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A physiological increase in LH may influence vascular permeability in the rat testis

A. Bergh*, J. E. Damber†‡ and A. Widmark†

*Departments of *Anatomy, *Pathology, †Physiology, and ‡Urology & Andrology, University of Umeå, 901 87 Umeå, Sweden*

Summary. Adult male rats were injected with different doses of hCG, or with 2·5 μg ovine LH subcutaneously, and other rats were mated with oestrous females. The animals were examined 4 h after treatments. Treatment with hCG resulted in a dose-dependent increase in leucocyte concentration in testicular blood vessels and in the number of blood vessels which could be labelled with intravenously injected carbon particles. Carbon leakage was not observed in control testes. Treatment with a low dose of ovine LH or inducing an endogenous LH peak by mating also resulted in leucocyte accumulation and vascular leakage of carbon in the testis. The magnitude of the response was considerably lower than after high doses of hCG. The physiological relevance of the discrete response observed after physiological LH stimulation is unknown but LH-induced changes in testicular microcirculation could be of interest for the understanding of the physiology and pathophysiology of the testis.

Keywords: testis; LH; hCG; vascular permeability; inflammation

Introduction

Treatment of adult male rats with human chorionic gonadotrophin (hCG) or luteinizing hormone (LH) in high doses results in inflammation-like changes in the testicular microcirculation (Bergh *et al.*, 1988). Within 4 h after treatment polymorphonuclear leucocytes (PMNs) accumulate in the blood vessels, interendothelial cell gaps are opened in post-capillary venules and via this route macromolecules leak and PMNs migrate into the interstitial space (Bergh *et al.*, 1986, 1987). The total testicular blood flow, the volume of interstitial fluid in the testis and the lymph flow are also increased after hCG treatment (Setchell & Sharpe, 1981).

The physiological significance of these vascular changes is, however, unknown. They have as yet only been documented after stimulation with gonadotrophin doses giving a maximal testosterone response (Bergh *et al.*, 1986, 1987). It is therefore possible that they are only a sign of testis malfunction induced by supramaximal stimulation. Indeed, focal tubule damage has been demonstrated within 24 h after a high dose of hCG (van Vliet *et al.*, 1988; Kerr & Sharpe, 1989). On the other hand, it cannot be excluded that the same type of vascular changes may be induced, although perhaps not of the same magnitude, after LH stimulation in the physiological range, and that such changes could be involved in the physiological control of the vascular permeability for macromolecules in the testis. In this paper we try to answer this question by studying the vascular response to hCG/LH in physiological doses and to the endogenous gonadotrophin release known to be induced by mating (Kamel *et al.*, 1977).

Materials and Methods

Dose–responses to hCG

Adult male Sprague–Dawley rats (350–400 g/body weight) were injected subcutaneously with saline (0·154 M-NaCl) or human chorionic gonadotrophin (hCG: Organon, Oss, The Netherlands) at doses of 6·25, 12·5, 25 or 50 i.u. (for details see below) and the following responses were studied.

Leucocyte accumulation. At 4 h after hCG treatment some of the animals were killed and their testes (and psoas muscle in control animals, see below) were fixed in Bouin's fluid, dehydrated and embedded in glycol methacrylate (Histo-Resin, LKB, Stockholm, Sweden). The volume density of polymorphonuclear leucocytes (PMNs) in testicular (and psoas) blood vessels was quantified morphometrically on 2 μm thick sections stained with haematoxylin–eosin as described earlier (Bergh *et al.*, 1986).

Vascular permeability. At 4 h after hCG treatment some of the animals were sedated with pentobarbitone sodium (40 mg/kg) and injected intravenously with a solution of colloidal carbon (Pelikan drawing ink, 1 ml/kg body weight) as described in detail previously (Bergh *et al.*, 1987). By this method, blood vessels with a major increase in vascular permeability (open interendothelial cell gaps) are labelled with carbon, whereas other blood vessels are unlabelled (Majno *et al.*, 1961; Bergh *et al.*, 1987). After an additional hour when the carbon had been removed from the circulation by uptake in the reticuloendothelial system, the animals were killed and the testes were fixed by immersion in a fixative containing 4% formaldehyde, 3% glutaraldehyde, 0·05% picric acid in 0·05 M-cacodylate buffer for at least 7 days.

