Aust. J. Biol. Sci., 1983, **36**, 285–93

Limitations Imposed by Testicular Blood Flow on the Function of Leydig Cells in Rats *in vivo*

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Abstract

Testis blood flow per testis closely follows testis weight in rats made aspermatogenic by a single exposure of the testis to 43°C for 30 min or 500 rad (5 Gy) of irradiation from a caesium source, or following ligation of the efferent ducts. Aspermatogenesis following these treatments was associated with only minor changes in the concentrations of testosterone in peripheral blood before stimulation with human chorionic gonadotrophin (hCG), and a reduced responsiveness to hCG when testis weight had fallen after heating. The concentrations of testosterone in testicular venous blood was normal or above normal during aspermatogenesis resulting from heat or irradiation, and only slightly reduced following efferent duct ligation.

Consequently testosterone production (defined as the product of plasma flow and the veno-arterial concentration difference for testosterone) was markedly reduced during aspermatogenesis, both before and after stimulation with hCG. It appears that the reduced blood flow limits the amount of testosterone leaving the testis, and while the Leydig cells are capable under some circumstances of compensating partially for this fall by increasing the concentration of testosterone in the testicular venous blood, this compensation is not complete when there are severe reductions in blood flow. Therefore one can conclude that the mass of the tubules is the main determinant of testis blood flow and the Leydig cells must manage with what the tubules require.

Introduction

In studies on the interrelationships between the Leydig cells and the seminiferous tubules, an apparent anomaly has arisen. Following a variety of treatments which 'selectively' damage the tubules (cryptorchidism—Risbridger *et al.* 1981*a*; fetal irradiation, vitamin A deficiency, administration of hydroxyurea—Rich *et al.* 1979; efferent duct ligation—Main and Setchell 1980; Risbridger *et al.* 1981*b*; local heating—Main and Setchell 1980; Galil and Setchell 1980, 1981; or local irradiation —Wang *et al.* 1983) the Leydig cells appear hypertrophic and, furthermore, if the aspermatogenic testes are decapsulated or Leydig cells are isolated from them and incubated *in vitro*, they produce normal or often greater than normal amounts of testosterone. On the other hand, the concentration of testosterone in the peripheral blood is normal or slightly subnormal despite substantial increases in the concentration of luteinizing hormone (LH) in the blood, and the aspermatogenic testes appear to be less able to respond to stimulation with injections of human chorionic gonadotrophin (hCG), at least as judged by the concentrations of testosterone in the peripheral circulation.

0004-9417/83/030285$02.00

In an attempt to resolve this anomaly, we have studied rats, both before and after stimulation with hCG, and measured the secretion of testosterone *in vivo* (defined as the product of plasma flow and the veno-arterial concentration differences for testosterone) by normal testes and by testes at various times after three treatments—local heating, irradiation and efferent duct ligation—which produce aspermatogenesis in different ways.

Materials and Methods

The procedures used have been described in detail elsewhere (Main and Setchell 1980; Wang *et al.* 1983). In brief, adult male rats weighing about 400 g were anaesthetized with pentobarbitone sodium (50 mg/kg) intraperitoneally and one or both testes were subjected either to local heating in a water-bath for 30 min, usually at 43°C (Galil and Setchell 1980), or the efferent ducts were ligated close to the testes (Setchell 1970) or the rat was placed inside a cylinder of lead and both testes, hindlegs and the tail exposed to approximately 500 rad (5 Gy) of irradiation from a caesium source in a Gammacell irradiator (Wang *et al.* 1983). The animals were allowed to recover, and at various times afterwards they were anaesthetized again. Testicular blood flow was measured with radioactive microspheres. Samples of blood were then collected from the testicular veins and the posterior vena cava; previous studies have shown that testosterone concentration in this blood, which is much easier to collect, is equivalent to that in arterial blood (B. P. Setchell and S. J. Main, unpublished observations). The testes were then removed and weighed. One testis was fixed by intra-arterial perfusion for histology. In some instances, the other testis was decapsulated and the cells were dispersed by forcing the parenchyma through a hypodermic needle. The dispersed cells were then centrifuged (8000 *g* for 5 min) to yield a cell fraction and a fluid fraction which will be referred to as 'testicular fluid'. It consists of a mixture of tubular fluid and testicular lymph with some cell contents from damaged cells and the proportions of these may vary if the testes are abnormal.

