The form and function of the Leydig cells in hypophysectomized rams treated with pituitary extract when spermatogenesis is disrupted by heating the testes


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ABSTRACT

The morphology and in-vivo function of the Leydig cells were studied in rams when spermatogenesis had been disrupted by a single exposure of the testes 20 days earlier to a temperature of about 42 °C for 45 min. To avoid complications due to changed negative feedback from the testes to the pituitary with consequent changes in the degree of gonadotropic stimulation, ten of the animals (five heated and five unheated) were surgically hypophysectomized when the testes were heated and then treated twice daily with pituitary extract. Six intact rams (three heated and three unheated) were also studied. The heat-affected testes were about half the size of the unheated testes, and blood plasma flow was closely related to testis weight. There were no differences in the testosterone concentrations in spermatogenic venous blood, testicular lymph or rete testis fluid, or in oestradiol in spermatogenic venous plasma from heated or unheated testes. Consequently, testosterone secretion by the heat-affected testes was markedly reduced, and the concentrations in jugular blood were also lower in the heat-affected rams than in controls. The volume of the interstitial tissue was less in absolute terms in the heat-affected rams, but it made up a greater fraction of the testes. The absolute volume of the blood plus lymph vessels, and their fraction of the interstitial tissue were lower in the heat-affected testes, although there was no effect on their volume as a fraction of the whole testis. The heat-affected testes of the hormone-treated rams had fewer Leydig cells, but each cell was larger; no equivalent difference was found in the intact rams. However, the dose of pituitary extract chosen was somewhat excessive, as there were higher than normal concentrations of FSH, LH and testosterone in jugular blood plasma, of testosterone and oestradiol in testicular venous blood plasma and of testosterone in rete testis fluid in the hormone-treated hypophysectomized rams. The testes of the unheated hypophysectomized rams increased in size by about 20% during treatment with pituitary extract, although testicular blood plasma flow was lower per unit weight of testis. The absolute volume of each Leydig cell and the total volume in absolute terms and as a fraction of the interstitial tissue was greater in the hormone-treated than in the untreated rams, but not the volume as a fraction of the whole testis. The total number of Leydig cells was higher in the hormone-treated unheated rams than in all the other rams taken together. It would therefore appear that when spermatogenesis was disrupted following heating of the testes and the gonadotropic stimulation was kept constant, the Leydig cells underwent hypertrophy, presumably because of a change in the secretion of paracrine factor(s) by the tubules. However, there was also a decrease in testosterone secretion, which was closely related to decreases in blood plasma flow through the testes, and bore little or no relation to the number, total volume or size of the Leydig cells, or the concentration of testosterone in the testicular venous blood.


INTRODUCTION

It used to be thought that Leydig cells are unaffected by a short period of moderate heating of the testes, sufficient to cause disruption of spermatogenesis (see Waites & Setchell, 1969; Waites, 1976; Setchell, 1978). However, there is now evidence in rats that heat-induced aspermatogenesis is associated with hyper-
trophy, but reduced in-vivo function of Leydig cells 
(Damber, Bergh & Janson, 1980; Jegou, Laws & de Kretser, 1984; Galil & Setchell, 1987a,b), similar to 
the changes seen during cryptorchidism (Bergh & 
Risbridger, Kerr & de Kretser, 1981; Bergh, Ason 
Bergh, Damber et al. 1984). There are also changes 
in the number, appearance and function of the Leydig 
cells in cryptorchid rams (Hochereau-de Reviers, 
Blanc, Cahoreau et al. 1979; Barenton, Blanc, Caraty 
et al. 1982; Lunstra & Schanbacher, 1988). It is 
commonly believed that the effects of cryptorchidism 
are largely if not entirely due to the increased tem-
perature to which the testis is exposed. However, 
there is the complication that the exposure to the elevated 
temperature persists up to and during the time of 
assessment of Leydig cell function, so that it is not 
possible to separate the effects due to temperature 
directly and those due indirectly to the changes to 
the seminiferous tubules. Furthermore, changes in feed-
back control of the pituitary by the testis may have 
influenced the Leydig cells by altering their level of 
stimulation by luteinizing hormone (LH) and other 
gonadotrophins. To avoid these complications, and 
to provide data in another species besides the rat, 
we have now studied the effects of disruption of 
spermatogenesis produced by prior local heating of 
the testis on Leydig cell morphology and function in 
hypophysectomized rams treated with pituitary 
extract to maintain testis functions.

