker 1995). In men following single measured doses of 6 Gy and above (Rowley et al. 1974) and low-dose fractionated irradiation of 1.5 Gy and above (Greiner 1982) irreversible damage of spermatogenesis can be expected. Monkey studies showed that proliferating and non-proliferating spermatogonia were most sensitive to irradiation (van Alphen et al. 1988a,b).

To date cryopreservation of semen before oncological therapy represents the only option for maintaining reproductive capacity (Kliesch et al. 2000). Theoretically, hormonal gonadal protection (Meistrich 1998) and retransplantation of germ cell stem cells (Schlatt 1999), preserved prior to chemotherapy, offer experimental methods to preserve spermatogenesis and fertility. Various clinical and preclinical studies for hormonal gonadal protection of the testes have been performed and most studies in rats have
shown efficacy via blocking gonadotropin secretion and lowering intratesticular testosterone (Delic et al. 1986, Kangasniemi et al. 1995a,b; Meistrich & Kangasniemi 1997, Meistrich et al. 1997, 1999, 2000) with treatments given prior to or even after toxic treatment. However, so far this principle has failed in oncological patients treated with gonadotropin-releasing hormone (GnRH) agonists (Johnson et al. 1985, Waxman et al. 1987, Krause & Pfuger 1989, Brennemann et al. 1994).

This failure is primarily attributed to the short treatment period and insufficient suppression of gonadotropins by GnRH agonists (Behre et al. 1992). Unlike the concept of suppressing gonadotropin secretion, in the rhesus monkey an opposing concept of gonadal protection was shown by stimulation of spermatogenesis via additional administration of follicle-stimulating hormone (FSH) (van Alphen et al. 1989b). The putative underlying mechanism is stimulation of the spermatogenic process, as previous studies have shown that FSH stimulates the number of proliferating spermatogonia in normal monkeys (van Alphen et al. 1988a,b; Weinbauer et al. 1992, Plant & Marshall 2001). In addition, the beneficial effect of FSH is proposed to act via increased activity of DNA repair mechanisms (van Alphen et al. 1989b).

Since controlled studies to evaluate the opposing concepts of stimulating or suppressing spermatogenesis for gonadal protection are impossible to perform in humans, this randomized controlled study was conducted in a nonhuman primate model, to clarify whether gonadal protection can be achieved by either concept.

Animals and methods

Animals and experimental design

Twenty adult intact male cynomolgus monkeys (Macaca fascicularis) from our monkey colony were used in the study (mean ± s.e.m. age 13·6 ± 1·0 years; mean ± s.e.m. weight 7·5 ± 0·4 kg). All animals were caged individually and were maintained in a temperature- and humidity-controlled room, with a 12 h light:12 h darkness period. They were fed a standard monkey pellet food supplemented with fresh fruit. Water was freely available. The monkeys had no contact with female animals. For all interventions (except the s.c. injections) including unilateral castration, monkeys were sedated by i.m. injection of ketamine hydrochloride (8–10 mg/kg) (Park-Advise, Munich, Germany). Maintenance and experimentation were performed in accordance with the German Law for Care and Use of Laboratory Animals.

Prior to randomization hormones (inhibin B, testosterone), semen analysis (weight, sperm count, sperm motility, eosin test, computer-assisted sperm analysis (CASA)), body weight and testicular volume were determined. In addition, at study day 0 testicular biopsies were performed as described previously (Weinbauer et al. 1998). The animals were randomly allocated to four groups (five monkeys in each group) according to testicular volume (initial maximal variation 7%). The investigators were blinded to the experimental status of the animals. Mean ± s.e.m. age was 16·2 ± 1·6 years in the GnRH antagonist, 15·2 ± 1·8 in the recombinant human FSH (rhFSH), 14·8 ± 1·6 in the vehicle and 17·2 ± 1·3 in the control group. Further examinations were performed on study days 14, 28 and 50 and from then onwards every 2 weeks until study day 540 and included semen analysis (except CASA every 4 weeks), body weight, testicular volume and hormone analysis. One examination at day 470 was not performed. Testicular biopsies were performed on study days 36 and 259 and unilateral castration on day 554.