Blood plasma was separated by centrifugation and analysed by radioimmunoassay for testosterone. The peripheral samples were also analysed by radioimmonoassay for FSH and LH and testosterone. The intra-assay and interassay coefficients of variation were 6·2, 6·1 and 6·1% and 15·7, 21·7 and 20·1% respectively and the sensitivities were 24·8, 1·6 ng/ml and 0·46 pmol/ml respectively. Details of the methods used are given elsewhere (Main and Setchell 1980). Plasma flow was calculated from blood flow by multiplying this flow by (1 − haematocrit).

The significance of differences between groups was assessed by the Mann–Whitney U-test.

Results

All three treatments produced a marked derangement in spermatogenesis and a fall in testes weight, but the time course of this fall varied according to the treatment used (Fig. 1). After heating, testis weight began to fall by 2 days ($P<0·01$), reached a nadir at about 21 days ($P<0·01$) and had recovered partially by 56 days ($P<0·05$), by which time the testes were histologically normal. Following irradiation, there was only a very small decrease in testis weight over the next 21 days ($P<0·05$ at 4 days, $<0·01$ at 14 days and $<0·001$ at 7 and 21 days), but by 40 days after irradiation, testis weight had fallen to less than half normal and the values at 52 days were even lower. By 80 days after irradiation, there was only a slight recovery in weight, although histologically most of the tubules at that time were normal and only a few showed persistent abnormalities. In contrast, after efferent duct ligation there was initially an increase ($P<0·01$) in testis weight due to the accumulation of fluid which normally passes into the epididymis. Then after about 36 h, the blood–testis barrier breaks down (B. P. Setchell and M. S. Laurie, unpublished observations), testis weight fell back towards control and there was a progressive disorganization of the germinal epithelium and eventually loss of almost all the germinal cells. By 21 days after ligation, the seminiferous tubules contained virtually no germinal cells, only

Sertoli cells and a few spermatogenia and testis weight had fallen to about two-thirds of control. No recovery took place up to 90 days after ligation.

With all three treatments, testicular blood flow fell in parallel with the testis weight (Fig. 1); there was no increase in testicular blood flow corresponding with the increase in testis weight immediately after efferent duct ligation. The only discrepancies between the falls in testis weight and blood flow occurred during the recovery phase. By 56 days after local heating the histological appearance of the testis was normal, the testicular blood flow (expressed as μl/min per testis) had returned to normal, although the testes were still appreciably smaller than control ($P<0{\cdot}05$).

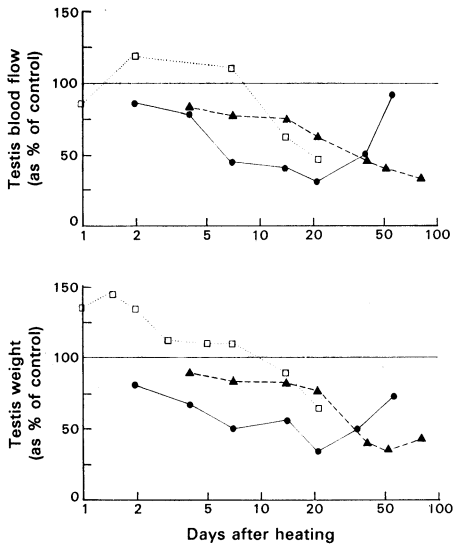

Fig. 1. Testis blood flow per testis (upper panel) and testis weight (lower panel) as percentages of control in rats at various times following unilateral and bilateral local heating of the testes (43°C for 30 min, ●), bilateral local irradiation (500 rad, ▲) or unilateral and bilateral efferent duct ligation (□). The effects of unilateral and bilateral heating were indistinguishable, as also were those for unilateral and bilateral efferent duct ligation. The data for each group have been pooled. Each value is the mean for a group of between five and eight rats.

In contrast, by 80 days after irradiation, when again testicular histology was essentially normal, both testicular blood flow ($P<0{\cdot}001$) and testis weight ($P<0{\cdot}001$) were still appreciably lower than control.

There was little consistent change in the concentration of testosterone in the peripheral blood of unstimulated rats after local heating or efferent duct ligation but there was a slight fall ($P<0{\cdot}05$) after irradiation (Fig. 2). The concentration of testosterone in the peripheral blood 2 h after injection of hCG was reduced when the testis weight was reduced after heating but not at the equivalent stage after irradiation.