The testes were decapsulated and the testicular surface was examined at × 50 magnification using a Zeiss stereomicroscope equipped with a square lattice (with 121 test points) in the eyepiece. In 25 randomly chosen areas measured per testis the average number of carbon leakage sites per unit area (6·25 mm^2) of testicular surface was calculated.

The testes were then cut into small pieces. Randomly chosen pieces were post-fixed in OsO_4, dehydrated and embedded in polybed 812 (Bergh *et al.*, 1987). Sections (1 μm) were stained with toluidine blue and the percentages of sectioned capillaries and post-capillary venules (diameter < 70 μm) with subendothelial carbon deposits were quantified using a light microscope at × 1250 magnification (Bergh & Damber, 1987). Altogether, 500 cross-sectioned microvessels were counted in each testis. Ultramicrotome sections (80 nm) were stained with lead and uranyl acetate and examined in a Jeol 100 electron microscope.

Effect of a low dose of LH

Adult male Sprague–Dawley rats (350–400 g) were injected subcutaneously with 2·5 μg ovine LH (oLH-25, NIDDK). At 4 h after treatment, the testes of some animals were fixed in Bouin's fluid and leucocyte concentration was quantified as described above, and the other rats were injected with carbon to study vascular leakage as described above.

Effect of mating

Adult, sexually experienced, male rats (350–400 g) which had been living isolated (separate room) from females for 1 week were mated for 30 min with oestrous females. The males that were seen to copulate and that produced a vaginal plug were studied 4 h after the end of mating as follows.

In some of the animals the testes and pieces of the psoas muscle were fixed in Bouin's fluid and the intravascular PMN–leucocyte concentration was determined as described above. Other mated rats were injected with carbon and vascular leakage was quantified as described above.

Statistics

Comparisons amongst groups were made using the Kruskall–Wallis one-way analysis of variance and for comparison between groups the Mann–Whitney U-test. A P value of $< 0{\cdot}05$ was considered statistically significant.

Results

Effect of different doses of hCG

On intravascular leucocyte concentration. The lowest hCG dose giving a significant increase in leucocyte concentration was 12·5 i.u. and higher doses gave an additional increase (Fig. 1). In rats treated with ⩾12·5 i.u. hCG, occasional PMN-leucocytes were also observed in the interstitial space but this was not observed in controls or in the animals treated with the lowest hCG dose.

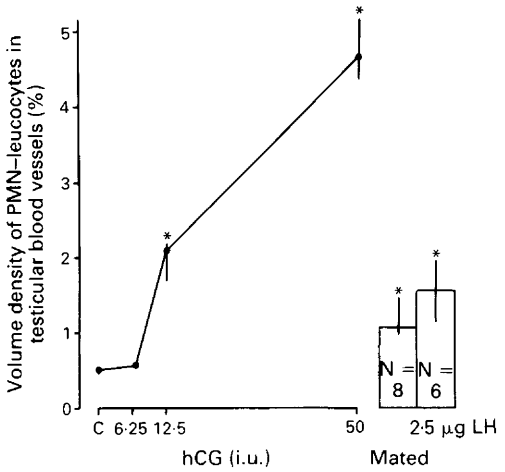

Fig. 1. Volume density of PMN-leucocytes in testicular blood vessels 4 h after treatment with hCG at different doses of 2·5 μg ovine LH and 4 h after mating. Values are median and 25- and 75-percentiles, for 5 animals/group, unless otherwise indicated. *Significantly different from basal value ($P < 0{\cdot}05$).

On vascular permeability. When sectioned testicular capillaries and post-capillary venules were examined in the light microscope at × 1250 or with the electron microscope no subendothelial deposits of carbon particles were observed in the control animals (Fig. 2a, and as described by Bergh *et al.*, 1987). In the stereomicroscope, however, a very low number of small blood vessels with darkly stained spots was detected (Table 1). When these particular vessels were embedded and studied in the light microscope at high magnification it was noted that these dark spots were caused by small intravascularly located carbon clumps and not by true carbon leakage (Fig. 2b). The number of such carbon aggregates was apparently very low and they were not observed in the randomly chosen 500 vessel profiles examined in each testis. In animals treated with ⩾12·5 i.u. hCG, subendothelial deposits of carbon particles were observed in relation to interendothelial cell junctions in post-capillary venules, and migrating leucocytes were occasionally observed (Fig. 2c, and as described by Bergh *et al.*, 1987). When the numbers of leakage sites detected in the stereo- or light microscope were plotted against the hCG dose given almost linear dose–response curves were obtained (Fig. 3, Table 1). The percentage of capillaries and post-capillary venules labelled with carbon as detected in the light microscope was highly correlated to the number of dark spots in blood vessels detected in the stereomicroscope (the linear correlation coefficient was $r = 0{\cdot}93$, $P < 0{\cdot}01$, $n = 25$ testes).