Starting on day 0 the groups (five animals each) were treated 29 days prior to irradiation according to established monkey protocols (van Alphen et al. 1989b, Weinbauer et al. 1998, 2000). However, as studies have shown efficacy of hormonal gonadal protection even if the treatment is administered after irradiation, the treatment was prolonged for 7 further days after irradiation until study day 36. Treatment group I was treated with twice-daily s.c. injections of 15 IU rhFSH (Gonal-F; Serono, Unterschleißheim, Germany). Group II was treated with once-daily s.c. injections of 450 μg/kg of the GnRH antagonist cetorelix (Cetrotide; ASTA Medica, Frankfurt, Germany) and one s.c. saline injection. The irradiated vehicle-treated group (group III) and the non-irradiated control group (group IV) received twice-daily s.c. saline injections.

On day 29, all groups except one saline group (non-irradiated control group) were exposed to X-ray irradiation of both testes in a 12 × 12 cm field using a Mevatron 77 accelerator (Siemens, Munich, Germany) applying 10 MeV photons. The build-up effect was due to an acrylic glass plate of 2·2 cm thickness. The dose applied at the surface was 4·0 ± 0·06 Gy. The exit dose at the testes was 3·84 Gy, the equivalent of a testicular dose of 3·8 ± 0·06 Gy at a depth of 1·5 cm. The maximum irradiation of the remaining body was estimated as 0·0006 Gy.

Nineteen animals remained healthy as evidenced by body weight, physical inspection and food consumption and completed the study. One monkey (non-irradiated control group) had to be killed as it developed a severe illness at study day 406. Histopathological examination revealed severe hepatic failure probably caused by a lymphosarcoma.

Measurements

Testes volumes Testis volume was determined as described previously by measuring the length and width of the testis using Vernier calipers (Weinbauer et al. 1998).
The formula for an ellipsoid was used to estimate testicular volume.

**Hormone assays** Prior to all other interventions, blood samples were collected during the morning hours by venipuncture of the saphenous vein. Blood was allowed to coagulate overnight at 4 °C, after which the serum was separated and stored at −20 °C until analyzed. Inhibin B was assayed using a two-site enzyme-linked immunosorbent assay (Serotec, Oxford, UK); sensitivity was 7.8 pg/ml and intra- and interassay coefficients of variation (CV) values were 3.6 and 8.4% respectively. Testosterone was assayed by an established RIA (Chandolia et al. 1991). Sensitivity of the testosterone assay was 0.68 nmol/l and intra- and interassay CV values were 6.3 and 8.4% respectively.

**Semen analysis** Ejaculates were obtained every 2 weeks by rectal probe electroejaculation, and evaluation of sperm numbers was performed as described previously (Weinbauer et al. 1994). Sperm counts were expressed per total ejaculate (exudate plus coagulum). Sperm morphology (Papanicolaou staining), sperm motility and eosin test assessments were performed every 2 weeks according to the WHO Manual (World Health Organization 1999). Epididymal sperm (only at unilateral castration on day 554) were collected from the mid-cauda region and dispersed in BMW medium. CASA for kinematic measurements of motile sperm from both the ejaculates and epididymis were performed as described (Yeung et al. 1996) using a newer version of the Hamilton Thorne CASA system.

**Testicular histology** On study days 0, 36 and 259 open testicular biopsies were performed. Tissue was fixed in Bouin’s solution, embedded in paraffin and cut at a section thickness of 3 µm for light microscopy as described previously (Weinbauer et al. 1998). Sections were stained with periodic acid Schiff’s base and haematoxylin and tubules were scored for the most advanced germ cells. In addition, the repopulation index (percentage of tubular cross-sections containing at least spermatogonia) was calculated as described previously (van Alphen et al. 1989b). In the biopsies all tubular cross-sections present were counted (mean 43 ± 4 S.E.M.). After unilateral castration 200 tubular cross-sections were counted. One biopsy (day 259) in a rhFSH-treated monkey could not be evaluated as it revealed only rete testes.

**Flow cytometry**

For flow cytometry analysis, dissected testicular tissue fragments were transferred into PBS and were stored in 80% ethanol at 4 °C. In a small modification of the previous protocol (Krishnamurthy et al. 1998, Weinbauer et al. 2001a), after gently shaking to resuspend the cells, 3 ml ethanol-fixed testicular cells were washed in PBS without Mg2+ and Ca2+, incubated with 0.5 ml pepsin solution (1% in PBS without Mg2+ and Ca2+) for 15 min at 37 °C and centrifuged at 400 g for 5 min. After propidium iodide treatment, cells were analyzed using a Coulter flow cytometer EPICS XL (Coulter, Krefeld, Germany) as described previously (Weinbauer et al. 2001a).