The concentration of testosterone in testicular venous blood of unstimulated rats was initially unaffected after local heating but then rose as the testis decreased in size ($P<0{\cdot}01$ at 28 days; $<0{\cdot}05$ at 56 days). In rats simulated with hCG 21 days after heating the testes, the concentration of testosterone in testicular venous blood

was also slightly above control ($P<0{\cdot}01$, Fig. 3). Following efferent duct ligation, the unstimulated concentration of testosterone in the testicular venous blood was slightly subnormal ($P<0{\cdot}05$) except at 14 days after operation. In rats stimulated

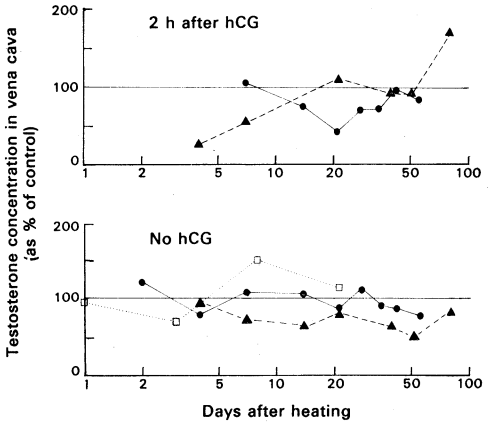

Fig. 2. Concentrations of testosterone in blood plasma from the posterior vena cava, as percentages of control in rats either before or 2 h after the injection of hCG (100 i.u. intravenously) at various times after bilateral local heating of the testes (●), bilateral local irradiation (▲) or bilateral efferent duct ligation (□).

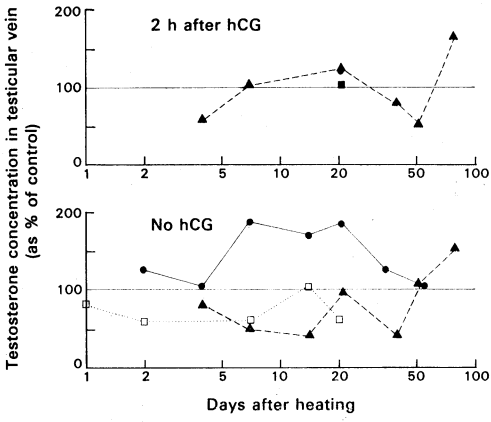

Fig. 3. Concentrations of testosterone in blood plasma from a testicular vein, as percentages of control in rats either before or 2 h after the injection of hCG (100 i.u. intravenously) at various times after unilateral or bilateral local heating of the testes (●), bilateral local irradiation (▲) or unilateral efferent duct ligation (□). Note the single points after hCG at 21 days for heating and efferent duct ligation. Provided that the comparison was made with appropriate controls, there were no differences between the effects of unilateral and bilateral heating, and the results have been pooled.

with hCG 21 days after ligation the concentrations of testosterone in testicular venous blood was similar to control (Fig. 3). After irradiation, the concentration of testosterone in plasma from the testicular vein of unstimulated rats fell initially ($P<0{\cdot}05$ at 7 days, $<0{\cdot}01$ at 14 days) but then recovered to normal or slightly

supranormal values as the testis decreased in size. The same pattern applied after stimulation with hCG, except that at 52 days after irradiation, there was no response to hCG in the concentration of testosterone in the testicular vein plasma (Fig. 3).

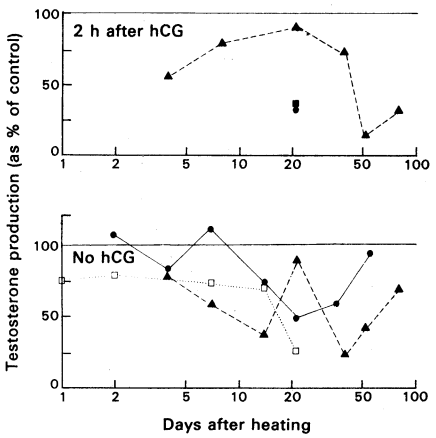

Fig. 4. Production of testosterone by the testes (defined as the product of plasma flow per testis and the veno-arterial concentration difference for testosterone) as percentage of control in rats either before or 2 h after the injection of hCG (100 i.u. intravenously) at various times after unilateral or bilateral local heating of the testes.

Table 1. Ratio of the concentration of testosterone in 'testicular fluid' to that in testicular venous blood plasma in rats either with or without hCG stimulation at various times after exposure of the testes to heat or irradiation

Treated rats were exposed to 43°C for 30 min or irradiation (500 rad) and compared by the Mann–Whitney U-test with pooled controls (i.e. with rats whose testes had been immersed in water at 33°C at the same time as for the various groups of heated rats and with unirradiated rats).