MATERIALS AND METHODS

Animals

Sixteen Ile-de-France rams were used between 
September and December 1987. They were between 2 
and 5 years old, weighed between 60 and 90 kg, and 
were divided into four groups, with similar pro-
portions of the different ages in each group. Under 
halothane anaesthesia, following induction with 
i.v. pentothal (0.75 g/animal) and atropine (40 mg/
animal), the pituitaries of ten rams were removed 
surgically by a parapharyngeal route, with the 
animals lying on their backs. The size of the testes was 
estimated at the time of operation by palpation with 
reference to a series of calibrated wooden spheroids. 
At the same time, the testes of five of these rams 
were heated by immersing the shaved scrotum in 
a controlled-temperature water bath kept at 45 °C, with 
the subcutaneous scrotal temperature monitored by a 
thermistor probe inserted through a small incision in 
the skin. The temperature of the subcutaneous tissue 
rise to between 42 and 43 °C over about 15 min and 
was maintained in this range for 45 min in four rams 
and 30 min in the other. Testicular temperature in 
the ram remains very close to subcutaneous scrotal 
temperature during scrotal heating because of the 
operation of countercurrent heat exchange in the 
spermatic cord (Waites & Moule, 1961), and therefore 
we have assumed that testicular temperature was also 
rised to between 42 and 43 °C, although it was not 
measured in the present experiments. The scrotas of 
the other five hypophysectomized rams were shaved 
and testes size estimated but nothing else was done. 
The other six rams remained intact, with three of them 
aanaesthetized for the testes to be heated as described 
above, two for 45 min and the other for 30 min. We 
became concerned that heating for 45 min was pro-
ducing too great a fall in testis weight, and therefore 
shortened the time for one hypophysectomized and 
one control ram. However, the shorter time did not 
have any consistently different effect on the change in 
testis weight, or any of the other estimations and the 
results with the two times of heating have therefore 
been combined.

Hormone treatment

The hypophysectomized rams were given cortisone 
acetate (Roussel, Paris; 4 ml on day 1, 3 ml on day 2, 
2 ml on day 3 and 1 ml on day 4 of a 25 mg/ml 
solution) and penicillin (Extancilline; Pharma Service, 
Anthony, France; 2.4 × 10⁹ units on day 1 and 
1.2 × 10⁹ units on days 2 and 3, i.m.) after 
the operation, to facilitate recovery from the stress 
of surgery in the absence of a pituitary; there seems to be 
little likelihood, given the interval between treatment 
and the detailed measurements on the testis that 
these treatments influenced the results found. In any 
case, both heated and control rams received similar 
treatment. The hypophysectomized rams were also 
jected i.m. twice daily with 2 ml of a saline suspen-
sion of a sheep pituitary acetone powder, prepared by 
M. M. de Reviers and M. Courto, INRA Station de 
Physiologie de la Reproduction, Nouzilly, by the tech-
nique of Jutisz, Hermier, Colonge & Courrier (1960), 
120 mg per dose, 1 mg powder being equivalent to 
40 µg NIH S1 follicle-stimulating hormone (FSH), 
determined by the assay of Steelman & Pohley (1953). 
Unfortunately, no information is available on the LH 
potency of the preparation used, but the FSH/LH 
atio in the blood plasma was similar in injected and 
injected animals.

Sampling schedule

Blood samples were collected from the jugular vein 
three times weekly and after hypophysectomy and/or 
heating, just before the morning injection of pituitary 
extract. On days 5 and 12 after the operations, blood 
samples were collected at hourly intervals beginning 
at 09.00 h, immediately before the morning injection.
of the pituitary extract. On day 20, the rams were anaesthetized as described above, and catheters were implanted in a testicular vein under the head of the epididymis and in a testicular vein above the spermatic cord of both testes, again with the ram on its back. Infusions of p-aminohippurate (PAH; 2% (w/v), 0.1 ml/min) into the testicular veins on the testes were started and 15, 30 and 45 min later blood samples were collected from both spermatic veins above the cords and from the jugular vein. Plasma was separated by centrifugation and analysed for PAH; blood flow was calculated as described by Laurie & Setchell (1979), Chandrasekhar, Holland, D’Occhio & Setchell (1985) and Chandrasekhar, D’Occhio & Setchell (1986). The remainder of the plasma was then frozen and kept for steroid analyses.

Cannulae were then placed in lymphatic vessels in the spermatic cord and testicular lymph collected usually for 30 min, when a further set of blood samples was taken. Then one rete testis was cannulated and rete testis fluid collected usually for 30 min, when a final set of blood samples was collected. The testes were then removed, weighed and portions fixed for histology and morphometry.