**Statistics**

Initially, differences between the study groups were evaluated by two-way ANOVA for repeated measurements. In the case of an overall significance in the ANOVA, ANOVA was followed by a Bonferroni post-hoc test for evaluating differences at every single time point. Variations over time within the study group were evaluated by Friedman ANOVA for repeated measurements. In the case of an overall P < 0.05 in the ANOVA, differences between baseline values and the following time points were tested by Dunn’s multiple comparison post-hoc test. Data given as percentages were arcsin square root transformed before analysis. All analyses were performed using the statistical software GraphPad Prism for Windows version 2.01 (GraphPad Software Inc., San Diego, CA, USA). Two-sided P values of 0.05 were considered significant. In general, results are given as means ± S.E.M.

**Results**

**Body weight and testes volume**

Body weight (Table 1) remained unchanged in the vehicle group and showed significant reductions in the cetrorelix group from day 64 until day 218 (with the exception of day 148).

Testicular volumes in the control group remained unchanged (Fig. 1a). Compared with baseline and with the control group in all irradiated groups a significant decrease of testicular volume was detected. Compared with baseline this difference was significant for the GnRH antagonist group from day 78 until day 334 (excluding days 273, 287 and 335), for the rhFSH group from day 64 until day 287 and for the vehicle group from day 92 until day 344 (excluding days 148, 190, 273, 322 and 335). Between the irradiated groups, testicular volumes in the rhFSH-treated group were significantly higher compared with the vehicle (only in the overall ANOVA and not in the post-hoc tests) and the cetrorelix group (day 28 and 50). In addition, testicular volumes in the cetrorelix group were significantly smaller than in the vehicle group (day 28).

**Hormones**

During the treatment phase, testosterone levels in the cetrorelix group were reduced to a mean 26% (day 14) and 21% (day 28) of initial values (Fig. 1c). However, in the overall ANOVA (but not in the post-hoc tests) not only
the cetrorelix group showed significant variations of testosterone levels; significant variations of testosterone levels could also be detected in the vehicle group while in both other groups testosterone levels remained unchanged. Between the groups, differences in testosterone levels could be detected for the vehicle group compared with all

Table 1 Body weight and ejaculate weight of the rhFSH, GnRH antagonist, vehicle (irradiated) and control groups (not irradiated) over time (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Mean baseline</th>
<th>Day 28</th>
<th>Day 50</th>
<th>Day 106</th>
<th>Day 162</th>
<th>Day 246</th>
<th>Day 330</th>
<th>Day 442</th>
<th>Day 540</th>
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<tbody>
<tr>
<td>Body weight (kg)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6.1 ± 0.4</td>
<td>6.0 ± 0.3</td>
<td>7.4 ± 0.7</td>
<td>6.1 ± 0.4</td>
<td>6.0 ± 0.4</td>
<td>6.2 ± 0.4</td>
<td>5.9 ± 0.4</td>
<td>5.8 ± 0.4</td>
<td>5.8 ± 0.4</td>
</tr>
<tr>
<td>Vehicle</td>
<td>8.2 ± 0.9</td>
<td>8.0 ± 0.9</td>
<td>8.0 ± 0.8</td>
<td>8.0 ± 0.7</td>
<td>7.7 ± 0.6</td>
<td>7.9 ± 0.6</td>
<td>7.6 ± 0.6</td>
<td>7.5 ± 0.7</td>
<td>7.8 ± 0.7</td>
</tr>
<tr>
<td>rhFSH</td>
<td>8.1 ± 0.7</td>
<td>8.1 ± 0.5</td>
<td>8.3 ± 0.5</td>
<td>8.1 ± 0.5</td>
<td>7.8 ± 0.5</td>
<td>7.8 ± 0.5</td>
<td>7.6 ± 0.4</td>
<td>7.6 ± 0.3</td>
<td>7.8 ± 0.3</td>
</tr>
<tr>
<td>GnRH antagonist</td>
<td>7.2 ± 0.5</td>
<td>6.8 ± 0.6</td>
<td>6.5 ± 0.5</td>
<td>6.1 ± 0.5</td>
<td>6.1 ± 0.4</td>
<td>6.6 ± 0.4</td>
<td>6.5 ± 0.4</td>
<td>6.9 ± 0.5</td>
<td>7.2 ± 0.6</td>
</tr>
<tr>
<td>Ejaculate weight (g)</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.7 ± 0.2</td>
<td>1.4 ± 0.6</td>
<td>0.4 ± 0.1</td>
<td>0.5 ± 0.2</td>
<td>0.8 ± 0.4</td>
<td>0.9 ± 0.3</td>
<td>1.0 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>1.7 ± 0.7</td>
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<td>Vehicle</td>
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<td>2.3 ± 0.9</td>
<td>2.4 ± 1.3</td>
<td>1.2 ± 0.5</td>
<td>1.8 ± 0.8</td>
<td>1.9 ± 0.8</td>
<td>2.0 ± 1.0</td>
<td>1.0 ± 0.4</td>
<td>1.6 ± 0.6</td>
</tr>
<tr>
<td>rhFSH</td>
<td>1.0 ± 0.3</td>
<td>1.4 ± 0.9</td>
<td>1.2 ± 0.4</td>
<td>0.9 ± 0.4</td>
<td>1.2 ± 0.4</td>
<td>1.0 ± 0.3</td>
<td>0.8 ± 0.3</td>
<td>1.2 ± 0.6</td>
<td>1.1 ± 0.6</td>
</tr>
<tr>
<td>GnRH antagonist</td>
<td>0.4 ± 0.2</td>
<td>0.1 ± 0.0</td>
<td>(2)</td>
<td>(3)</td>
<td>(3)</td>
<td>0.1 ± 0.2</td>
<td>1.6 ± 0.5</td>
<td>0.7 ± 0.3</td>
<td>0.9 ± 0.2</td>
</tr>
</tbody>
</table>

