Reduction of long-term effects of local heating of the testis by treatment of rats with a GnRH agonist and an anti-androgen

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Heating the testes of anaesthetized adult rats to 43°C for 30 min in a waterbath was followed by a large decrease in testis and epididymis mass and number of spermatozoa 35 days later. These parameters had recovered to some extent, but not completely, by days 70 and 97 after heating, but had decreased again in rats examined on day 182. There were no consistent effects of heating on androgen status, as determined by the concentrations of testosterone in blood and testis fluids, or by seminal vesicle mass, and interstitial fluid volume was increased in the heated testes. Treatment of rats with an implant of a GnRH agonist and daily injections of an anti-androgen for 14 days (sufficient in itself to cause large temporary decreases in tissue mass,

number of spermatozoa and androgen status) did not reduce the initial decrease in testis mass or number of spermatozoa seen after heating, but reduced the later decreases in mass and number of spermatozoa significantly. These findings indicate that, as well as causing damage to spermatocytes and spermatids, as previously reported, heating also reduces the ability of spermatogonia to repopulate the seminiferous tubules at longer intervals after heating. Furthermore, it appears that this effect on the spermatogonia can be reduced by treating the animals with a GnRH agonist and anti-androgen, a treatment similar to that shown by other authors to improve recovery of the testis from irradiation or drug treatment.

Introduction

The damaging effects of whole body or local heating on the testes of mammals are well known and, although recovery begins about 40 days after a single exposure, even 60 or more days later, there is still a significant reduction in testis mass (for review, see Setchell, 1998). Repeated applications of heat to the testes of rats causes a reduction in testis mass that lasts many months (Bowler, 1972).

The effects of the drugs cyclophosphamide or procarbazine on spermatogenesis can be reduced by pretreatment of the rats with an antagonist or an agonist of GnRH combined with the anti-androgen flutamide (Kangasniemi *et al.*, 1995a,b; Meistrich *et al.*, 1995). Similarly, the effects of irradiation on spermatogenesis can be minimized by pretreatment of the rats with GnRH analogues, either agonists or antagonists (Shetty *et al.*, 2000; Shuttleworth *et al.*, 2000), and treatment with a GnRH agonist is effective even when given after the radiation (Meistrich and Kangasniemi, 1997). In rats with suppressed spermatogenesis resulting from administration of 2,5-hexanedione, treatment with a GnRH agonist results in reversal of the testicular atrophy and normalized stem cell factor expression (Blanchard *et al.*, 1998).

In the present study the response of the testis to local

heating in rats treated with a GnRH agonist and an anti-androgen was examined, and the time of observation was extended to investigate whether the testis would eventually make a full recovery.

Materials and Methods

Animals

A total of 80 adult Sprague–Dawley strain rats (B and K Universal, Sollentuna) aged about 50 days and weighing about 280 g at the start of the experiment were used. The rats were anaesthetized with pentobarbitone sodium (Apoteket, Stockholm, 50 mg kg⁻¹ i.p.) and the scrotum and hind legs of 48 of them were immersed for 30 min in a waterbath held at 43°C. Immediately after the heating, half the heated animals and half the controls were given an implant of a GnRH agonist Zoladex (3.6 mg, Zeneca Pharmaceuticals, Macclesfield), and the first of 14 daily s.c. injections of an anti-androgen, flutamide (20 mg ml⁻¹ in sesame oil:ethanol 50:50, 1 ml kg⁻¹, Schering-Plough, Stockholm). The animals were weighed and checked at frequent intervals until sampling.

Sampling

At various times afterwards (Table 1), groups of rats from the four treatment groups were anaesthetized again and a blood sample was removed from the surface veins of one testis, which was then removed, weighed and decapsulated.

