Effect of efferent duct ligation on the function of the blood–testis barrier in rats

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The function of the blood–testis barrier has been assessed from the ratio of the Cr-EDTA space in the parenchyma to the measured interstitial volume in the testes of rats at various times after unilateral ligation of the efferent ducts. The barrier remained effective during the phase of fluid accumulation and testicular mass gain, which was linear for at least 24 h, but the testis mass began to decrease between 32 and 40 h after efferent duct ligation, and the Cr-EDTA space at 40 and 48 h after efferent duct ligation exceeded the volume of the interstitial tissue. This finding indicated that, at these times, the barrier to Cr-EDTA, which is normally excluded from the tubules, had broken down and the marker was entering the tubules. Thereafter, the Cr-EDTA space decreased again to be less than the interstitial tissue volume, indicating a restoration of the barrier function, although degeneration of the seminiferous epithelium continued to become more obvious. The present study is the first report of a reversible breakdown of the barrier, but the relevance of the breakdown to the effects on spermatogenesis requires further study.

Introduction

It has been known for many years that ligation of the efferent ducts (EDL) leading from the testis to the epididymis causes retention in the testis of the fluid secreted there, leading initially to distention and turgidity of the testis, and eventually to degeneration of the germinal epithelium and cessation of spermatogenesis (for early references, see Waites and Setchell, 1969; Setchell 1970a). Normal spermatogenesis requires a fully functional blood–testis barrier (Setchell, 1980), which is established only at puberty (Setchell et al., 1988). In the only functional study of the effect of EDL on the blood–testis barrier (Setchell, 1986), the space of distribution in the testis of Cr-EDTA (a marker normally excluded from the seminiferous tubules, but passing readily into interstitial extracellular fluid) remained less than the measured interstitial tissue volume at 24 h after EDL, but exceeded it at 48 and 72 h after EDL, indicating that the barrier was less efficient in excluding the marker from the tubules at these times. By 21 days after EDL, the testis had decreased in size to about 60% of controls; spermatogenesis had been disrupted (Main et al., 1978); and the measured interstitial space had increased because of shrinkage of the seminiferous tubules. The present study is the first report of a reversible breakdown of the barrier, but the relevance of the breakdown to the effects on spermatogenesis requires further study.

The Cr-EDTA spaces were measured in the whole testis, including the capsule, which has subsequently been shown to accumulate much more 125I-labelled IgG than the parenchyma of the testis (Pollanen and Setchell, 1989), and subsequent measurements of the Cr-EDTA space in the parenchyma (Setchell et al., 1996) are considerably lower than earlier values for the whole testis including the capsule (Setchell et al., 1988). Furthermore, the measurements were made in animals in which the ureters were ligated to maintain a steady concentration of the marker in the circulation, an assumption which has subsequently been found to be unfounded and, therefore, probably resulted in exaggerated estimates of the Cr-EDTA space. In addition, the Cr-EDTA spaces and the measurements of the interstitial tissue volume were made in different animals and, for the measurements of interstitial tissue volume, fixed tissues were used, in which the seminiferous tubules would have shrunk, leading to an overestimate of the volume of the interstitial tissue. Nevertheless, the results of this preliminary study contrasted with results from studies on the effectiveness of the barrier using electron opaque markers that indicated that the barrier remained effective after EDL (Ross, 1977; Osman and Ploen, 1978; Anton, 1982; but compare with Neaves, 1973).

Therefore, the present study was undertaken to clarify these discrepancies, to define precisely when the barrier broke down and when it was re-established, and to determine whether the disruption to spermatogenesis that follows EDL could be attributed to a failure in the function of the blood–testis barrier.