Histological and morphometric techniques

Pieces of testis tissue about 5 mm thick, with a total volume of about 2 ml, from the equatorial zone of the testis, were fixed in Bouin–Holland fixative for 2 weeks, then, after progressive dehydration with ethanol from 70% onwards, the blocks were treated with celloidine (1% in methyl benzoate) for 1 day, paraffin-butanol and finally pure paraffin (1-5 days); sections were cut 10 μm thick, stained with Schiff reagents (Feulgen reaction) and alcian blue to show up the cell membrane of the Leydig cells. Using standard morphometric techniques as described in detail by Schanbacher, Pelletier & Hochereau-de Reviers (1987), estimates were made of the volume of the interstitial tissue and blood plus lymph vessels (with no distinction between them), as percentages of the whole testis and in cm³/testis, the total number of Leydig cells per testis, their mean surface area and volume and the total volume of these cells. The degree of shrinkage with this technique determined from the volume of a piece of testis was 44%, a value very similar to that seen in semi-thin sections cut from tissue fixed for electron microscopy, and all values have been corrected to allow for this amount of shrinkage. The ratio of nucleus to cytoplasm was similar (0.3 to 0.4) in Leydig cells in the present sections and in semi-thin (2 μm) sections, provided the latter included the nucleus and nucleolus of the cell, and it seems most likely that shrinkage would not modify the ratio of nucleus to cytoplasm.

The relative volume of the Leydig cells in the intertubular tissue was determined with an ocular integrator (25 point) on 20 fields per animal and the total volume of the Leydig cells per testis was then calculated from the total volume of intertubular tissue and the relative volume of the Leydig cells, after correction for histological shrinkage. The cellular cross-sectional area of perivascular Leydig cells was measured on 20 cells per animal by a graphic tablet planimeter, and their mean individual volume calculated, assuming the cells were spherical. Leydig cells in rams are not perfectly spherical, but any error introduced by assuming that they were would be important only if the elongation coefficient is large, and the variation in the area was tenfold or greater; in both experimental and control rams in the present experiments, there was less than a twofold variation. The total number of Leydig cells per testis was then calculated from their total and individual volumes.

Hormone assays

Testosterone and oestradiol were determined by radioimmunoassay in plasma, lymph and rete testis fluid, after extraction in ethyl acetate–cyclohexane (1:1), using the methods of Hochereau-de Reviers, Copin, Seck et al. (1990) and Thibier & Saumande (1975) respectively. The minimal detectable amount of testosterone in plasma was 0.7 nmol/l and in rete testis fluid 1.7 nmol/l and the minimal detectable amount of oestradiol was 14 pmol/l. The intra-assay coefficient of variation for testosterone was 3.6% for 11 nmol/l (n = 20) and the interassay coefficient was 12% (n = 4); the values for oestradiol were 12% and 12% for 57 pmol/l and 9% and 6% for 350 pmol/l.

Plasma FSH and LH concentrations were measured by radioimmunoassay; for FSH, using a kit from the NIADDK, Bethesda, MD, U.S.A. (purified hormone for iodination: oFSH S 1-1, AFP-5679C; reference preparation: oFSH-RP-1 with antisemur AFP-C5288113 at a dilution of 1/80000); the sensitivity was 0.5 ng/ml and the intra-assay and interassay coefficients of variation were 12 and 14-1% respectively. For LH, we used an antisemur prepared as described by Pelletier, Garnier, de Reviers et al. (1982), at a final dilution of 1/900 000, and a reference preparation (oLH-1051, kindly supplied by Dr Y. Combarnous, INRA Station de Physiologie de la Reproduction, Nouzilly; 1 mg of this preparation is equivalent to 2-1 mg LH-NIH-S1). The minimal amount detectable was 0.1 ng/ml, and the intra- and interassay coefficients of variation were 9-7% and 7-5%.

Statistical analyses

All results were evaluated by two- or three-way analysis of variance, with hypophysectomy–hormone
treatment, heating and, where appropriate, side (left or right) as factors, and with Schaffé multiple range tests (Snedecor & Cochran, 1980) to assess the significance of the difference between individual groups. No significant differences between the left and right testes were detected.

RESULTS

Testis weight and blood flow

The heated testes of both the hypophysectomized hormone-treated rams and the intact rams decreased by about 50% in size during the 20 days between heating and removal of the testes. Details of the histological changes in the seminiferous tubules will be reported in a separate paper. Total blood plasma flow was significantly \( (P<0.01) \) reduced in both groups after heating (Fig. 1), but there was no significant effect of heating on blood flow per unit weight of testis (Table 1) because of the difference in testis size.