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Table 1. Mass of one testis and one epididymis of rats after heating of the scrotal region (H) or treatment with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

	Days after heating	Number of rats	Treatment (number of rats)			
			C (4 or 2)	H (6 or 3)	HZ (6 or 3)	Z (4 or 2)
Mass of testis (g)	35	4 or 6	1.62 ± 0.06	0.70 ± 0.03*	0.30 ± 0.02 [†]	0.38 ± 0.01 ^{***}
	70	2 or 3	1.83 ± 0.09	1.03 ± 0.06 ^{aa}	1.01 ± 0.07 ^{aa}	0.87 ± 0.08 ^{††}
	97	2 or 3	1.69 ± 0.01	1.18 ± 0.11 ^{aaa}	0.95 ± 0.09 ^{††, aa}	1.40 ± 0.03
	140	4 or 6	1.73 ± 0.09	1.11 ± 0.10 ^{aaa}	1.21 ± 0.10 ^{††, aaa}	1.63 ± 0.02
	182	4 or 6	1.84 ± 0.02	0.88 ± 0.05 ^{***, bb}	1.38 ± 0.09 ^{†, aaa}	1.66 ± 0.08
Mass of epididymis (mg)	35	4 or 6	440 ± 28	150 ± 39	160 ± 38	100 ± 23 ^{†††}
	70	2 or 3	510 ± 20	310 ± 37 ^a	170 ± 3	170 ± 15 ^{††}
	97	2 or 3	560 ± 0	420 ± 64 ^{aaa}	200 ± 18 [†]	380 ± 20 [†]
	140	4 or 6	660 ± 18	360 ± 43 ^{*, aa}	480 ± 41	540 ± 22
	182	4 or 6	620 ± 6	310 ± 18 ^{aa}	440 ± 68	550 ± 38

Values are means ± SEM.

All values for heat-treated testis or epididymis (H) are significantly less than controls (C).

Significantly different from day 35: ^a*P* < 0.05; ^{aa}*P* < 0.01; ^{aaa}*P* < 0.001.

Significantly different from day 97 or 140: ^{bb}*P* < 0.01.

Significantly different from HZ: ^{*}*P* < 0.05; ^{***}*P* < 0.001.

HZ significantly different from Z: [†]*P* < 0.05; ^{††}*P* < 0.01.

Significantly different from C: ^{*}*P* < 0.05; ^{††}*P* < 0.01; ^{†††}*P* < 0.001.

The interstitial fluid was removed by irrigation with 4 ml isotonic mannitol; the parenchyma was then forced through a 21 gauge needle with a 5 ml syringe, and a cell and fluid fraction was separated by centrifugation at 10 000 *g* for 15 min (Setchell *et al.*, 1976; Turner *et al.*, 1984). The other testis was also removed, weighed and fixed by pumping 0.9% (w/v) sodium chloride followed by 4% (v/v) glutaraldehyde in cacodylate buffer for about 5 min at a rate of 700 µl min⁻¹ with a peristaltic pump through a 25 gauge needle inserted into the testicular artery on the surface of the testis. In some of the smaller testes, the perfusion was not successful, and these testes were then fixed by immersion in the same fixative. All testes were stored in the same fixative until processing for histology. The seminal vesicles and epididymides were then removed and weighed, and the epididymides were frozen for determination of number of spermatozoa. Finally, a blood sample was removed by syringe and needle from the posterior vena cava, and the animal was killed with an overdose of anaesthetic.

Cubes of parenchyma (about 1 mm³) were cut from near the centre of each fixed testis, post-fixed in osmium tetroxide, dehydrated and embedded in Agar 100 resin. Semithin (about 1 µm) sections were cut on glass knives and stained with buffered toluidine blue. For preliminary examination, the slides were numbered so that the treatment was not apparent to the observer. At least six, and usually ten or more suitably orientated tubular cross-sections from each animal were used for measurement of tubular diameter, which was done using a microscope fitted with a digital video camera and an image-analysis system. The number of suitable transverse tubular cross-sections

depended on the actual size of the piece of tissue taken and the orientation of the tubules within the section. At the same time, each tubular cross-section was classified as normal if all generations of germ cells were present and there were no degenerating cells, or abnormal, if some cell types were missing or degenerating cells were present. The sections were then examined at higher magnification to determine the most advanced stage of spermatogenesis in the abnormal tubular cross-sections.

