Inhibin B is a more sensitive marker of spermatogenetic damage than FSH in the irradiated non-human primate model

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Abstract

This study evaluated the effect of bilateral testicular irradiation (2 Gy) on reproductive hormones, testicular volume (TV) and sperm parameters in six adult cynomolgus monkeys. Hormone levels (FSH, inhibin B and testosterone (T)) were determined to find the most valuable endocrine marker of irradiation-induced damage. All parameters were analysed at weekly intervals for 14 weeks. Histological evaluation of both testes was performed at week 14 after irradiation when one monkey was castrated and at week 27 when the remaining five monkeys were bilaterally biopsied. A decrease in body weight, TV (30% of the pre-treatment size) and sperm count was observed after irradiation. Severe oligozoospermia was achieved throughout the study but azoospermia was recorded only occasionally. Histological evaluation revealed a heterogeneous picture with patchy arrangement of seminiferous tubules containing advanced germ cell types. An increase (P<0.05) in FSH levels and, to a lesser degree also in T levels, occurred several weeks after irradiation. Inhibin B levels showed a sharp decline (P<0.001) as soon as 1 week after irradiation. FSH and inhibin B did not return to baseline levels during the observation period. A negative correlation was found between FSH and inhibin B values (r = -0.35, P<0.001). Inhibin B correlated positively with testis volume (r=0.73, P<0.001) and sperm counts (r=0.55, P<0.01). In conclusion, this study shows that inhibin B represents an early and more sensitive marker of testicular damage than FSH. Furthermore, the rapid fall of inhibin B after irradiation suggests that this hormone is a direct parameter of premeiotic germ cell proliferation.

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Introduction

The testis, presenting many rapidly proliferating cells, is an ideal site to investigate the effects of cytotoxic agents such as radiation and chemotherapeutic drugs (van Alphen et al. 1988a,b, 1989). However, irradiation affects mainly proliferating premeiotic germ cells while spermatocytes and spermatids are generally less sensitive to cytotoxic agents and are lost from the testis by maturation depletion rather than suffering direct damage (van Alphen et al. 1988a). In oncological patients, testicular irradiation doses as low as 0.35 Gy can cause azoospermia, which is usually reversible. After higher irradiation doses (>2 Gy) reappearance of sperm in the ejaculate is observed after 1 year, if at all. A recovery to pre-treatment sperm counts may be reached after 3–4 years (Berthelsen 1984, Meistrich & Kangasniemi 1997). Leydig cells are more resistant to irradiation, being affected by doses larger than 15 Gy (Ogilvy-Stuart & Shalet 1993).

Inhibin is a heterodimeric glycoprotein hormone consisting of an α and either a βA (inhibin A) or a βB subunit (inhibin B) which is released from the testis (de Kretser & Robertson 1989). Its subunits are produced in Sertoli cells and germ cells (Roberts et al. 1989, Andersson et al. 1998). The discussion on the prognostic value of inhibin B for spermatogenic function was recently renewed. It was shown that inhibin B serum levels are a good endocrine predictor for identifying subfertile patients (Byrd et al. 1998, Mahmoud et al. 1998) and that it correlates with follicle-stimulating hormone (FSH), testis volume, sperm counts and the presence of Sertoli cell only (SCO) tubules in testicular biopsies (Bergmann et al. 1994, Andersson et al. 1998, Pierik et al. 1998). Furthermore, inhibin B levels are age-dependent with an increase during pubertal development (Byrd et al. 1998, Raivio et al. 1998) and a decrease in aging men (Byrd et al. 1998). Interestingly, prepubertal and postpubertal germ cells and Sertoli cells play different roles in the regulation of serum inhibin B levels (Andersson et al. 1998). While in the prepubertal testis inhibin beta-B and alpha subunits are produced by Sertoli cells, in the adult testis inhibin beta-B subunits are present in germ cells at the spermatocyte and spermatid
stage. Thus, Sertoli cell maturation leads to a change in the cellular inhibin B, but not inhibin alpha expression. This suggests that the correlation of inhibin B levels and spermatogenesis is due to the fact that inhibin B is a joint product of Sertoli and germ cells (Andersson et al. 1998).