If not all monkeys of one group could be analyzed, actual numbers of animals analyzed are given in parentheses.

*Significantly different from baseline.

Figure 1 Total testicular volume (a), inhibin B serum concentration (b) and serum testosterone concentrations (c) in the GnRH antagonist group I, rhFSH group, vehicle group and control group. The treatment phase is indicated as a hatched box. The time point of irradiation is indicated by the arrow. Results are given as means ± S.E.M.
other groups and for the rhFSH group. However, none of these differences were significant in the post-hoc tests at a single time point.

No differences in inhibin B serum levels were detected for the control group during the whole study. Within all irradiated groups a significant decrease of inhibin B serum levels was seen, which followed an initial stimulation to a maximal 177% (day 28) of baseline values in the rhFSH group (Fig. 1b). Compared with baseline this difference was significant for the GnRH antagonist group from day 92 until day 308, for the rhFSH group from day 78 until day 204 and for the vehicle group from day 78 until day 246 (excluding days 148, 204, 218 and 332). Compared with the control group, differences in inhibin B serum levels for the GnRH antagonist (from day 50 until day 322 plus days 344 until 393 and days 420, 434, 483 and 540) and vehicle group (day 50 until day 322 plus days 344 until 399 and days 420, 434, 483 and 540) were, with exceptions, significant from the nadir until the end of the study, while for the rhFSH group differences were mostly only evident around the nadir of the experiment (from day 78 until day 148 plus days 176, 218 and 287). Between the irradiated groups, inhibin B levels in the rhFSH group were significantly higher compared with the GnRH antagonist-treated group (days 14 and 28) and vehicle group (day 28) during the treatment phase, which were themselves not significantly different.

**Semen parameters**

In the GnRH antagonist group the majority of monkeys did not ejaculate on days 28, 50, 64, 78, 92, 106 and 120 respectively. These days were omitted from the statistical calculations. Except for a significant reversible decrease in the GnRH antagonist group, in all other groups no significant variations of ejaculate weight could be detected compared with baseline (Table 1). Between the groups, ejaculate weight in the GnRH antagonist-treated group was significantly lower compared with all other groups, while in the vehicle group it was significantly higher than in all other groups. However, none of the observed overall significant differences in the ANOVA became significant in the post-hoc tests.

All irradiated groups showed a sharp significant decrease of sperm count (GnRH antagonist from day 50 until day 218; rhFSH from day 106 until day 232 plus days 273, 363, 420 and 526; vehicle from day 92 until day 218 plus day 273) compared with baseline with a gradual recovery afterwards (Fig. 2a). Except for a significant increase of sperm count on study day 258, no differences could be detected for the control group. Compared with the control group, all irradiated groups showed a significant reduction of sperm count (GnRH antagonist: days 64, 162, 204–287, 393 and 525; rhFSH: days 162, 204, 218, 246–287, 393 and 525; vehicle: day 258). Significant differences in sperm counts between the groups could be detected in the GnRH antagonist-treated group compared with the rhFSH– (day 50) and vehicle-treated (day 50 and 64) groups during the early phases of recovery. In addition, sperm counts in the vehicle group were significantly higher during the treatment phase and early phases of recovery (days 28 and 50) compared with the rhFSH-treated group.