![Graph](image)

**Figure 1.** Testis weight and testicular blood plasma flow (determined by p-aminohippurate (PAH) dilution) in intact rams (open symbols) and surgically hypophysectomized rams after treatment daily for 20 days with pituitary extract (solid symbols). The testes of some of the rams were heated by immersion in a temperature-controlled water bath at the time of hypophysectomy or 20 days before the measurements in the intact rams (circles) or left unheated (squares). The lines represent the calculated regressions for testis blood plasma flow (TBPF) on testis weight (TW): TBPF = \(-2.03 + 0.195 \times TW\), \(r=0.956\), for intact rams, TBPF = \(-1.58 + 0.117 \times TW\), \(r=0.803\) for hypophysectomized rams; the slopes of the two lines are not significantly different.

The testes of the hypophysectomized hormone-treated unheated rams increased in size from about 270 g by about 20% in the 20 days of treatment with pituitary extract (Fig. 1), so that, at the end of the experiment, the testes of the treated group were significantly \( (P<0.05) \) larger than those of the intact controls, which did not change appreciably. Blood flow through the testes of the hypophysectomized hormone-treated rams was significantly \( (P<0.001) \) less, expressed per unit weight of testis, than in the intact rams (Table 1).

Hormone concentrations and production rates

Testosterone levels in jugular blood plasma from heated hormone-treated hypophysectomized rams were significantly less than in unheated animals 2 days after heating and from 12 days onwards, although the pituitary extract injections produced very similar gonadotrophin levels in the two groups (Fig. 2). There was also a shorter response in testosterone concentration to the morning injection of pituitary extract after 12 days of treatment (Fig. 3). In contrast, the concentrations of testosterone in blood from a testicular vein above the spermatic cord were similar in the heated and unheated hypophysectomized rams treated with pituitary extract (Table 1). Consequently, there were large differences in testosterone secretion rates between the heated and unheated testes, which were entirely due to differences in total blood flow. The concentrations of testosterone in testicular lymph and rete testis fluid were also similar in heated and unheated rams. In the intact rams, the concentration of testosterone in jugular venous blood was reduced from day 5 onwards if the testes had been heated (Fig. 2) but again this difference was not reflected in differences in concentration in blood from the testicular vein above the cord and, consequently, secretion rates were reduced roughly in proportion to the reduction in blood plasma flow (Table 1).

There was significant secretion of oestradiol by the testes, but there were no significant differences in the concentrations in peripheral or testicular venous plasma or in secretion rates between heated and unheated testes (Table 1).

The concentrations of FSH and LH were substantially \( (P<0.001) \) elevated by the treatment with pituitary extract after hypophysectomy, the FSH to about three times, and the LH to about seven times prehypophysectomy values; levels rose for about the first 5 days of treatment and then plateaued (Fig. 2). Following the morning injection of pituitary extract, the FSH levels rose by about 45% of preinjection values; although most of the rise occurred in the first hour, maxima were reached between 4 and 5 h later; the rises in LH were more variable, averaging 57% in the non-heated and 23% in the heated rams. Again most of the rise occurred in the first hour, with the maxima at 4 and 1 h respectively after injection.
TABLE 1. Testis weight, plasma flow and concentrations of testosterone and oestradiol in blood plasma, lymph and rete testis fluid (with calculated secretion rates of the hormones). Rams were surgically hypophysectomized and treated for 20 days with pituitary extract and/or their testes were heated briefly to between 42 and 43 °C by immersion in warm water. Values given are means ± s.e.m. with number of observations in parentheses.