This study describes the effect of testicular irradiation on testis morphology and sperm parameters in a well-established preclinical model of spermatogenesis, the cynomolgus monkey (*Macaca fascicularis*) and addresses the question whether inhibin B or FSH is the earlier and more sensitive marker for the detection of irradiation-induced damage.

**Materials and Methods**

**Animal experiments**

A total of six adult cynomolgus monkeys (*Macaca fascicularis*), aged 8–9 years, and with body weights ranging from 7·2 to 11·0 kg were included in the investigation. The animals were housed in individual cages under defined environmental conditions as described previously (Weinbauer et al. 1998). Standardised monkey diet supplemented with fresh fruit was provided twice daily and tap water was available *ad libitum*. Maintenance and handling of the monkeys were performed in accordance with the German Federal Law on the Care and Use of Laboratory Animals. This study was performed in preparation of a larger investigation on the effects of testicular germ cell transplantation which is still ongoing. The additional treatment the monkeys received for the transplantation study were a bilateral biopsy 3 weeks before irradiation for retrieval of transplantable germ cells and a unilateral injection of germ cell preparations into the right testis 40 days after irradiation. Repopulation of the testis with germ cells is a very slow process in rodents and will only occur months after the infusion of germ cells (Russell et al. 1998). Therefore, we assumed that the effects of germ cell transplantation would not interfere with the irradiation response during the period of the study.

Body weight and testicular volumes (TV) of all animals were recorded at weekly intervals. TV 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 the testicular volume: $TV = \text{width}^2 \times \text{length} \times \frac{\pi}{6}$.

Blood samples were collected at weekly intervals by venepuncture of the saphenous vein under ketamine sedation (Ketavet, 5–8 mg/kg, i.m., Parke Davis, Munich, Germany). The blood was allowed to coagulate overnight at 4°C, then the serum was separated and stored at −20°C until analysis. Ejaculates were obtained from sedated monkeys by rectal probe electroejaculation and the evaluation of sperm concentration was performed as described previously (Weinbauer et al. 1994). Sperm counts were expressed per total ejaculate (exudate plus coagulum). Azoospermic samples as estimated after light microscopy on semen smears were centrifuged at 2000 rpm for 5 min, and the sediment was subsequently examined for the presence of sperm. Sperm morphology assessment was performed by Papanicolaou staining according to WHO criteria (1992).

**Testicular irradiation**

At 40 days after collecting baseline data and 20 days after taking bilateral biopsies all monkeys underwent a single 2 Gy X-ray irradiation of both testes. Local irradiation of the testes was performed in a 5–10-cm field using a Mevatron 77 accelerator providing 10 MeV photons (Siemens, Munich, Germany). A 2-cm plexiglass layer was put directly on the scrotal skin to compensate for the build-up effect. Prior to irradiation, measurements of the absorbed dose were performed with a calibrated ionisation chamber inside a tissue equivalent phantom. The dose rate was 3 Gy/min. Assuming a 5-cm tissue diameter of the testes, the maximum dose variation in the testis was estimated to be about −18%. This indicates that each testis received a dose of between 1·64 and 2 Gy. If the testes had been only 3 cm in diameter there would be a dose reduction of −9% at the lower edge of the *tunica albuginea*. The maximum irradiation of the remaining body was 1·5% of the dose, i.e. 3 cGy.