Within the control group, progressive sperm motility remained unchanged and significantly higher compared with all irradiated groups (GnRH antagonist: days 190, 273, 335 and 363; rhFSH: days 78 and 162; vehicle: days 106 and 162). Except the rhFSH group, in all irradiated groups significant reductions of progressive sperm motility compared with baseline were observed in the overall ANOVA while the post-hoc tests did not show any significant differences at single time points (Fig. 2b).

All irradiated groups showed significant reductions in normal sperm morphology compared with baseline and control at numerous time points (data not shown).

From day 28 until day 316 an eosin vitality test was possible only occasionally in all irradiated groups due to missing values because of azoospermia or severe oligozoospermia or anejaculation. Within the groups, significant variations could be observed for the vehicle and cetorelix group in the overall ANOVA (no significances in the post-hoc tests) while within the rhFSH group and in the control group no differences compared with baseline could be observed (Fig. 2c). In the ANOVA, between the groups significant reductions of vital cells could be detected for all irradiated groups compared with control, which were significant at the post-hoc tests only for the cetorelix group (at days 14, 335 and 363).

CASA for kinematic parameters of motile sperm from the mean of the two pretreatment ejaculates and the mean of the samples on study days 512 and 540 revealed no differences compared with baseline or with control (data not shown). In addition, CASA of epididymal sperm obtained at hemicastration showed no differences between the groups (data not shown).

**Testicular histology**

Results of testicular histology and flow cytometric analysis are shown in Table 2. No significant differences could be detected for the percentages of cells in the S-phase of the cell cycle within or between the study groups (data not shown).

**Influence of age**

Analyzing the data there appeared to be a minor age effect in all irradiated groups; the recovery of testicular volume (Fig. 3a), inhibin B (Fig. 3b), sperm parameters (Fig. 3c) and testicular histology (Fig. 4a and b), was a little better in the younger (<12 years) vs the older (17–20 years) monkeys. The only exceptions were testicular volume,
sperm count and the repopulation index in the rhFSH group, where better recovery of the older monkeys could be detected. However, due to the low numbers of two or three younger animals in each treatment group, valid statistical analysis of the effects of treatment and age were not possible.

Discussion

Testicular irradiation in mice, rats, monkeys and men uniformly decreases the number of differentiating spermatogonia and later depletes the more advanced spermatogenic cells (Rowley et al. 1974, Clifton & Bremner 1983, van Alphen et al. 1988a, 1989a, b, Meistrich et al. 2000, Shuttlesworth et al. 2000). In rhesus monkeys and humans after single doses of 4 and 6 Gy it takes 5 years or more for spermatogenesis to return to preirradiation germinal cell numbers and sperm concentrations (Rowley et al. 1974, van Alphen et al. 1988a). Others have used doses of 1–4 Gy for the depletion of the seminiferous epithelium or studies on gonadal protection in rhesus monkeys (van Alphen et al. 1988a, b, 1989a, b). Previously we used an irradiation dose of 2 Gy in cynomolgus monkeys which was sufficiently high to induce azoospermia for more than 200 days (Schlatt et al. 2002). Surprisingly, following a dose of 4 Gy used in this study only one monkey in the vehicle group developed temporary azoospermia while all monkeys in the GnRH antagonist group and two monkeys in the rhFSH group developed temporary azoospermia. In all monkeys, sperm could be detected in the ejaculate at study end, but only two irradiated monkeys developed stable sperm concentrations in the range of the baseline values, while most others presented with oligozoospermia. None of the irradiated monkeys achieved baseline testicular volumes and only one monkey in each group reached baseline inhibin B values. The great variability seen within each group is comparable with the human situation where irradiation also leads to variable changes in testicular histology and