<table>
<thead>
<tr>
<th>Testis weight (g)</th>
<th>H vs U</th>
<th>HyH vs HyU</th>
<th>Hy vs I</th>
<th>IH vs IU</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heated (H)</td>
<td>142 ± 14 (10)</td>
<td>324 ± 12·3 (10)</td>
<td>249 ± 6·6 (6)</td>
<td>134 ± 12 (6)</td>
</tr>
<tr>
<td>Unheated (U)</td>
<td>13±0 ± 3·3 (5)</td>
<td>39·8 ± 5·5 (5)</td>
<td>42·7 ± 4·8 (3)</td>
<td>23·5 ± 3·5 (3)</td>
</tr>
<tr>
<td>Testosterone (nmol/l)</td>
<td>***</td>
<td>**</td>
<td>*</td>
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<tr>
<td>Jugular vein plasma</td>
<td>116 ± 39 (5)</td>
<td>162 ± 42 (5)</td>
<td>4·4 ± 0·73 (3)</td>
<td>2·2 ± 1·28 (3)</td>
</tr>
<tr>
<td>Left spermatic vein plasma</td>
<td>1006 ± 302 (5)</td>
<td>1194 ± 538 (5)</td>
<td>33·3 ± 7·3 (3)</td>
<td>29·0 ± 4·9 (3)</td>
</tr>
<tr>
<td>Right spermatic vein plasma</td>
<td>1232 ± 499 (5)</td>
<td>1312 ± 725 (5)</td>
<td>32·0 ± 6·3 (3)</td>
<td>32·9 ± 7·4 (3)</td>
</tr>
<tr>
<td>Plasma flow (ml/min): left</td>
<td>12±3 ± 2·9 (5)</td>
<td>30·2 ± 1·3 (5)</td>
<td>48·6 ± 0·4 (3)</td>
<td>23·3 ± 3·8 (3)</td>
</tr>
<tr>
<td>Plasma flow (ml/min): right</td>
<td>92·5 ± 14·1 (10)</td>
<td>112·6 ± 10·4 (10)</td>
<td>185·4 ± 9·3 (6)</td>
<td>175·5 ± 1·5 (6)</td>
</tr>
<tr>
<td>Plasma flow (ml/min per min)</td>
<td>19·2 ± 1·2 (5)</td>
<td>144·2 ± 10·3 (5)</td>
<td>62·4 ± 8·5 (3)</td>
<td>55·2 ± 7·5 (3)</td>
</tr>
<tr>
<td>Plasma flow (μl/g per min)</td>
<td>92·5 ± 14·1 (10)</td>
<td>112·6 ± 10·4 (10)</td>
<td>185·4 ± 9·3 (6)</td>
<td>175·5 ± 1·5 (6)</td>
</tr>
<tr>
<td>Testosterone secretion</td>
<td>8·7 ± 1·4 (10)</td>
<td>*</td>
<td>1·30 ± 0·22 (6)</td>
<td>0·75 ± 0·16 (6)</td>
</tr>
</tbody>
</table>

*P<0·05. **P<0·01. ***P<0·001 (analysis of variance and Schaffe's multiple range test).

ND, not determined.
However, the levels of both hormones were still significantly raised above preinjection values 7 h later, and the profile of the change in no way resembled a spontaneous LH peak (Fig. 3). Surprisingly, the concentration of FSH and LH did not rise in the heated intact rams, and at no stage were they different from the unheated intact controls.

The concentration of testosterone in jugular venous blood was also significantly raised by the treatment with pituitary extract in the hypophysectomized rams, although no change was seen in testosterone levels after 2 days of treatment; significant differences were found between hypophysectomized rams injected with pituitary extract and intact animals 5 days after the operation, with a second unexplained rise after 16 days of treatment. The concentration of testosterone in blood from a testicular vein above the spermatic cord was also greater in the hypophysectomized rams treated with pituitary extract than in the intact rams. No lymph values were available for the intact rams,
but the rete testis fluid testosterone levels were considerably higher in the hypophysectomized pituitary-treated animals than in the untreated intact rams (\( P < 0.001 \)), although the increase was less than in the testicular venous blood. Oestrogen levels in both peripheral and spermatic venous blood plasma and oestradiol secretion rates were greatly increased by the hormone treatment (Table 1).

**Morphology of interstitial tissue and Leydig cells**

The volume of the interstitial tissue was reduced following heating to about the same extent in absolute terms in hypophysectomized hormone-treated and intact rams; the decrease was not as great as the fall in testis weight, and, as a consequence, interstitial tissue as a percentage of the testis was higher in the heat-affected testes. The volume of the blood and lymph vessels was reduced in the heat-affected testes, when expressed as a percentage of the interstitial tissue or in absolute terms, but not when expressed as a fraction of the whole testis (Table 2).

In the heated hormone-treated hypophysectomized rams, the mean volume and surface area of each Leydig cell was appreciably greater than in the unheated group, but the number of cells was considerably less. Consequently, the total volume of the Leydig cells in the heated hormone-treated hypophysectomized rams was not significantly different from the equivalent unheated animals. When expressed as a percentage of the interstitial tissue, the proportion of the testis made up by Leydig cells in the hypophysectomized pituitary extract-treated rams was increased after heating. There was no effect of heating on the numbers of Leydig cells in the intact rams, or on their size or surface area (Table 2).

The hormone-treated hypophysectomized and intact rams had similar percentages of interstitial tissue in their testes, but because treatment of the hypophysectomized rams with pituitary extract had caused an increase in testis size if they were not heated, the absolute volume of the interstitial tissue in these animals was also increased (\( P < 0.05 \)). The volume of the blood plus lymph vessels, expressed as fractions of the interstitial tissue or of the whole testis was slightly lower in the hormone-treated than the intact rams.