**Histological techniques**

For histological analysis of the testis one monkey was bilaterally orchidectomised under general anaesthesia 14 weeks after irradiation. Both testes were weighed and immersion fixed in Bouin’s solution, routinely embedded in paraffin and cut at a section thickness of 5 µm for light microscopy. The other five monkeys were biopsied 13 weeks later (week 27) for histological analysis of irradiation in the left testis. The tissue was retrieved as an open biopsy under general anaesthesia of the monkeys as previously reported (Weinbauer et al. 1998) and was embedded and handled as described above. As the left testis served as saline-injected controls for comparison with the contralateral site, into which germ cells were injected, the left testes were considered as normal and could therefore be analysed for the presence of germ cells after irradiation. Sections were stained with periodic acid Schiff’s base (PAS) and haematoxylin. A systematic randomised histological evaluation was performed. In the castrated monkey 500 tubules per testis were scored for the most advanced germ cell type. Sections from eight different regions of the testis were analysed. A total of 100 seminiferous tubules were scored in cross-sections of the biopsies. In two of the ten biopsies analysed only 71/90 seminiferous tubule cross-sections were present. The results were expressed as the relative number of tubules.

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showing SCO, spermatogonia, spermatocytes, round and elongated spermatids. PAS and haematoxylin-stained testis sections from three normal age-matched cynomolgus monkeys of the institutional colony were used as controls. Histological evaluation was independently performed by two different observers (LF & SS) who obtained comparable results (r=0.80, P<0.0001).

Hormone assays
FSH was assayed by a heterologous radioimmunoassay as reported earlier (Weinbauer & Nieschlag 1993). The sensitivity was 0.156 ng/ml and intra- and interassay coefficients of variation were 6-2 and 7% respectively. Inhibin B was assayed by a two-site enzyme-linked immunoassay (Serotec, Kidlington, Oxford, UK). Sensitivity was 7.8 pg/ml and intra- and interassay coefficients of variation were 3.1 and 13.7% respectively. Serial dilutions of monkey serum gave analytical responses parallel to the standard curve which showed the suitability of the system for the measurement of inhibin B in monkey serum. Testosterone (T) was assayed by an established radioimmunoassay (Khurshid et al. 1991). Sensitivity was 0.068 nmol/l and intra- and interassay coefficients of variation were 5.8 and 13.1% respectively.

Statistical analysis
Evaluation of body weight, TV, sperm counts, FSH, inhibin B and T data was performed by one-way analysis of variance (ANOVA) followed by multiple comparison test (Bonferroni). Because of the skewed distribution of the

Figure 1 Irradiation-induced changes in testis volume (A; expressed as percentage mean baseline values), sperm counts (B), inhibin B (C) and FSH (D). Irradiation (2 Gy) was performed at day 0. Each time point represents the means ± s.e.m. of six monkeys.
sperm count data, the calculation was performed on log-transformed data. Correlations between FSH, inhibin B, TV and sperm counts were performed by Spearman analysis. Data were expressed as means ± s.e.m. Statistical significance was assumed at P<0.05.

Results

Clinical observations and body weight

No serious side effects after irradiation were observed in any of the monkeys, except for a slight reddening of the scrotal skin which lasted for 1 week. At the end of the study we recorded a slight, but significant reduction of 8–10% (P<0.0001) in body weight with respect to mean pre-treatment values.

Testis volume and sperm count

After irradiation, a significant reduction (P<0.001) in mean testis size (right and left testis) was observed in all monkeys (Fig. 1A). The nadir was reached 70 days after irradiation (mean pre-treatment: 22.7 ± 2.5 ml, day 70: 7.2 ± 0.9 ml, P<0.001). Thereafter, no further significant changes were recorded (Fig. 1A). The regression of both testes was similar.

Mean pre-treatment sperm concentration was 60.3 ± 15.5 x 10^6/ejaculate. Individual values ranged from 20 to 107 x 10^6/ejaculate. The sperm concentration started to decrease significantly 35 days after irradiation (day 35: 9.2 ± 3.5 x 10^6/ejaculate, P<0.05) (Fig. 1B). Sperm counts decreased further until 60 days after irradiation; thereafter the values showed fluctuations on a low level (Fig. 1B). Azoospermia were only occasionally recorded in all six monkeys (the first time at 2 months after irradiation). No significant reduction in the ejaculate volume was recorded. Sperm morphology showed a slight reduction in the percentage of normal forms 6 weeks after irradiation (71.4 ± 9.9%) compared to pre-treatment values (89.8 ± 3.0%). Due to the very low number of sperm retrieved, sperm morphology could not be assayed at later time points.