In addition, the use of data from a greater number of time
points after EDL provided an opportunity to resolve differences in the estimates of the time for which the fluid retained in the testis by the ligature results in an increase in the mass of the rat testis, which has been reported to be linear in young rats up to 30 h after EDL (Setchell, 1970b) and up to 48 h after EDL (Jegou et al., 1982). However, in older rats, Smith (1962) reported that testis mass did not begin to increase until 6 h after EDL, but from then to 36 h after EDL, the increase in weight was approximately linear. By contrast, Jegou et al. (1983) reported that mass gain was linear up to 6 h after EDL, with a slower but also linear increase between 6 and 24 h. As the mass gain has been used as an estimate of fluid secretion by the testis (Setchell, 1986; Hinton and Setchell, 1993), it is important to confirm the period over which mass gain is linear, and to confine sampling to this period.

Materials and Methods

Experiment 1

A total of 75 mature Porton rats, body weight 480 ± 8 g from the University of Adelaide Central Animal House were used. The rats were anaesthetized with Avertin (8 ml kg⁻¹ i.p.; Dyer et al., 1981), and the efferent ducts of the left testis of each animal were ligated with silk thread as close as possible to the testis, as described by Setchell (1970b) and Main et al. (1978). At various times (0, 2, 4, 8, 12, 16, 24, 32, 40, 48, 56, 64 and 72 h) after the operation, groups of rats (4–8 per time point) were anaesthetized again, jugular catheters were inserted and a priming dose of 3 µCi ⁵¹Cr-EDTA (Amersham, Bucks) followed by a sustaining infusion (0.4 µCi min⁻¹) was given with a peristaltic pump, for 20 min. In some rats, blood samples were removed from a catheter in a femoral artery at 2, 5, 10 and 15 min to monitor the concentration of Cr-EDTA in the blood. As reported by Setchell et al. (1996), this injection schedule gave Cr-EDTA concentrations in blood plasma that were constant between 10 and 20 min after the start of the infusion, ensuring that a valid estimate of the Cr-EDTA space was obtained. At the end of the 20 min infusion, a blood sample was removed from the posterior vena cava of all rats; both ligated and control testes were removed and weighed, and one half of each testis was frozen in liquid nitrogen and the other half was decapsulated. Radioactivity was measured in the parenchyma from the decapsulated half of the testis, in the capsule, the caput, corpus and cauda epididymidis, the prostate and in a measured volume of blood plasma, using a gamma-counter (Wallac 1282 Compgamma, Wallac LKB, Turku, Finland), and the volume of distribution or ‘space’ for Cr-EDTA was calculated using the mean concentration of Cr-EDTA in the plasma during the infusion. When only a final blood sample was taken, mean concentration was estimated using a factor determined from those rats in which a full set of samples had been obtained. The frozen half of each testis was cut into slices about 4 mm thick and sectioned on a cryostat for measurement of the volume of the interstitial tissue, as described by Setchell et al. (1996).

Experiment 2

A total of 36 adult male Wistar rats, weighing 320–450 g, from the animal house of the Institute of Animal Physiology, Babraham, Cambridge were used. Efferent duct ligation was performed in the same way as in Expt 1 but under pentobarbitone sodium anaesthesia and, at various times afterwards, the animals were anaesthetized again. Samples of blood for testosterone analysis were collected from testicular veins on both testes and from an artery, and blood flow through ligated and control testes was measured using radioactive microspheres, as described by Galil and Setchell (1988) and Wang et al. (1983).

Statistical analyses

Comparisons between the ligated and control testes were made by paired t tests (Statworks, Cricket DSoftware, Philadelphia, PN) and between times after EDL by analysis of variance (Super-ANOVA, Abacus Concepts, Berkeley, CA). Regression lines were obtained using the Statworks program and their standard deviations calculated from the square root of the variance of the slope of the regression lines was estimated by calculating a value for t, the ratio of the difference to its SD. The SD of the difference was obtained by taking the square root of the variance of the slopes of the two regression lines, Vₓ = Vₓ₁ + Vₓ₂.