Effect of a low dose of ovine LH

On intravascular leucocyte concentration. At 4 h after treatment, the PMN concentration was increased 3·5-fold over the value in controls (Fig. 1). It was possible to detect PMNs in the interstitial space but their number was very low.

On vascular permeability. The number of carbon-stained segments of testicular blood vessels detected in the stereomicroscope was increased 3·3-fold over the control value (Table 1), and a small number of subendothelial carbon deposits was detected in post-capillary venules by light or electron microscopy (Fig. 3).

Effect of mating

On intravascular leucocyte concentration. In the control animals intravascular leucocyte concentration in the testis and psoas muscle were 0·46 ± 0·04 and 0·46 ± 0·03 respectively

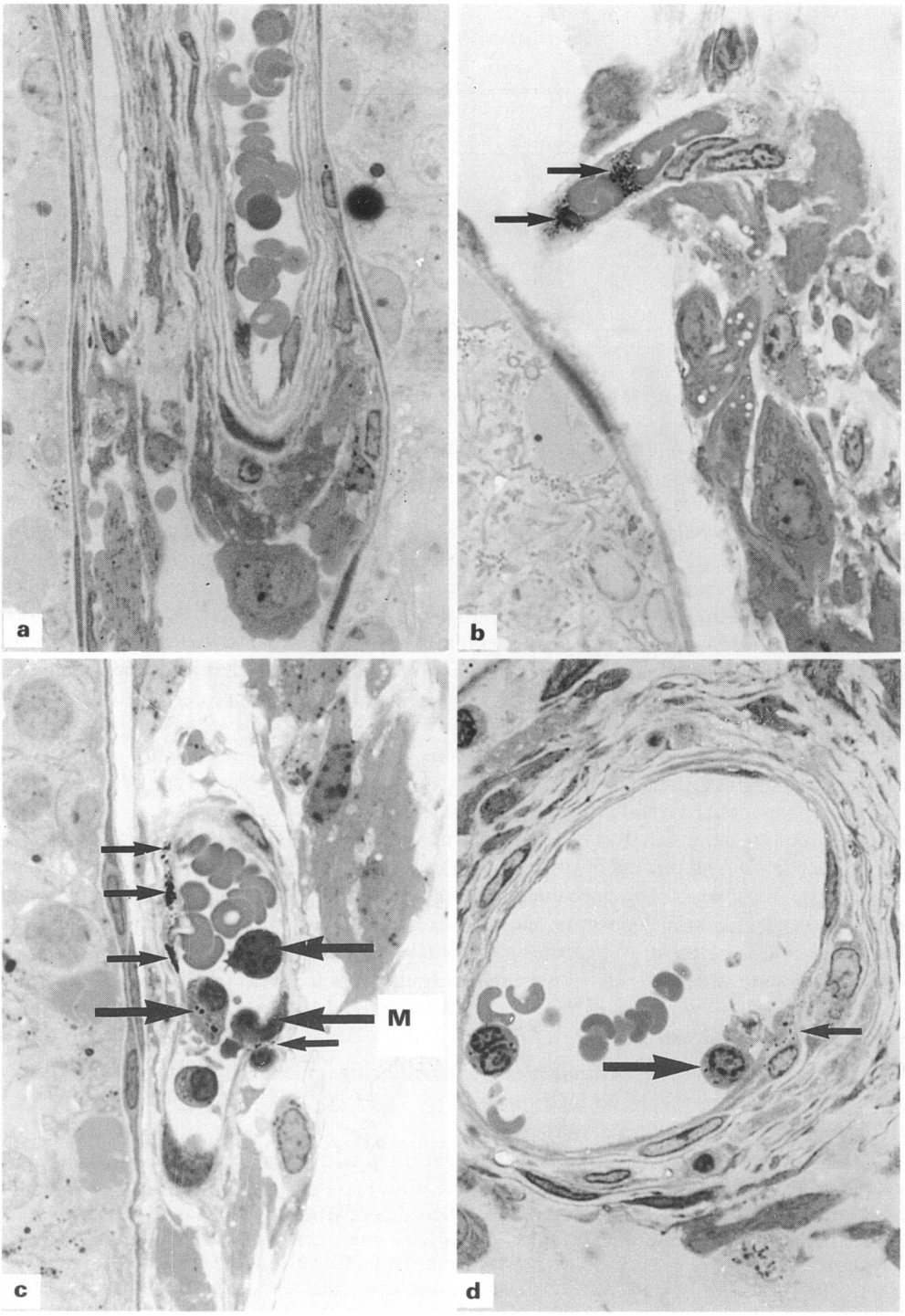

Table 1. Number of carbon-stained blood vessel segments per unit area of testicular surface (6·25 mm^2) 4 h after subcutaneous injection of hCG at different doses or 2·5 μg ovine LH, and 4 h after mating

	No. of rats	Median	25–75 percentiles
Control	5	0·40	0·40–0·43
hCG 6·25 i.u.	5	0·60	0·60–0·70
12·5 i.u.	5	2·3	1·0–2·5*
25 i.u.	5	35	34–36*
50 i.u.	5	76	46–91*
LH 2·5 μg	7	1·7	0·9–2·0*
Mating	8	1·0	0·8–1·3*

*Significantly different from the basal value ($P < 0·05$).

(mean ± s.e.m., $n = 5$). At 4 h after mating a 2·8-fold increase was observed in the testis (Fig. 1) but the concentration in the psoas was unaffected (0·49 ± 0·03, mean ± s.e.m., $n = 8$).

On vascular permeability. At 4 h after mating it was occasionally possible to find post-capillary venules with subendothelial carbon deposits but the number was low (Fig. 2d; Fig. 3), and they were generally smaller than those observed after high doses of hCG (Fig. 2c; also Bergh *et al.*, 1987). By focussing the light microscope up and down through thick (approximately 100 μm) unstained sections, vessels with carbon staining could easily be visualized (Fig. 4). Electron microscopy verified that carbon accumulated in the subendothelial space in relation to inter-endothelial cell junctions. When the number of carbon-stained vascular segments on the testis surface was counted in the stereomicroscope, a 2·5-fold increase over the basal value was observed (Table 1).