The number of Leydig cells in the unheated hormone-treated hypophysectomized rams was greater than the other three groups taken together (\( P < 0.01 \)), although there was no significant difference between the hormone-treated and intact unheated animals. The mean volume (\( P < 0.01 \)) and surface area (\( P < 0.01 \)) of the Leydig cells were higher in the unheated hormone-treated hypophysectomized rams compared with unheated intact rams, and there were significant differences between all hormone-treated and intact animals (Table 2); the total volume of Leydig cells, both in absolute terms and as a fraction of the interstitial tissue and of the whole testis was increased by the hormone treatment.

**DISCUSSION**

The results of the present experiments are relevant to two different problems. The first is the relation between seminiferous tubule and Leydig cell function in the face of constant stimulation by pituitary hormones, which was the question we set out to answer. The second is the effect of high doses of gonadotrophins on testis physiology, which was inadvertently addressed because an excessive dose of pituitary extract was used. The high dose used does not affect the conclusions reached concerning the first and main objective of the study, which depended on having a constant, not necessarily normal, level of gonadotrophic stimulation.

Our results show unequivocally that hypertrophy of the Leydig cells but reduced steroid secretion by the testis are associated with deranged spermatogenesis even when the degree of pituitary stimulation is held constant. In many of the earlier experiments which suggested an effect of damaged tubules on the form or function of Leydig cells, the cause of the damage to the tubular cells was still operating and may have directly affected the Leydig cells (e.g. the increased temperature in cryptorchidism). A more general problem was that a change in the level of pituitary stimulation could not be excluded; when spermatogenesis was disrupted in both testes, changes in gonadotrophin secretion would be almost certain (Main, Davies & Setchell, 1978) but, even with unilateral heating, changes in FSH and LH levels in blood often occur (Galil & Setchell, 1987b). It would only be when disruption of spermatogenesis was focal, as in the experiments of Aoki & Fawcett (1978), that Leydig cell changes could most safely be attributed to direct effects of the damaged tubules. However, even in these experiments, an effect of the damaged tubules on the level of secretion of one or more pituitary hormones could not be excluded. It was to avoid this possibility that we decided to study rams surgically hypophysectomized and treated with a standard dose of hormones.

It is important to distinguish between the functional and anatomical changes in the Leydig cells in the heat-affected testes. Functional activity can best be assessed by measuring the amount of testosterone being secreted by the testis as a whole in vivo. Testosterone secretion by the heat-affected testes was clearly decreased in the present experiments, but this was almost entirely as a result of the decrease in blood
TABLE 2. Morphometric characteristics of interstitial tissue, Leydig cells and blood plus lymph vessels in the testes of rams which were hypophysectomized and treated for 20 days with pituitary extract and/or whose testes were heated briefly to between 42 and 43 °C by immersion in warm water. Values given are means ± S.E.M. with number of observations in parentheses.