Results

The mass of the ligated testis in Expt 1 was significantly higher than that of the contralateral unligated testis of the same animal by 2 h after EDL, and the difference increased linearly for up to 24 h (mass difference = 56.009 + 22.408 × time after EDL(h); r² = 0.981, SD of b = 1.404). There was no significant difference in testis mass between 24 and 32 h after EDL, but from 32 h onwards, it decreased again, and had returned to the same mass as the unligated testis at 56 h and thereafter. The coefficient of variation in the difference in mass between the two testes of the same animal was much greater between 32 and 64 h after EDL than up to 24 h after EDL (Fig. 1). The masses of the caput, corpus and cauda epididymidis, and of the lobes of the ventral prostate showed no consistent changes with time or between sides of the animals (data not shown). There was a very similar increase in testis mass up to 24 h after EDL in Expt 2, but the subsequent decrease was later than in Expt 1, occurring between 48 h and 7 days after EDL. From 14 days after EDL onwards, testis mass was significantly lower than on the unligated side, and there was no sign of recovery even 63 days after EDL (Table 1).

The Cr-EDTA space in the testicular parenchyma from both ligated and unligated testes decreased slightly but significantly between 2 and 12 h after EDL; thereafter, the value for the control testis returned to pre-ligation values or slightly above, while the space in the parenchyma of the
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Table 1. Blood flow in ligated (L) and contralateral control (C) rat testes after unilateral efferent duct ligation (EDL)

<table>
<thead>
<tr>
<th>Days after EDL</th>
<th>Number of rats</th>
<th>Testis mass (mg)</th>
<th>Testis blood flow</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>L</td>
<td>C</td>
</tr>
<tr>
<td>1</td>
<td>4</td>
<td>2296 ± 51***</td>
<td>1706 ± 70</td>
</tr>
<tr>
<td>2</td>
<td>6</td>
<td>2212 ± 91***</td>
<td>1628 ± 37</td>
</tr>
<tr>
<td>7</td>
<td>5</td>
<td>1725 ± 130</td>
<td>1573 ± 45</td>
</tr>
<tr>
<td>14</td>
<td>4</td>
<td>1420 ± 97**</td>
<td>1733 ± 23</td>
</tr>
<tr>
<td>21</td>
<td>9</td>
<td>1042 ± 57***</td>
<td>1612 ± 72</td>
</tr>
<tr>
<td>42</td>
<td>4</td>
<td>972 ± 98**</td>
<td>1627 ± 111</td>
</tr>
<tr>
<td>63</td>
<td>4</td>
<td>953 ± 97**</td>
<td>1650 ± 39</td>
</tr>
</tbody>
</table>

Significantly different from corresponding C by paired t test: *P < 0.05, **P < 0.01, ***P < 0.001.

...and showed no consistent changes with time or between ligated and unligated testes (data not shown). The Cr-EDTA spaces in the whole testes, including the capsule as well as the parenchyma, showed a similar pattern to that in the parenchyma (Fig. 2a), but the values for the whole testes were all higher, because of the large spaces in the capsules. The Cr-EDTA space in the caput epididymidis (192 ± 16 ml g\(^{-1}\)) was also larger than in the parenchyma of the testis, decreased slightly between 2 and 48 h and showed a small but significant increase on the ligated side at 56, 64 and 72 h after EDL to 140, 130 and 132%, respectively, that of the contralateral tissue. The Cr-EDTA space in the corpus and cauda epididymidis (234 ± 14 and 151 ± 8 ml g\(^{-1}\), respectively) also showed a slight temporary decrease with time, but there were no differences between the ligated and unligated sides. The Cr-EDTA space in the lobes of the ventral prostate (586 ± 52 ml g\(^{-1}\)) was the largest of the tissues examined, and showed no consistent changes with time or between left and right sides (data not shown).

The volume of the interstitial tissue of the unligated testis did not change significantly between 2 and 24 h after EDL, although it was slightly larger at 40 h. However, in the ligated testis, there was a significant decrease between 4 and 12 h after EDL, and then an increase between 32 and 72 h. The volume of the interstitial tissue per gram of testis was lower in the control testis than on the ligated side between 40 and 72 h after EDL (Fig. 3a). Consequently, the ratio of the Cr-EDTA space to the volume of the interstitial tissue decreased initially (between 4 and 12 h after EDL) in both testes, but then increased, to be higher than the contralateral control unligated testis at 40 and 48 h after EDL, and higher than the initial value in the ligated testis between 40 and 72 h (Fig. 3b).