Blood samples were centrifuged at 3000 *g* for 15 min to separate plasma, which was stored frozen. The degree of dilution of the interstitial fluid in the isotonic mannitol was estimated by analysing it for sodium concentration by flame photometry, assuming that the concentration in the interstitial fluid was the same as in serum, as has been shown in other animals (Maddocks and Setchell, 1988; Zupp *et al.*, 1988). Some of these diluted interstitial fluids, the tubular fluid fraction from the dispersed tubular cells and the plasma samples were analysed for testosterone by a fluorometric immunoassay (Delfia, Pharmacia, Stockholm, kindly performed by Karolinska Hospital Klinisk Kemi Department).

Spermatozoa were counted with a haemocytometer after maceration with an Ultraturax of the epididymides and the cellular fractions of the testicular parenchyma in 0.9% sodium chloride containing 0.05% (v/v) Triton X-100 (5 ml per tissue, followed by a further 1:10 dilution; Robb *et al.*, 1978).

Statistical analysis

Data were analysed by analysis of variance, followed by least mean square test (Superanova; Abacus Concepts,

Table 2. Mass of paired seminal vesicles with contents and initial body weight and daily body weight gain in control rats (C), and rats whose scrotal region had been heated (H) or treated with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

		Treatment			
		C	H	HZ	Z
Seminal vesicles (g)	35	1.66 ± 0.20	1.39 ± 0.08***	0.07 ± 0.005	0.06 ± 0.005***
	70	1.37 ± 0.04	1.60 ± 0.03***	0.83 ± 0.040	0.67 ± 0.020**
	97	1.63 ± 0.07	1.74 ± 0.09**	1.20 ± 0.12	1.49 ± 0.05
	140	2.26 ± 0.18 ^a	1.92 ± 0.09 [†]	1.56 ± 0.08 [†]	1.87 ± 0.10**
	182	2.01 ± 0.14 ^a	1.96 ± 0.09	1.87 ± 0.08	1.75 ± 0.13
Initial body weight (g)	0	284 ± 4	275 ± 2	279 ± 3	276 ± 3
Weight gain (g day ⁻¹)	0–7	6.1 ± 0.4	5.6 ± 0.1	4.7 ± 0.2	4.8 ± 0.2**
	7–17	6.1 ± 0.2	6.1 ± 0.2***	4.5 ± 0.3	3.4 ± 0.3**
	17–36	3.4 ± 0.2	3.2 ± 0.2	3.0 ± 0.1	2.4 ± 0.1***
	36–70	3.2 ± 0.1	3.1 ± 0.1	3.5 ± 0.1	3.3 ± 0.1
	70–97	1.6 ± 0.2	1.7 ± 0.2	2.2 ± 0.2	2.3 ± 0.1**
	97–140	1.4 ± 0.1	1.5 ± 0.2	1.8 ± 0.1	1.7 ± 0.1 [†]
	140–182	0.9 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	1.2 ± 0.3

Values are means ± SEM.

^aPooled data for days 140 and 180 after treatment are significantly different from pooled data for days 35, 70 and 97.

Significantly different from HZ on same day after heating: ** $P < 0.01$; *** $P < 0.001$.

Significantly different from Z on same day after heating: [†] $P < 0.05$

Significantly different from C on same day after heating: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Berkeley, CA) for individual comparisons, by Mann–Whitney U test when the data were not normally distributed or by chi-squared test.

Results

Masses of testes, epididymides and seminal vesicles

Thirty-five days after heating, the mass of the testes of rats heated but not treated with GnRH agonist and anti-androgen (H group) was less than half that of controls. There then followed a period of recovery up to day 97 after heating, when the mass of the testes had reached about 70% of that of controls. There was no change in the mass of the testes between day 97 and day 140 after heating. Thereafter, testis mass decreased again, so that by day 182 after heating, the mass of testes was again only half that of controls. At days 140 and 182 after heating, there was considerable variation between the masses of the two testes of individual rats and among rats within the group. The testes of the rats treated with GnRH agonist and anti-androgen (Z group) were reduced markedly in size at day 35 from the beginning of treatment, but recovered thereafter so that, by day 140, they were not significantly different from controls. The heated testes of the rats that were treated with GnRH agonist and anti-androgen (HZ group) were even smaller than those of the rats in group Z at day 35 and remained so at days 97 and 140. At days 70 and 182 after heating, the difference in size between HZ and Z rats was not significant, largely because of considerable variability in the HZ group. The testes of the H group were significantly smaller than those of the HZ group at day 182 after heating (Table 1).