<table>
<thead>
<tr>
<th></th>
<th>Hypophysectomized and hormone treated (Hy)</th>
<th>Intact (I)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Heated (H)</td>
<td>HyH vs HyU</td>
</tr>
<tr>
<td>Interstitial tissue volume (total ml/testis)</td>
<td>*** 26·3 ± 2·3 (5)</td>
<td>*** 47·7 ± 3·6 (5)</td>
</tr>
<tr>
<td></td>
<td>** 18·5 ± 1·31 (5)</td>
<td>* 14·3 ± 1·87 (5)</td>
</tr>
<tr>
<td>Leydig cells total number (× 10⁴/testis)</td>
<td>* 6·70 ± 1·04 (5)</td>
<td>* 11·4 ± 1·22 (5)</td>
</tr>
<tr>
<td>Mean surface area (μm²/cell)</td>
<td>*** 88·3 ± 1·3 (10)</td>
<td>*** 72·0 ± 2·0 (10)</td>
</tr>
<tr>
<td>Mean volume (μm³/cell)</td>
<td>*** 624 ± 14 (10) *** 460 ± 19 (10) *** 356 ± 24 (6) 323 ± 12 (6)</td>
<td></td>
</tr>
<tr>
<td>Total volume (ml/testis)</td>
<td>4·12 ± 0·70 (5)</td>
<td>5·18 ± 0·77 (5)</td>
</tr>
<tr>
<td>(% of interstitial tissue)</td>
<td>15·6 ± 1·8 (5)</td>
<td>11·2 ± 1·9 (5)</td>
</tr>
<tr>
<td>(% of testis)</td>
<td>2·92 ± 0·41 (5)</td>
<td>1·63 ± 0·28 (5)</td>
</tr>
<tr>
<td>Blood plus lymph vessels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total volume (ml/testis)</td>
<td>*** 6·91 ± 0·94 (5)</td>
<td>*** 15·8 ± 1·13 (5)</td>
</tr>
<tr>
<td>(% of interstitial tissue)</td>
<td>*** 26·0 ± 1·32 (5)</td>
<td>** 33·3 ± 0·45 (5)</td>
</tr>
<tr>
<td>(% of testis)</td>
<td>4·77 ± 0·31 (5)</td>
<td>4·85 ± 0·23 (5) ** 5·63 ± 0·38 (3) 6·14 ± 0·28 (3)</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001 (analysis of variance and Schaffé's multiple range test).
flow. Similar results have already been published for heat-affected and irradiated testes in rats (Galil & Setchell, 1987a; Wang, Galil & Setchell, 1983), but these rats all had intact pituitaries and consequently the results could have been affected by changes in gonadotrophin secretion, which were also reported. We have no explanation for the close relationship between testis weight and blood flow, except that most tissues appear to adjust their blood flow to their metabolic needs if tissue mass decreases. A similar relationship between testis weight and total flow was found in the intact and hormone-treated groups, although the flow per unit weight of testis was different (see below). The values for blood plasma flow per gram of testis were very similar to those reported with the same and other techniques in anaesthetized rams of other breeds (Fleet, Laurie, Noordhuizen-Stassen et al. 1982; Chandrasekhar et al. 1985, 1986; Noordhuizen-Stassen, Charbon, de Jong & Wensing, 1985). Blood flow was measured in all the animals in the present experiment with the animals lying on their backs, and posture has been shown to have an important effect on testicular blood flow. However, when posture is taken into consideration, the values for blood plasma flow in the present experiments are very similar to those which can be calculated (assuming a haematocrit of 30%) from the values for blood flow through the testes of conscious standing rams (Setchell & Waites, 1964; Laurie & Setchell, 1979), so anaesthesia is unlikely to have influenced our conclusions. It also seems most unlikely that the reduced blood flow per se would have an effect on the Leydig cells, as blood flow per gram of testis was unchanged. The availability of LH to the Leydig cells is also not likely to be affected even if blood flow per unit weight of testis had been decreased, as the uptake of a molecule of the size of LH is probably diffusion- or transport-limited, not flow-limited (see Setchell, 1990).

Turning now to the anatomical criteria of Leydig cell activity which, in contrast to the in-vivo functional criteria, suggest normal or enhanced function. Some degree of Leydig cell hyperactivity in the heat-affected testes of the hormone-treated rams in the present experiments was suggested by the finding of increased Leydig cell size, as previously described in cryptorchid rams by Lunstra & Schanbacher (1988), but not by Barenton et al. (1982), and in rats after heating (Damber et al. 1980) and during cryptorchidism (Kerr et al. 1979; Risbridger et al. 1981); these morphological changes in rats are in accord with the greater in-vitro production of testosterone by the Leydig cells from heat-damaged testes in this species (Jegou et al. 1984; Galil & Setchell, 1987a). On the other hand, there were fewer Leydig cells in the heat-damaged testes of hormone-treated rams, but this was because there was no increase in Leydig cell numbers in the heat-treated testes during hormone treatment. It should be remembered that a decrease in Leydig cell numbers was found in the testes of cryptorchid rams (Barenton et al. 1982; Lunstra & Schanbacher, 1988) and in the abdominal testis of unilaterally cryptorchid pigs (van Straaten & Wensing, 1977), although no decrease is seen in cryptorchid mice (Iguchi, Ohta & Takasugi, 1986). The absence of changes in the number or size of the Leydig cells in intact rams after heating is perhaps surprising, although it should be emphasized that smaller numbers of intact rams were used. We believe that the finding of hypertrophied Leydig cells in the testes of heated, hormone-treated rams is unequivocal evidence for the increased secretion of a stimulatory factor (or a decrease in an inhibitory one) by the heat-damaged tubules.