Hormones

Inhibin B levels decreased strongly (P<0.001) as soon as 1 week after irradiation and did not recover until the end of the study (Fig. 1C). FSH levels increased significantly (P<0.05) 3 weeks after irradiation and reached the zenith after 2 months (Fig. 1D). A significant overall increase (P<0.001) in T levels was observed (Fig. 2). A significant negative correlation existed between FSH levels and inhibin B values (r = -0.35, P<0.001; Fig. 3A). Inhibin B values were significantly correlated with TV (r = 0.73, P<0.001; Fig. 3B) and sperm counts (r = 0.55, P<0.001; Fig. 3C). FSH values correlated with TV (r = -0.25, P<0.01; Fig. 3D) and sperm counts (r = -0.42, P<0.0001; Fig. 3E).

Histological evaluation

At 14 weeks after irradiation testes from the orchiec-tomised monkey showed the following pattern of seminiferous tubules in respect to the most advanced germ cell type (means ± s.e.m. of left, saline-injected control testes; data for the second observer are shown in parenthesis): SCO or spermatogonia only: 49.3 ± 2.3% (32.2 ± 3.0%), spermatocytes 25.2 ± 2.9% (32.7 ± 2.8%), round spermatids 15.4 ± 2.1% (21.2 ± 1.9%), elongated spermatids: 6.8 ± 2.1% (10.2 ± 1.9%). No Leydig cell hyperplasia or fibrosis was observed. In the three age-matched control testes analysed, round or elongated spermatids were observed in all tubules examined. Analysis of the left testis biopsies obtained at week 27 after irradiation showed the following pattern: SCO or spermatogonia only: 66.0 ± 12.1% (61.2 ± 11.89%), spermatocytes 25.3 ± 6.9% (11.2 ± 5.0%), round spermatids 6.8 ± 2.1% (6.3 ± 2.8%), elongated spermatids: 12.0 ± 3.7% (15.9 ± 4.5%). Representative micrographs of the testicular histology are shown in Fig. 4A–F.

Discussion

The mechanism by which spermatogenesis is impaired following gonadotrophin withdrawal induced by
gonadotrophin-releasing hormone (GnRH)-antagonist treatment differs from that following irradiation. The antagonist induces an immediate and precipitous decline of bioactive luteinising hormone (LH) and T (Pavlou et al. 1986, Behre et al. 1992) as well as FSH (Khurshid et al. 1991). This is followed by an immediate interruption of premeiotic germ cell development leading to a depletional wave of germ cells (Zhengwei et al. 1998); here the key spermatogenic lesion is caused by an inhibition of type A-spermatogonial mitosis (Weinbauer et al. 1998, Zhengwei et al. 1998). In contrast, irradiation acts directly on the proliferating target cells of the testis, i.e. the differentiating spermatogonia by killing this X-ray-sensitive generation of cells. Unlike after antagonist treatment, high FSH and normal T levels are present after irradiation, as has been shown here and previously by van Alphen et al. (1988a).