In general, the masses of the epididymides showed a similar pattern, with those of the H group decreased at day 35, recovering somewhat up to day 97, after which time there were no further changes and epididymal mass remained less than that of controls for the rest of the experiment. In the HZ and Z groups, after the initial decrease, there was a steady recovery for the rest of the experimental period (Table 1).

The masses of the seminal vesicles were not different between the control and H groups, except for a small but significant reduction in the H group at day 140 after heating. In the HZ and Z groups, there was an initial decrease in seminal vesicle mass, indicating suppression of androgenic activity of the testis, and then recovery. After day 140, seminal vesicle masses were not different from those of uninjected rats. In control rats, there was a small increase in seminal vesicle mass with age (Table 2).

Body weight gain up to day 35 after treatment was significantly lower in rats treated with GnRH agonist and anti-androgen, but thereafter, there was a faster weight gain (Table 2) so that at the end of the experiment, there were no significant differences in body weight among the groups (data not shown).

Number of spermatozoa in testes and epididymides

Numbers of spermatozoa in the testes and epididymides were reduced markedly after heating, showed a partial recovery up to day 97 and then decreased again. Numbers of spermatozoa were also reduced initially in both the HZ and Z groups, and although the numbers of spermatozoa in the HZ group were often less than those in controls, they

Table 3. Numbers of spermatozoa ($\times 10^6$) in one testis and one epididymis of control rats (C) and rats heated (H) or treated with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

	Days after heating	Treatment			
		C	H	HZ	Z
Testis	35	140 $\pm$ 7	3 $\pm$ 1	2 $\pm$ 1	1 $\pm$ 0.2 ^{***}
	70	161 $\pm$ 5	43 $\pm$ 30 ^{aaa}	28 $\pm$ 12	39 $\pm$ 32
	97	141 $\pm$ 2	73 $\pm$ 40	13 $\pm$ 4 ⁺⁺⁺	122 $\pm$ 4
	140	165 $\pm$ 1	38 $\pm$ 22	63 $\pm$ 25 ⁺⁺	127 $\pm$ 11
	182	209 $\pm$ 4	11 $\pm$ 4 ^{**b}	112 $\pm$ 32 ⁺⁺	168 $\pm$ 20
Epididymis	35	258 $\pm$ 33	3 $\pm$ 1	7 $\pm$ 3	1 $\pm$ 0.3 ^{***}
	70	280 $\pm$ 10	52 $\pm$ 45 ^{aa}	4 $\pm$ 2	5 $\pm$ 4 ⁺⁺
	97	296 $\pm$ 12	129 $\pm$ 64	1 $\pm$ 1 ⁺⁺	168 $\pm$ 23
	140	358 $\pm$ 22	94 $\pm$ 56	212 $\pm$ 44	289 $\pm$ 14
	182	359 $\pm$ 20	61 $\pm$ 3 ^{bc}	157 $\pm$ 71	281 $\pm$ 30

Values are means $\pm$ SEM.

All values for heat-treated testis or epididymis (H) are significantly less than controls (C) ($P < 0.001$) except on day 97.

Pooled values for days 70 and 97 are significantly different from values for day 35: ^{aa} $P < 0.01$; ^{aaa} $P < 0.001$.

Values for day 182 are different from pooled data for days 70 and 97: ^b $P < 0.05$.

Values for day 182 are different from pooled data for days 70, 97 and 140: ^c $P < 0.05$.

Significantly different from HZ on same day after heating: ^{**} $P < 0.01$.

Significantly different from Z on same day after heating: ⁺⁺ $P < 0.01$; ⁺⁺⁺ $P < 0.001$.