Discussion

We have previously shown that hCG in supraphysiological doses induced leucocyte accumulation in the rat testis, probably as a result of local secretion of a leukotactic factor(s) and in the formation of large leakage sites in post-capillary venules (Bergh *et al.*, 1987, 1988). Leakage sites are caused by large gaps (up to the size permitting leucocyte migration) between the endothelial cells. Such leakage sites can be detected by injection of carbon particles (size approximately 20–30 nm). The carbon particles are partly restricted by the basement membrane and the site of leakage is therefore stained black. Carbon particles are too large to penetrate and label normal blood vessels in which the interendothelial cell clefts are closed (Majno *et al.*, 1961; Cotran *et al.*, 1967), as in the testis during basal conditions (Bergh *et al.*, 1987). Carbon labelling has been used extensively to study the

Fig. 2. Sections from testes of rats injected with colloidal carbon (× 1000): **(a)** and **(b)** are from control animals; **(c)** from a rat given 50 i.u. hCG 4 h before carbon injection; **(d)** from a rat mated 4 h before carbon injection. In control testes (a) subendothelial deposits of carbon were not observed. In control animals and those in all treatment groups a very low number of vessels contained carbon particles in the lumen (b), but this could easily be distinguished from true carbon leakage as seen in (c). In the testes from the hCG (50 i.u.)-treated rat (c) large sub-endothelial carbon deposits (small arrows), and intravascular and migrating (M) leucocytes (large arrows) are observed in a post-capillary venule. Intravascular leucocyte accumulation (large arrow) in relation to subendothelial deposits of carbon (small arrows) was also found in mated animals (d).

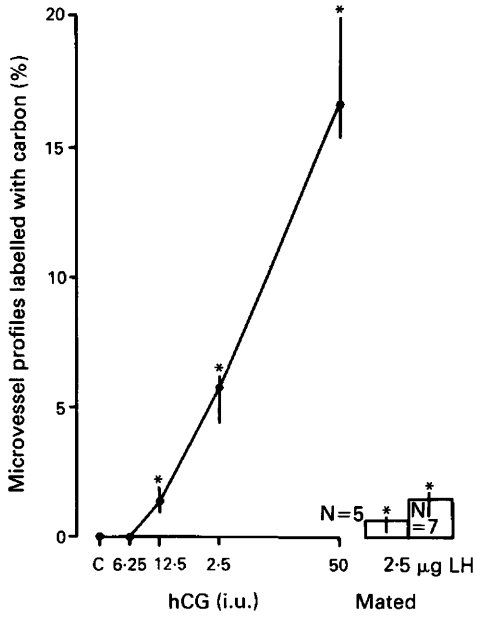

Fig. 3. Percentage of sectioned testicular microvessel profiles (capillaries and post-capillary venules with a diameter of $<70\,\mu m$) with subendothelial carbon deposits 4 h after treatment with different doses of hCG or 2·5 μg ovine LH and 4 h after mating. Values are median and 25- and 75-percentiles, for 5 animals/group, unless otherwise indicated.