Blood flow to the testis, whether expressed per whole testis or per gram of testis, was not significantly different between ligated and unligated testes up to 14 days after EDL but, at 21 and 42 days, blood flow through the ligated testis was lower (Table 1).

The concentrations of testosterone in arterial blood did not vary significantly with time after EDL, except that the values on day 14 were significantly (P < 0.05) lower than on the other days (11.0 ± 2.5, 7.0 ± 1.0, 5.7 ± 0.9, 8.2 ± 1.1, 0.9 ± 0.2).
and 4.8 ± 1.5 ng ml⁻¹ for days 0, 1, 2, 7, 14 and 21 after EDL, respectively) and a similar pattern was seen with the values for testicular venous plasma from the unligated testes (165 ± 24, 119 ± 26, 117 ± 5.4, 242 ± 38, 37 ± 12 and 123 ± 31 ng ml⁻¹). However, the values for testicular venous plasma from the ligated side were significantly (P < 0.05) lower on days 2, 14 and 21 than they were on day 0, and lower than on the unligated side on days 2, 7 and 21 (170 ± 24, 97 ± 24, 72 ± 11, 150 ± 14, 39 ± 13 and 84 ± 23 ng ml⁻¹).

**Discussion**

The present study used a technique validated for assessing the function of the blood–testis barrier (Setchell et al., 1996) to show that the barrier functions normally in the initial stages after ligation of the efferent ducts, while the testis is increasing in mass as a result of the retained secreted fluid. However, when the mass of the ligated testis began to decrease, between 32 and 40 h after EDL, there were large increases in the Cr-EDTA space and at 40 and 48 h after EDL, these increases exceeded the changes seen in the volume of the interstitial tissue (IT), so that the ratio of the Cr-EDTA space in the parenchyma to the IT volume exceeded one, indicating that Cr-EDTA must have been entering the tubules at these times. Thereafter, both Cr-EDTA space and IT volume remained higher than the initial values, as the tubules shrank again, but the ratio returned to a value less than unity, indicating that the marker was being excluded from the tubules as the barrier began to function again. Despite the much higher values for Cr-EDTA space in the capsule, which made the space in the whole testis significantly greater than in the parenchyma, the same pattern of changes was seen in the values for the whole testis. Previous studies (Setchell, 1986) reported that, by 21 days after EDL, when spermatogenesis is grossly disrupted, the IT volume is markedly increased, as is the Cr-EDTA space in the testis, but the ratio is less than one, indicating that, at this time, the marker is excluded from the tubules and the barrier is functional again.

The present results indicate that the blood–testis barrier is
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non-functional for as little as 8 h (between 40 and 48 h after EDL) and for no more than 24 h (between 32 and 56 h after EDL). It is perhaps surprising that such a short-term breakdown of the barrier would be sufficient to cause disruption of spermatogenesis to the extent that is seen after EDL, but clear histological evidence of pyknotic nuclei in spermatocytes can be found as early as 48 h after EDL (Smith, 1962) and, thus, a temporary breakdown of the barrier may initiate changes that cannot be reversed, even when the barrier becomes functional again. An even shorter exposure of the testis to heating to 43°C can also produce gross disruption of spermatogenesis, although the function of the barrier does not appear to be grossly affected (Setchell et al., 1996), as judged using the same technique as used in the present study.