Significantly different from C on same day after heating: ^{**} $P < 0.01$; ^{***} $P < 0.001$.

Table 4. Testosterone concentrations (nmol l⁻¹) in blood plasma from the posterior vena cava (PVC) and testicular veins (TV) and interstitial (IF) and tubular (STF) fluids from the testes of heated (H) and control (C) rats

Days after heating	Treatment	PVC	TV	IF	STF
35	H	7.69 $\pm$ 1.79	1830 $\pm$ 380	2270 $\pm$ 460	1060 $\pm$ 176 ⁺⁺
	C	9.17 $\pm$ 2.56	1200 $\pm$ 250	2150 $\pm$ 470	540 $\pm$ 97
97	H	4.65 $\pm$ 1.12	1010 $\pm$ 260	1500 $\pm$ 227	558 $\pm$ 125
	C	2.70 $\pm$ 0.19	785 $\pm$ 72	1260 $\pm$ 152	417 $\pm$ 63
182	H	3.57 $\pm$ 0.32	1320 $\pm$ 146 [†]	1370 $\pm$ 250	772 $\pm$ 39 [†]
	C	4.83 $\pm$ 1.22	722 $\pm$ 77	844 $\pm$ 116	499 $\pm$ 55

Values are means $\pm$ SEM.

Values for heated rats are significantly greater than those for controls for pooled data for three time points for TV, IF or STF by ANOVA: $P < 0.05$.

Pooled values for IF are significantly greater than pooled values for TV, which are significantly greater than pooled values for STF by ANOVA: $P < 0.001$.

Significantly different from C on same day after heating: [†] $P < 0.05$; ⁺⁺ $P < 0.01$.

were higher than in the H group at day 182 after treatment (Table 3).

Testosterone concentrations

The concentrations of testosterone in the blood plasma from the posterior venae cavae were not different between the H and control groups. The concentrations of testosterone in testicular venous blood plasma, testicular interstitial fluid and seminiferous tubular fluid were all higher than they were in the posterior vena cava plasma and, in general, the concentrations found in the heated rats were slightly higher than those found in controls, although the differences were significant only for tubular fluid at days 35 and 182 and testicular venous blood plasma at day 182 after heating. The concentrations of testosterone in the interstitial fluid were slightly but significantly higher than

those in the testicular venous blood, and both were higher than they were in the tubular fluid (Table 4).

Fluid volumes

The volume of interstitial fluid in the testis was increased initially after heating, then decreased, and increased again at days 140 and 182. A similar pattern was seen in the HZ rats, but there were no significant differences in rats from the Z group compared with the control group (Table 5).

The volume of tubular fluid was about 250 μ l g⁻¹ testis and showed no consistent differences among groups or with time (data not shown).

Histological observations.

The diameters of the seminiferous tubules were reduced by heating and by hormone treatment, and the combined

Table 5. Interstitial fluid volumes ($\mu\text{l g}^{-1}$ testis) in testes of control (C) rats and rats that had been heated (H) or treated with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

Days after heating	C	H	HZ	Z
35	85 $\pm$ 7	215 $\pm$ 41 ^{##}	153 $\pm$ 9	116 $\pm$ 11
70	80 $\pm$ 11	140 $\pm$ 12	100 $\pm$ 6	103 $\pm$ 3
97	93 $\pm$ 5	128 $\pm$ 30	145 $\pm$ 12	101 $\pm$ 11
140	86 $\pm$ 12	215 $\pm$ 52 [†]	173 $\pm$ 27	113 $\pm$ 10
182	100 $\pm$ 12	350 $\pm$ 47 ^{###,a}	282 $\pm$ 60 ^{##,a}	194 $\pm$ 64

Values are means $\pm$ SEM.

Values are significantly greater than controls: [†] $P < 0.05$; ^{##} $P < 0.01$; ^{###} $P < 0.001$.

^aValue significantly greater than at days 35, 70, 97 or 140: $P < 0.05$.

Pooled values for H and HZ on days 35, 140 or 182 are significantly greater than pooled values for C and Z by two-factor ANOVA: $P < 0.05$.