* $P<0{\cdot}005$. ** $P<0{\cdot}01$. *** $P<0{\cdot}001$

Treatment	Days after treatment	No. of observations	No hCG	2 h after hCG
Irradiation	Control	7	1·09	1·03
	4	6	1·33	1·29
	7	6	2·54	1·34
	14	6	3·05*	2·90*
	21	6	1·37	1·26
	40	6	4·97**	3·27*
	52	6	1·10	4·29*
	80	6	2·20	2·07
Heat	Control	37	1·46	0·97
	7	6	4·59**	1·73
	14	6	2·25*	1·60
	21	13	2·77**	1·98***
	35	6	2·01	1·92***
	42	6	2·33	0·73

When the secretion of testosterone was calculated, the results showed that there was a consistent decrease in production after all three treatments at the time of the decrease in testis weight, both before and after stimulation with hCG (Fig. 4). There

was also a decrease at 7 ($P<0{\cdot}05$) and 14 ($P<0{\cdot}01$) days after irradiation—i.e. before the testis weight had fallen appreciably.

In the control animals, the concentration of testosterone in the testicular fluid was only slightly greater than that in venous blood, but this ratio rose as the testis weight fell, after heating or irradiation, either with or without hCG stimulation (Table 1). No values were obtained for the concentration of testosterone in testicular fluid after efferent duct ligation.

In rats whose testes had been heated to various temperatures for 30 min, testis weight was reduced 21 days later only when temperatures above 41°C were used. Again testis weight and blood flow were closely correlated ($r = 0{\cdot}88$, $P<0{\cdot}01$ for bilateral heating; $r = 0{\cdot}69$, $P<0{\cdot}001$ for unilateral heating) and again there was a partial compensation for the reduced blood flow by increased concentrations of testosterone in the plasma from the testicular vein, which were not sufficient to maintain testosterone production within the normal range (Fig. 5).

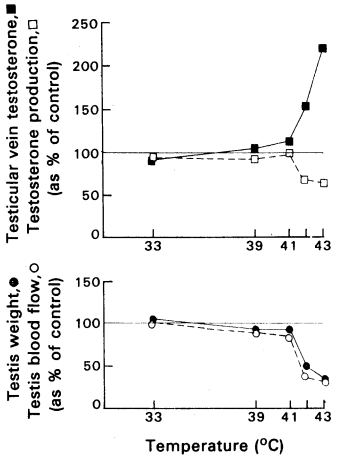

Fig. 5. Testis weight (●), testis blood flow per testis (○), concentration of testosterone in blood plasma from a testicular vein (■) and the production of testosterone (□), defined as in Fig. 4, in rats 21 days after bilateral exposure for 30 min of the testes to the temperatures shown. Each point is the mean of a group of five rats.

Following bilateral heating, irradiation or efferent duct ligation, there were increases in serum FSH and LH, similar to those described earlier (Main *et al.* 1978). Following unilateral heating, there were smaller rises in serum FSH ($P<0{\cdot}05$) than after bilateral heating ($P<0{\cdot}01$), but the pattern of the response was similar. Surprisingly, there were also rises ($P<0{\cdot}01$) in serum LH after unilateral heating; these rises were rather variable in size but they were not consistently less than those seen after bilateral heating and occurred at the same times after heating (data not shown). Unfortunately no gonadotrophin measurements were made after unilateral efferent duct ligation.

Discussion

These results demonstrate clearly that the Leydig cells in an aspermatogenic testis are capable of producing a normal or supranormal local concentration of testosterone, but the amount of testosterone entering the general circulation is reduced by a reduction in testicular blood flow. This is so whether the derangement of spermatogenesis was produced by efferent duct ligation, local heating or irradiation and these three treatments operate in quite different ways. Irradiation, at the dose

used, kills the dividing spermatogonia; the more advanced germinal cells are not immediately affected but are subsequently lost by maturation depletion (Dym and Clermont 1970). Local heating damages the pachytene spermatocytes and early spermatids (Chowdhury and Steinburger 1964; Collins and Lacy 1969; Setchell and Waites 1972) and again the rest of the cells are lost by maturation depletion. However, as later cells in the spermatogenic process are affected initially by heat, the fall in spermatid numbers occurs much earlier than after irradiation. As the spermatids constitute a larger part of the tubular tissue than any other cells, their numbers are the most important determinant of testis weight, with the spermatocytes making a significant contribution. Consequently, these different patterns of cell loss are reflected in a different timing of the decrease in testis weight.