Figure 2 Total sperm count (a), progressive sperm motility (WHO a+b) (b) and vital cells (unstained with eosin) (c) in the GnRH antagonist group I, rhFSH group, vehicle group and control group. The treatment phase is indicated as a hatched box. The time point of irradiation is indicated by the arrow. Results are given as means ± S.E.M. During days 28–92 most of the monkeys of the GnRH antagonist group failed to produce an ejaculate.
Table 2  Testicular histology and flow cytometry data of the rhFSH, GnRH antagonist, vehicle (irradiated) and control groups (non-irradiated) prior to treatment (day 0) and 7 (day 36), 230 (day 259) and 525 days (day 554) after irradiation (means ± S.E.M.)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Group</th>
<th>Day 0</th>
<th>Day 36</th>
<th>Day 259</th>
<th>Day 554</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tubuli containing elongated spermaticids (%)</td>
<td>Control</td>
<td>72 ± 7</td>
<td>74 ± 4</td>
<td>54 ± 2</td>
<td>73 ± 9</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>67 ± 8</td>
<td>64 ± 8</td>
<td>14 ± 6b</td>
<td>48 ± 10</td>
</tr>
<tr>
<td></td>
<td>rhFSH</td>
<td>63 ± 6</td>
<td>73 ± 4</td>
<td>20 ± 2b</td>
<td>55 ± 7</td>
</tr>
<tr>
<td></td>
<td>GnRH antagonist</td>
<td>72 ± 5</td>
<td>61 ± 4</td>
<td>24 ± 11</td>
<td>34 ± 13b</td>
</tr>
<tr>
<td>Tubuli containing spermatogenesis up to the round spermatid level (%)</td>
<td>Control</td>
<td>90 ± 8</td>
<td>86 ± 2</td>
<td>70 ± 3</td>
<td>77 ± 7</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>84 ± 8</td>
<td>78 ± 16</td>
<td>21 ± 7b</td>
<td>52 ± 10</td>
</tr>
<tr>
<td></td>
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<td>80 ± 8</td>
<td>86 ± 3</td>
<td>26 ± 2b</td>
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<tr>
<td></td>
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<td>87 ± 1</td>
<td>27 ± 11b</td>
<td>37 ± 13b</td>
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<tr>
<td>Tubuli containing spermatogenesis up to the spermatocyte level (%)</td>
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<td>98 ± 2</td>
<td>92 ± 4</td>
<td>84 ± 6a</td>
<td>97 ± 3</td>
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<tr>
<td></td>
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<td>87 ± 6</td>
<td>78 ± 8</td>
<td>25 ± 6b</td>
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<td>84 ± 3</td>
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<td>46 ± 16b</td>
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<td>Repopulation index (%) compared with day 0</td>
<td>Control</td>
<td>100 ± 0</td>
<td>94 ± 1 9</td>
<td>86 ± 2 7a</td>
<td>98 ± 1 3b</td>
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<td>Vehicle</td>
<td>100 ± 0</td>
<td>95 ± 16 5</td>
<td>32 ± 3 8a</td>
<td>71 ± 1 12b</td>
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<tr>
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<td>48 ± 6 7b</td>
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<td>GnRH antagonist</td>
<td>100 ± 0</td>
<td>80 ± 1 4 6</td>
<td>36 ± 14 9ab</td>
<td>54 ± 21 3b</td>
</tr>
<tr>
<td>Flow cytometry, HC1 and 1C2 cells (%)</td>
<td>Control</td>
<td>68 ± 1 ± 3</td>
<td>680 ± 2 1</td>
<td>641 ± 2 8</td>
<td>670 ± 2 7</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>66 ± 8 ± 1</td>
<td>673 ± 1 0</td>
<td>489 ± 4 5b</td>
<td>658 ± 3 2</td>
</tr>
<tr>
<td></td>
<td>rhFSH</td>
<td>65 ± 5 ± 1</td>
<td>664 ± 1 1</td>
<td>55 ± 2 1</td>
<td>716 ± 1 4</td>
</tr>
<tr>
<td></td>
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<td>690 ± 1 ± 2</td>
<td>684 ± 1 7</td>
<td>51 ± 1 5</td>
<td>584 ± 4 7</td>
</tr>
<tr>
<td>Flow cytometry, 2C (diploid) cells (%)</td>
<td>Control</td>
<td>16 ± 4 ± 1</td>
<td>156 ± 0 5</td>
<td>17 ± 1 0</td>
<td>17 ± 2 0 4</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>16 ± 6 ± 0 5</td>
<td>166 ± 0 6</td>
<td>33 ± 4 0b</td>
<td>20 ± 8 2 0</td>
</tr>
<tr>
<td></td>
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<td>26 ± 2 5b</td>
<td>16 ± 5 0 6</td>
</tr>
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<td></td>
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<td>188 ± 1 2</td>
<td>32 ± 5 3b</td>
<td>27 ± 0 4 0</td>
</tr>
<tr>
<td>Flow cytometry, 4C (tetraploid) cells (%)</td>
<td>Control</td>
<td>8 ± 2 ± 0 7</td>
<td>10 ± 4 ± 1 5</td>
<td>12 ± 1 1 5</td>
<td>9 ± 2 ± 2 1</td>
</tr>
<tr>
<td></td>
<td>Vehicle</td>
<td>10 ± 8 ± 0 5</td>
<td>10 ± 7 ± 1 3</td>
<td>12 ± 0 ± 1 0</td>
<td>8 ± 0 ± 8</td>
</tr>
<tr>
<td></td>
<td>rhFSH</td>
<td>10 ± 4 ± 0 7</td>
<td>9 ± 2 ± 1 1</td>
<td>9 ± 8 ± 0 2</td>
<td>6 ± 9 ± 0 6</td>
</tr>
<tr>
<td></td>
<td>GnRH antagonist</td>
<td>7 ± 7 ± 0 7</td>
<td>6 ± 1 ± 0 6d</td>
<td>10 ± 5 ± 7a</td>
<td>8 ± 7 ± 0 4</td>
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</table>