In fact, during the phase of linear increase in testis mass after EDL, both the measured interstitial volume and the Cr-EDTA space both decreased slightly in both the ligated and control testes, and this decrease was greater in the Cr-EDTA space than in the volume of the interstitial tissue, so the Cr space:IT volume ratio also decreased. This decrease was presumably an effect of the anaesthetic, akin to that reported by Wang et al. (1985) on testicular blood flow. The Cr space and the Cr space:IT volume ratio was also lower at this time in the ligated testis than in the control, presumably due to compression of the interstitial tissue by the swollen tubules, which also causes the testis to become turgid (Smith, 1962, Setchell, 1970b). The linear increase in testis mass difference between ligated and control sides of 20.3 mg h⁻¹ up to 24 h after EDL seen in the present study contrasts with the study of Jegou et al. (1983), in which an increase of about 40 mg h⁻¹ for the first 8 h after EDL, and a slower rate (20 mg h⁻¹) between 8 and 24 h after EDL was reported. Therefore, in the present study regression coefficients were calculated for mass difference against time after EDL for pooled data from a number of sources, including Jegou et al. (1983), but excluding Smith (1962), whose data were omitted because they showed no increase for 6 h, and the increase continued for up to 36 h after EDL, indicating that the ligature might not have been applied close enough to the testis in these experiments. The regression slopes for 0–10 and 12–24 h after EDL were significantly different (25.0 ± 2.71 (sd) and 15.0 ± 2.67 mg h⁻¹, respectively; t = 2.64, P < 0.02), but the slopes of lines for 0–16 and 0–24 h after EDL (24.0 ± 1.25 and 20.3 ± 1.53 mg h⁻¹, respectively) were not significantly different from the slope for 0–10 h after EDL (t = 0.32 and 1.47, respectively). This finding confirms the validity of using the mass gain during the period up to 16 h after EDL as a measure of fluid secretion.

The finding that blood flow through the testis is unaffected or only slightly reduced during the period immediately after EDL confirms the findings of Tuck et al. (1970) and Wang et al. (1985), although it is somewhat surprising that the obvious increased turgidity of the testis at that time does not appear to have any effect. The decrease in blood flow at 21 days after EDL also confirms earlier findings (Main and Setchell, 1980, Wang et al., 1985), and this decrease is greater than the decrease in testis mass, so that blood flow per unit mass of testis also decreases, as does the flow per testis. This situation contrasts with that seen after heating or irradiation of the testis, where blood flow decreases as spermatogenesis is disrupted, in proportion to the decrease in testis mass (Wang et al., 1983; Galil and Setchell, 1988). The significance of this finding in relation to interactions between tubules and interstitial tissue functions has not been explored.

The normal peripheral concentrations of testosterone, despite the reduced blood flow and testosterone concentrations in testicular vein plasma 21 days after EDL confirm earlier observations (Main and Setchell, 1980; Wang et al., 1985) but, in the present experiments, there was no evidence that secretion of testosterone by the unligated testis had increased to compensate for the reduced secretion by the ligated testis. The mechanism responsible for this compensatory increase remains obscure, and LH concentrations do not change after bilateral or unilateral EDL (Main et al., 1978; Main and Setchell, 1980). The number of animals used in the present study was small; the testosterone concentrations in testicular venous blood are always variable from day to day; and there were no sham-operated controls sampled on the same day for a truly valid comparison, as there were in the earlier study (Main and Setchell, 1980). Therefore, it is likely that the previous evidence is more reliable. However, these considerations are relevant only to changes with time after EDL, and do not apply to comparisons between a ligated testis and the contralateral control testis of the same animal.

The changes in Cr-EDTA space in the caput epididymidis after EDL are another indication of the influence on this part of the epididymis of factors carried there by the flow of fluid from the testis through the efferent ducts (see Sujarit et al., 1990; Setchell et al., 1994).

In conclusion, this is the first report, to the authors’ knowledge, that the blood–testis barrier can be reversibly disrupted experimentally, but the relation between this disruption and the subsequent failure of spermatogenesis needs further investigation. After cadmium administration, one of the first changes to be observed is that the blood–testis barrier to rubidium becomes less effective, but this is followed by an increase in vascular permeability and a catastrophic decrease in blood flow (Waite and Setchell, 1966; Setchell and Waites, 1970), leading to complete irreversible necrosis of the tubules.

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