Efferent duct ligation operates in a quite different manner. Initially the fluid secreted by the testis is retained by the ligature leading to a linear increase in weight and turgor of the testis (Setchell 1970) then the blood testis barrier breaks down (B. P. Setchell and M. S. Laurie, unpublished observations), the seminiferous epithelium becomes disorganized, cells are sloughed into the lumen and finally all germ cells are lost (Smith 1962). No recovery was observed after ligation during the period of the observations reported here, in contrast to the situation after heat and X-irradiation, where the epithelium is repopulated from undamaged spermatogonia or stem spermatogonia respectively.

However, there is an interesting contrast between these two treatments during the recovery phase. After heating, the testis blood flow per testis returns to normal values, even while the testis is still about 20% smaller than normal. In contrast, during the recovery after irradiation, the testis weight and blood flow remain substantially below control, even when the histological appearance of the testis is largely normal. This latter observation would support the idea that the amount of blood flowing through the testis is determined largely by the mass of the tubules, rather than by some specific factor from the tubules, secreted at a rate depending on whether the tubules were normal or not. There is nothing unusual in a tissue regulating its blood flow in accord with its mass of metabolically active tissue, and as the tubules constitute about 90% of the testis in the rat, it is obvious that unless specific factors from the interstitial tissue are involved, the mass of the tubules will be the main determinant of the flow, with the Leydig cells making do with what the tubules require.

It is not clear why the Leydig cells can only partially compensate for this decrease in flow by increasing the concentration of testosterone in testicular vein plasma. Darney and Ewing (1981) have shown that testosterone production by the isolated perfused testis is inhibited by concentrations of testosterone similar to the higher values found in the testicular venous plasma in the present experiments, so product inhibition may be involved.

It is also puzzling that apparently normal circulating concentrations of testosterone can be maintained after bilateral heating or irradiation when the production rates of testosterone by the testes are apparently decreased. We have no real explanation for this anomaly and can only conclude that the pattern of LH and testosterone secretion in the animal just before anaesthesia may be somewhat different from that measured under anaesthesia. The former would probably be the main determinant of the peripheral levels of testosterone, and it is unfortunate that anaesthesia is necessary to measure testicular blood flow and testosterone production, in view of its well-known effect on gonadotrophin secretion.

The observation that the testicular fluid to venous plasma ratios for testosterone (Table 1) change during aspermatogenesis might be taken to imply that the characteristics of the vascular endothelium of the testis are changed during aspermatogenesis. However, the fluid is a mixture of extracellular interstitial fluid and tubular fluid and the proportions of these may change during tubular degeneration. Further studies are needed in which extracellular interstitial and tubule fluids are evaluated separately. Other studies have shown that the blood vessels in the aspermatogenic testes increase their permeability to albumin after treatment with hCG (Galil and Setchell 1983; Wang *et al.* 1983) as in normal testes (Setchell and Sharpe 1981).

Nevertheless the testicular capillaries do show a number of peculiarities: they are second only to brain capillaries in their levels of alkaline phosphatase and γ-glutamyl transferase and these enzymes appear only at puberty (Kormano 1967; Niemi and Setchell 1983); testicular capillaries are the only unfenestrated capillaries in endocrine tissues in the rat (Wolff and Merker 1966); they are unusually sensitive to cadmium salt (Waites and Setchell 1966; Setchell and Waites 1970); the endothelial cells are involved in amino acid transport in the testis (Bustamante *et al.* 1982); they maintain considerable gradients of conjugated steroids between lymph and venous blood in the pig (Setchell *et al.* 1983) and, as we have already mentioned, their permeability to albumin is altered by stimulation by hCG.

One mystery remains. Why should the Leydig cells become hypertrophic when the tubules degenerate? It cannot be a response to the increase in gonadotrophins in the blood as when the treatment is unilateral, hypertrophy occurs only in the aspermatogenic testis and not in the contralateral control testis. Tubular factors have been suggested, but another possibility is that the Leydig cells are responsive to steroids. Oestrogen receptors have been demonstrated on these cells (Brinkmann *et al.* 1972) and there may also be some androgen receptors (Mulder *et al.* 1976) as well as LH receptors. Therefore it is at least possible that the hypertrophy of the Leydig cells may be a response to the high levels of steroids found locally in the aspermatogenic testis, and it will be interesting to measure the concentrations of oestrogens in the testes of these animals. The steroids could originate either in the Leydig cells or in the tubules. For reasons discussed in detail elsewhere (Setchell *et al.* 1983) it now seems most likely that in mature testes all the steroids produced, including the oestrogens, originate in the Leydig cells and not in the tubules. It must be admitted that this would mean that the Leydig cells have the unusual property of responding to a hormone which they themselves produce, and there appears to be no other example of this known.

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Manuscript received 5 November 1982, accepted 9 March 1983