Table 6. Tubular diameters (μm) in sections of testes of control rats (C) and those that had been heated (H) or treated with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

Days after heating	C	H	HZ	Z
35	303 $\pm$ 6.3	193 $\pm$ 6.1 ^{###}	163 $\pm$ 8.8	187 $\pm$ 3.3 ^{###}
70	335 $\pm$ 15	250 $\pm$ 26 ^a	240 $\pm$ 6 ^a	255 $\pm$ 15 [†]
97	300 $\pm$ 20	243 $\pm$ 28	283 $\pm$ 9	290 $\pm$ 0
140	300 $\pm$ 10	242 $\pm$ 22 ^a	248 $\pm$ 24	307 $\pm$ 3
182	308 $\pm$ 6.3	190 $\pm$ 8.6 ^{###,b}	213 $\pm$ 18 ^{###,c}	317 $\pm$ 6.7

Values are means $\pm$ SEM.

^aValue significantly different from that for day 35: $P < 0.05$.

^bValue significantly different from that for days 70 and 140: $P < 0.05$.

^cValue significantly different from that for day 97: $P < 0.05$.

Significantly different from HZ on same day after heating: ^{##} $P < 0.01$.

HZ significantly different from Z on same day after heating: ^{###} $P < 0.001$.

Significantly different from C on same day after heating: [†] $P < 0.05$; ^{###} $P < 0.001$.

Table 7. Numbers of tubular cross-sections and percentages of normal tubules in testes of control rats (C) and those that had been heated (H) or treated with GnRH agonist and anti-androgen (Z), or both treatments (HZ)

Days after heating	C		H		HZ		Z	
	Number of tubules	Percentage normal (%)	Number of tubules	Percentage normal (%)	Number of tubules	Percentage normal (%)	Number of tubules	Percentage normal (%)
35	62 (8–24)	100	111 (6–50)	0	246 (15–68)	0	126 (18–49)	0
70	12 (6–6)	100	45 (8–25)	36	31 (10–11)	58	28 (14–14)	61
97	39 (18–21)	100	82 (22–31)	51	40 (9–18)	57 ^{###}	46 (19–27)	100
140	56 (9–25)	84 ^a	110 (10–42)	42	143 (10–43)	44 ^{###}	64 (10–26)	100
182	73 (14–28)	100	172 (9–38)	6 ^{###}	110 (20–30)	30 ^{###}	42 (12–18)	100

Ranges of the numbers of suitable tubules per rat are given in parentheses.

^aAbnormal all from one rat.

All percentage normal values for heated testes (H) were less than those for controls (C).

^{###}Value significantly lower than that for HZ by chi-squared test: $P < 0.001$.

^{##}Value significantly lower than that for Z by chi-squared test: $P < 0.001$.

treatments had a greater effect than either alone. In the H and HZ groups, the diameters returned towards normal, but decreased again at day 182 after heating (Table 6).

There was considerable variability in the histological appearance of seminiferous tubules within the same testis and among rats in the same treatment group. Detailed cell counts have not yet been completed but preliminary observations indicate that, in some abnormal tubular cross-sections, both meiotic and post-meiotic cells were absent,

with only spermatogonia and Sertoli cells present. In other abnormal tubules, the most advanced spermatogenic cells were spermatocytes, in others, round spermatids, and in others, elongated spermatids up to stage 16. The testes of the rats in group H showed disrupted spermatogenesis in all rats at day 35 after heating. Thereafter, there was recovery in some tubules in most animals, and a subsequent deterioration at days 140 and 182 after heating (Fig. 1), although in most rats, complete spermatogenesis could be observed in