What is most difficult to explain is why the hypertrophied or normal sized Leydig cells did not produce testosterone at a greater or equal rate, so that, with the lower blood flow, higher concentrations of testosterone would be produced in the interstitial fluid and venous blood. This would mean that secretion rate would increase or remain the same, the movement of testosterone from the testis to the circulation being flow-limited (see Setchell, 1990). The concentrations of testosterone in testicular venous blood, lymph and rete testis fluid in the rams in the present experiment were not increased at all when spermatogenesis was deranged, even in the intact rams. In contrast, in the rat, the testosterone concentrations in testicular venous blood are actually raised in the heat-affected testes, although not sufficiently to prevent a fall in total output of hormone (Galil & Setchell, 1987a). However, this difference between rams and rats could be explained by the different gonadotrophin response in the two species (see below). There is evidence for some local product feedback control of testosterone production (Darney & Ewing, 1981), which could prevent the Leydig cells from continuing to produce testosterone at the same rate despite the fall in blood flow. However, if this is the explanation, then comparison of the testosterone levels in spermatic venous blood from intact and hormone-treated rams show that the level at which this control operates can be drastically altered by gonadotrophins.

A direct effect of heat on the Leydig cells is unlikely 20 days after a single exposure, although it cannot be excluded. However, the fact that testosterone concentrations in peripheral plasma were lower in the heated rams than in the equivalent unheated animals as early as 2 days after heating may imply that heat has a direct early effect on the Leydig cells, since there would be little effect on most of the tubular cells (Waites & Ortavant, 1968) or on testis size (J. L. Zupp & B. P. Setchell, unpublished results) at that time. Changes in the function (Main et al. 1978; Galil &
Secretion of oestradiol by the testis has been demonstrated in several species (see Setchell & Brooks, 1988), but apparently not previously in the ram. An acute response to human chorionic gonadotrophin (hCG) has been found in rats (de Jong, Hey & van der Molen, 1973), bulls (Amann & Ganjam, 1975) and boars (Setchell et al. 1983), although there was no change in oestradiol levels in testicular venous blood of rats after 4 days treatment with hCG (de Jong et al. 1973). The response of our rams to treatment with pituitary extract for 20 days was much greater than any of the acute responses.

Surprisingly, testicular blood flow was actually decreased in the hormone-treated animals. Human chorionic gonadotrophin causes a fall between 4 and 6 h and increases in testicular blood flow between 12 and 48 h after injection in the rat (see Setchell, 1990). No other studies appear to have been done on the effects of prolonged treatment with gonadotrophins on testicular blood flow. It is interesting that steroid secretion and testis size increased although the regime of treatment meant that there were virtually no pulses of LH secretion. Other studies (e.g. Martin, Sutherland & Lindsay, 1987) have suggested that LH pulse frequency is an important determinant in the degree of stimulation of the testis, but it is clear from our results that stimulation can also occur with a continually high level of LH. This is in agreement with the findings of Chase, Schanbacher & Lunstra (1988) on rams and Gibson-Berry & Chase (1990) on rats, who found that continuous infusions of LH were as effective as pulsatile administration of the same dose in animals immunized with gonadotrophin-releasing hormone.

The greater number of Leydig cells in the unheated hypophysectomized pituitary extract-treated rams suggests that the rate of multiplication or degeneration of the Leydig cells is affected by the high levels of gonadotrophins in the blood of these animals. This is consistent with the changes in Leydig cell numbers seen in rats after prolonged treatment with hCG (Christensen & Peacock, 1980; Teerds, de Rooij, Rommerts & Wensing, 1988; Teerds, Closet, Rommerts et al. 1989a; Teerds, Rommerts, van de Kant & de Rooij, 1989b); DNA labelling in rat Leydig cells was also enhanced after hCG or LH treatment (Abney & Carswell, 1986).

The other unexpected finding was that in the intact rams neither FSH nor LH concentrations increased after heating of the testis, despite a fall in testis weight of about 50%. The associated changes in spermatogenesis, which will be described elsewhere should have been sufficient to reduce feedback by inhibin and increase gonadotrophin levels in plasma, as is seen after cryptorchidism in this species (Schanbacher & Ford, 1977; Blanc, Cahoreau, Cour et al. 1978;
Schanbacher, 1980) and in rats with various types of aspermato genesis including that following heating of the testis (see Schell, Davies & Main, 1977; Main et al. 1978; Rich, Kerr & de Kretser, 1979; Jegou et al. 1984; Gaill & Schell, 1987a,b). As rete testis fluid from the ram was one of the first sources of a substance with the correct characteristics for inhibin (Setchell & Sirinathsinghji, 1972; Schell & Jacks, 1974) this is rather puzzling. Furthermore, it suggests that inhibin may be even less important quantitatively in the adult ram than in the adult rat, where it has been estimated by Main et al. (1978) that testosterone is the major control, with inhibin as a significant but minor factor.

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