A similar reduction in TV was observed after GnRH antagonist treatment (Cetrorelix), (Weinbauer et al. 1994) compared to irradiation in this study with less time required to reach the nadir than after antagonist treatment. Azoospermia was achieved in all GnRH antagonist-treated animals, while after irradiation azoospermia was only occasionally recorded and advanced germ cells were observed in testis tissue sections of all six monkeys 14 and 27 weeks post irradiation. In the same period after
Figure 4 Representative micrographs showing the testicular histology of a monkey 14 weeks after irradiation (A,B), in an intact control monkey (C,D) and in biopsies obtained from the left testis at week 27 (E, F). Figure B, D and F (objective magnification: ×25) are larger magnifications of A, C and E (objective magnifications: ×10) (PAS and haematoxylin staining, all scale bars represent 100 μm). The arrows indicate SCO tubules, the arrow heads indicate tubules with normal spermatogenesis or advanced germ cells.
GnRH-antagonist treatment, the percentage of tubules containing advanced germ cells (i.e., spermatocytes and spermatids) was zero or close to zero (Weinbauer & Nieschlag 1993), indicating efficient elimination of stem cell proliferation or premeiotic germ cell survival (Zhengwei et al. 1998). Our findings confirm that doses of 0.5–4 Gy are sufficient to induce severe testicular damage in monkeys (van Alphen et al. 1988a,b). The persistence of the hormonal milieu might explain why the few germ cells which have not been killed by irradiation are able to repopulate small areas of the testis.

Significantly increased FSH levels were observed from 3 weeks after irradiation. We observed negative correlation of FSH to TV and sperm counts, confirming that FSH is an endocrine indicator of damage to the germinal epithelium (Bergmann et al. 1994). The baseline levels of inhibin B (794–6–2202 pg/ml) were similar to data previously reported for rhesus monkeys (Plant et al. 1997). As in men, inhibin B levels correlated positively with sperm counts and testis volume and negatively with FSH levels (Pierik et al. 1998). This confirms its role as a feedback signal for FSH production and provides strong evidence that inhibin B is also an important marker of the competence of Sertoli cells and spermatogenesis in non-human primates. The rapid and pronounced decline of inhibin B levels 1 week after irradiation could be ascribed to a fast functional change of Sertoli cells in consequence of irradiation. The premeiotic germ cells, but not the Sertoli cell, are most likely affected by the irradiation. Therefore, the damage to germ cells (i.e., loss of the proliferating capacity) caused by irradiation might signal Sertoli cells to stop inhibin B production. At week 6 post irradiation sperm counts were still around 10 million per ejaculate, indicating that advanced germ cells continued maturing during the first few weeks after irradiation and that the germ cell loss occurs mainly by maturation depletion. This indicates that Sertoli cells support meiotic and postmeiotic germ cells despite low inhibin B values, identifying inhibin B as a marker for intact premeiotic germ cell development.

The most prominent drop in sperm counts occurred between weeks 4–6. The time span of a spermatogenetic cycle in the cynomolgus monkey is approximately 10 days (Fouquet & Dadoune 1986, Weinbauer et al. 1998). Between 8 and 10 days is the period for epididymal passage in a macaque (Amann et al. 1976). In consequence, the germ cell type which will mature into mature sperm 30–40 days later must have been the target for the radiation. Considering these time intervals it is most likely that the actively proliferating premeiotic germ cells were the target cells for irradiation since an immediate loss of B–spermatogonia would lead to a depletion of sperm after three spermatogenetic cycles (30 days).

Previous findings in adult non-human and human primates showed positive immunoreactivity for inhibin alpha and beta subunits both in Sertoli cells and Leydig cells, but not in germ cells (Vliegen et al. 1993). On the other hand, recent results in humans have demonstrated that immature Sertoli cells show a positive staining both for inhibin alpha and beta subunits, while in the adult testis the fully differentiated Sertoli cells express only the α-subunit; the β-subunit was present in germ cells from pachytene spermatocytes to early spermatid stages and to a lesser degree in Leydig cells (Andersson et al. 1998). We propose that Sertoli cells are the source for both subunits in the monkey (Vliegen et al. 1993) and that they respond to the loss of spermatogonia by ceasing to produce inhibin B. Further studies are needed to specifically address the cellular production site of the inhibin subunits and its regulation.

Finally, the earlier response of inhibin B in comparison to FSH suggests a greater sensitivity of the former to testicular damage. As a direct product of Sertoli cells, it might be a better marker for testicular function than FSH which depends on hypothalamic function as well as testicular factors and steroid hormones (Pierik et al. 1998).

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