Fig. 1. Photomicrographs of sections of testes from rats treated 35 days earlier with GnRH agonist plus anti-androgen (group Z; a) and those that had also been heated at 43°C for 30 min (group HZ) at day 35 (b) or day 182 after heating and treating (d,f), and from rats 182 days after heating that had not been treated with hormones (group H; c,e). Note that at day 35 after GnRH agonist and the start of anti-androgen treatment (a), spermatogenesis was disrupted, and the most developed cells were acrosome-phase spermatids. In hormone-treated rats heated 35 days earlier (b), few germ cells remained and there were numerous lipid droplets, with virtually no lumina. In rats examined at day 182 after heating, there was great variability in the appearance of the testis, both among animals and within one testis. One of the least and some of the most affected tubules in one heated rat in group H are shown (c,e) and a similar range of effects is shown in rats in group HZ (d,f). However, in (d) there are some step 19 spermatids, whereas in (c), there are only a few elongated spermatids around step 13 or 14, and there were many more severely affected tubules in the rats in group H. Scale bar represents 100 µm.

some tubules. The percentage of tubular cross-sections showing a normal appearance increased to day 97 and then decreased again to a value < 10% at day 182 after heating, which was significantly lower than that in the HZ group at the same time. Spermatogenesis was also arrested at day 35 after heating in the HZ and Z groups, but all tubular cross-sections were normal in the Z groups by day 97 and thereafter. In the HZ group, there was a steady improvement in the percentage of normal tubules up to day 140 after heating, with a slight but significant deterioration thereafter. However, the percentage of normal tubules remained lower than it did in rats in the Z group from day 97 after treatment onwards (Table 7).

Discussion

The most surprising finding in this study was the decrease in testis and epididymal masses and in the number of spermatozoa in the testis and epididymis in heated rats towards the end of the experiment, associated with a significant deterioration in the histology. This finding indicates that, as well as causing degeneration of spermatocytes and round spermatids, as has been reported by Chowdhury and Steinberger (1964, 1970) and Collins and Lacy (1969), a single short exposure of the testis to a moderate heat stress also inhibits spermatogonial recruitment and possibly the number of stem cells. Studies are currently being undertaken to test this contention by investigating the number of spermatogonia and other types of cell at various times after heating. An effect of heat on spermatogonia in mice had already been reported by Reid *et al.* (1981), who performed studies on the repopulation of seminiferous tubules after irradiation. Differentiation of spermatogonia has also been reported to be arrested in cryptorchid mice (de Rooij *et al.*, 1999) and heating the testes of sheep caused a reduction in the production of A₁-spermatogonia 20 days later, with no effect on the numbers of A₀-spermatogonia (Hochereau-de Reviers *et al.*, 1993). Miraglia and Hayashi (1993) reported a decrease in the percentage of tubules with spermatogonia from 100 to 89% at day 15 after heating the testes of rats to 43°C for 30 min, as in the present study. This small but significant decrease was not as great as the decreases in the percentages of tubules with early or late spermatids (from 100 to 84 and 69%, respectively). There was no decrease in the percentage of tubules with primary spermatocytes at that time.

The present results on the slow and incomplete recovery of testis mass after a single heat exposure are consistent with earlier studies. For example, Setchell *et al.* (1996), Setchell and Waites (1972), Galil and Setchell (1988), Main *et al.* (1978) and Bartlett and Sharpe (1987) found testis mass reduced to 68%, 70%, 47%, 57% and 44% at days 63, 60, 56, 50 and 42 after heating, respectively, which were the longest times examined in these studies. In most of these studies, testis mass reached a minimum of about 30–40% of controls between day 21 and day 28 after heating, although

Bartlett and Sharpe (1987) reported a decrease in testis mass up to day 42 after heating.

It is less surprising that inhibition of spermatogenesis immediately after heating by treatment of rats with a GnRH agonist and an anti-androgen did not reduce the decrease in testis mass and number of spermatozoa seen on day 35 after heating. The homogenization-resistant spermatid nuclei counted at this time would have been zygotene spermatocytes at the time of heating, assuming a spermatogenic cycle duration of 12.9 days (Russell *et al.*, 1990) and there is no evidence to indicate that this treatment influences the degeneration of the spermatocytes.