*aSignificantly different from baseline; bSignificantly different from control; cSignificantly different from rhFSH; dSignificantly different from vehicle.

Most of the studies performed in rats and one study in two baboons have shown that blocking gonadotropin secretion can lead to hormonal protection even if the treatment is applied after irradiation (Clifton & Bremmer 1983, Lewis et al. 1985, Meistrich 1998, Meistrich et al. 2000, Shuttlesworth et al. 2000). In our study we used a GnRH antagonist which was successfully used in rat hormonal protection studies (Shuttlesworth et al. 2000) and showed superior gonadotropin suppression compared with GnRH agonists (Weinbauer et al. 1994, 1998). As a result of GnRH antagonist treatment, testosterone levels were decreased by up to 21% of initial values during the treatment phase (Fig. 1c), which resulted in significant reversible reductions of body weight (possibly as a catabolic effect of androgen deficiency), ejaculate weight (possibly as a consequence of impairment of accessory gland function) and temporary anejaculations (Table 1). In addition, on the day prior to irradiation in the cetrorelix group inhibin B values and testicular volume were decreased to 57 and 58% of baseline values respectively, as a consequence of inhibition of type A to type B spermatogonial proliferation (Weinbauer et al. 1998, Zhengwei et al. 1998a,b). In contrast to the majority of rat studies, and in agreement with studies with GnRH agonists (Johnson et al. 1985, Waxman et al. 1987, Krause & Pfuger 1989, Bremennann et al. 1994) or gestagens (Fossa et al. 1988) for hormonal gonadal protection in oncological patients, we failed to achieve hormonal gonadal protection. The suppressive effects of the GnRH antagonist produced an additive effect on the irradiation-induced suppression, sperm concentrations (Rowley et al. 1974, Clifton & Bremmer 1983). Probably because of the great individual variability to cytotoxic treatments, at the end of the study the mean number of sperm per ejaculate in the vehicle group was non-significantly higher than in both other irradiated groups, a fact caused by extraordinarily good recovery of one single monkey in this group. Without this monkey, the mean sperm count in the vehicle group would be comparable with the GnRH antagonist group.
resulting in more severe effects on testicular volume, sperm count, sperm morphology and spermatogonial proliferation, as indicated by the 4C to 2C ratios, which overall were significantly reduced in the GnRH antagonist-treated group in comparison with the vehicle groups, especially in the early phases of recovery. Whether longer treatment with cetrorelix after irradiation might have given rise to different results remains speculative. While in the human studies, the failure of the blocking approach to hormonal protection could be attributed primarily to the insufficient gonadotropin and spermatogenesis suppression of the GnRH agonists and short duration of hormonal pretreatment (3–16 days), our study suggests different/additional reasons for this failure. In rodent studies, hormonal protection is supposed to act primarily via lowering intratesticular testosterone levels to 3–10% of baseline values, although it is not known how testosterone induces this effect (Clifton & Brenner 1983, Lewis et al. 1985, Meistrich et al. 1996, 2000, Shetty et al. 2000, Shuttlesworth et al. 2000). In monkeys and humans, however, after gonadotropin withdrawal, high amounts of testicular androgens remain, which should not severely affect spermatogenic regulation in primates (Morse et al. 1973, Huhtaniemi et al. 1987, Weinbauer et al. 1988, 2001b, Zhengwei et al. 1998b, McLachlan et al. 2002). A further reason for the failure of the GnRH analogues might be different hormonal and spermatogenetic regulation in rodents and primates, as in rats testosterone seems to be the dominant hormone for rat spermatogenesis (Awoniyi et al. 1992, Zirkin 1993) while in the cynomolgus monkey and humans the combination of testosterone/luteinizing hormone and FSH activity is mandatory for stimulating qualitatively and quantitatively normal spermatogenesis (Weinbauer & Nieschlag 1998, Weinbauer et al. 2000, 2001b, Plant & Marshall 2001, McLachlan et al. 2002). In addition, the less favorable results of the cetrorelix group might in part be explained by the generally smaller recovery of the older monkeys, i.e. three older animals in the cetrorelix group, but only two older animals in the vehicle and rhFSH group (Figs 3 and 4).
Figure 4 Tubuli containing spermatogenesis up to elongated spermatids (a) and percentage of tubuli showing repopulation (b) in the GnRH antagonist group I (circles), rhFSH group (squares), vehicle group (triangles) and control group (diamonds). Old monkeys (17–20 years of age) are shown as filled symbols, young animals are shown as open symbols.
In contrast to rats, in primates the number of surviving spermatogonia determines the grade of spermatogenic recovery after irradiation (Rowley et al. 1974, van Alphen et al. 1988a, 1989a). Administration of FSH in rhesus and cynomologus monkeys has been shown to increase the total number of spermatogonia within 28 days via increased spermatogonial proliferation (van Alphen et al. 1988a, Weinbauer et al. 1992, Foresta et al. 1998, Plant & Marshall 2001). In a study for hormonal gonadal protection in four rhesus monkeys after 16 days of FSH stimulation (twice-daily 15 IU) the number of total A spermatogonia and Apale spermatogonia increased to 117 and 153% respectively compared with controls (van Alphen et al. 1989b). During the treatment phase we also observed a stimulatory effect of FSH on early spermatogenic proliferation, as evidenced by a 77 and 13% increase in inhibin B and testicular volume in the rhFSH-treated group respectively (Fig. 1a and b). Due to the radiation a sharp decrease of inhibin B and bi-testicular volume to 19 and 35% of baseline values respectively was evident, which was only slightly different in both other irradiated groups.

However, there is evidence that the initial stimulatory effect of rhFSH on spermatogenesis during the treatment phase was also maintained after irradiation as inhibin B (Fig. 1b) and total bi-testicular volume (Fig. 1a) in the rhFSH-treated group were not suppressed as long as in both other irradiated groups. This beneficial effect of FSH is only small, tends to disappear with increasing study duration and was not seen in all parameters investigated. Irrespective of the treatment performed, the younger monkeys showed a minor tendency for a generally better recovery compared with the older monkeys, which appears to be reversed by the treatment in the older monkeys of the rhFSH group (Figs 3 and 4). A similar better recovery has also been described in younger vs older mice after exposure to ionizing radiation (Meistrich et al. 1978). The mechanisms underlying this tendency for a better recovery in the younger animals and older monkeys treated with FSH are unclear. However, as in humans, decreased endocrine reserve capacity of the pituitary and Leydig cells as well as impaired spermatogenic efficiency (Rolf & Nieschlag 2000) might contribute to the lower recovery rate of the older monkeys, which might be reversed by FSH treatment. However, as the magnitude of the benefits observed in the older monkeys of the rhFSH group is comparable with the better recovery seen in the younger monkeys of the other irradiated groups, overall it remains questionable whether the beneficial effect of FSH has any clinical relevance for younger oncological patients.

In conclusion, GnRH antagonist treatment did not provide gonadal protection in the irradiated nonhuman primate model. In agreement with others, FSH treatment resulted in slight advantages in terms of recovery of inhibin B, testicular volume and histology (as evidenced by faster restoration compared with controls), which were probably of no or only minor clinical relevance. However, whether these beneficial effects of FSH warrant a clinical study for gonadal protection remains questionable, as a similar recovery of the investigated parameters could be observed in the younger vs the older monkeys in the other irradiated groups.

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