The very low seminal vesicle masses, barely above those found in castrated animals, in the Z and HZ groups at day 35 after treatment confirm that treatment with GnRH agonist and anti-androgen reduced androgenic activity to negligible amounts. The sustained reduction in seminal vesicle mass in these animals indicates that there was a sustained suppression of androgen activity up to day 140 after the start of treatment, which was longer than expected. The decreased rate of gain of body weight during and immediately after the hormone treatment was also presumably a result of suppression of androgen concentrations in the circulation, but this effect persisted only until between day 70 and day 97 after treatment, indicating that body weight gain is less sensitive than the seminal vesicles to small reductions in androgenic activity. The higher concentrations of testosterone in the blood of the testis vein in heat-treated rats, despite the lack of any effect of heating on the concentration of testosterone in the peripheral blood, is also in accord with earlier studies (Galil and Setchell, 1988). Whether these higher concentrations of testosterone in the blood of the testis vein, mirrored in the concentrations in the interstitial and tubular fluids, are high enough to inhibit spermatogonial recruitment, as suggested by Meistrich *et al.* (1996, 2000) remains to be determined. The finding that the concentration of testosterone in testicular interstitial fluid was only slightly higher than that in testicular venous blood is in agreement with earlier studies in which interstitial fluid was collected with a push–pull catheter (Maddocks and Setchell, 1988, 1989a,b) and contrasts with the much higher values published for so-called interstitial fluid collected after death. The present values for seminiferous tubular fluid may be slight underestimates, because of a small dilution of this fluid with the mannitol solution used to remove the interstitial fluid, but this is not likely to be sufficient to explain the difference reported here between tubular fluid and interstitial fluid. A lower value for tubular fluid than interstitial fluid contrasts with the findings of Comhaire and Vermeulen (1976), who found similar concentrations in tubular and interstitial fluid collected by micropuncture. The present results also differ from those of Turner *et al.* (1984), who reported lower concentrations of testosterone in testicular venous blood plasma than in tubular fluid collected by micropuncture. The validity of the present technique for collecting interstitial fluid is supported by the estimates of volume

collected, which are in good agreement with estimates of the volume of distribution for Cr-EDTA, a marker that is confined to the interstitial extracellular fluid and excluded from the tubular fluid (Setchell *et al.*, 1996; Tao *et al.*, 2000), and also for the volume of fluid that can be collected after death (Bartlett and Sharpe, 1987). Bartlett and Sharpe (1987) also reported an increase in the volume of interstitial fluid after heating of the testis, as observed in the present study. The reason for this increase, or its significance, has yet to be determined, but may simply be due to the reduced volume of the seminiferous tubules.

Administration of a potent and long-acting GnRH agonist effectively suppresses testosterone secretion by suppressing LH and FSH release for at least 28 days, by which time 92% of the depot preparation has been released (Furr and Hutchinson, 1992; Kangasniemi *et al.*, 1995a). In the present study, the effect of any initial surge of testosterone was neutralized by the daily injection of a potent anti-androgen during the first 14 days of GnRH agonist treatment. In terms of the return to normal of testicular and seminal vesicle masses, and normalized testicular histology, the effects of the medication were undetectable by day 97 after treatment, and this finding is in agreement with the report that testis mass and the number of spermatozoa returned to normal by day 63 after the start of treatment (Kangasniemi *et al.*, 1995b). Thus, the temporary suppression of the action of testosterone appears partially to protect spermatogenesis from the long-term damage caused by the heating. Whether this protection is due to reduced testosterone or gonadotrophins, or both, cannot be determined from the present results. It is possible that during the period of cell degeneration after heating, and the production of potentially toxic cytokines, stimulation of spermatogenesis by FSH and testosterone actually increases the damage.

The present results add to the previous observations of a protective effect of GnRH plus anti-androgen treatment on acute damage to spermatogenesis. Taken together, these studies give a background for prospective clinical trials, aiming at the protection of spermatogenesis during or after cancer treatment.

The authors are grateful to Zeneca Pharmaceuticals, Macclesfield, Cheshire for supplying the Zoladex implants and to Schering Plough AB, Stockholm for supplying the flutamide, to Dr Lars Olof Hansson for the testosterone assays and to the Nobel Foundation for financial support.

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Received 22 November 2000.

First decision 22 January 2001.

Final revision received 30 March 2001.

Accepted 5 April 2001.