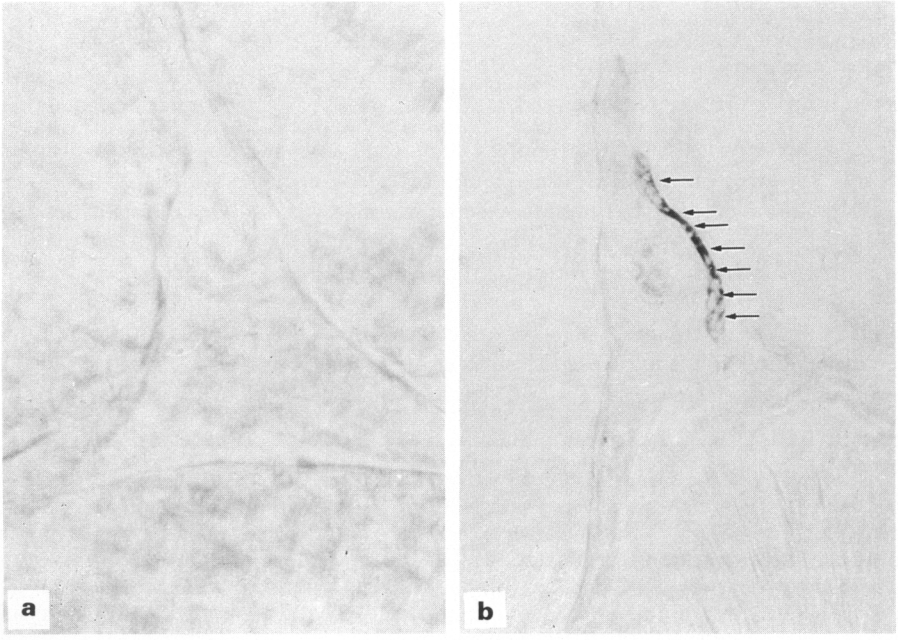

Fig. 4. Light micrographs of a 100 μm thick unstained section of testicular tissue from **(a)** a control rat and **(b)** a rat injected with colloidal carbon 4 h after mating. In (b) a post-capillary venule with numerous leakage sites (arrows) is seen in the interstitial space. ×40.

increase in vascular permeability caused by inflammation mediators and the number of labelled vessels is related to the dose of the inflammation mediator administered (O'Donnell *et al.*, 1987). In this study we observed that the degree of vascular leakage can probably be estimated easily by counting the number of carbon-stained blood vessels on the surface of the decapsulated testis using a stereomicroscope, since the results obtained by this technique are correlated with those obtained by the considerably more cumbersome counting of labelled vessel profiles in tissue sections. However, a small number of blood vessels are apparently embolized by the injected carbon particles (as described for other tissues, Cotran *et al.*, 1967) and staining caused by this can only be discriminated from true leakage in tissue sections. However, since the number of carbon emboli is very low, this is only a problem when detecting very discrete effects.

In the present study we demonstrate that the magnitudes of the two hCG-induced vascular responses, leucocyte accumulation and formation of major leakage sites, are clearly dose-dependent and hCG doses which are known to give a submaximal testosterone response (see Hodgson & de Kretser, 1982; Veijola *et al.*, 1984; Bergh *et al.*, 1986) are also effective. Moreover high doses of hCG resulted in a maximal testosterone response within 1 h, whereas the peak response is not observed until 6–24 h after treatment with lower ($\sim$10 i.u.) doses (Hodgson & de Kretser, 1982; Veijola *et al.*, 1984). To exclude vascular effects of low hCG doses it is therefore necessary to examine the effect at additional times after treatment. The dose–response curves for leucocyte accumulation and carbon leakage 4 h after hCG treatment were similar, supporting our previous suggestion that they are causally interrelated, i.e. leucocytes are probably involved in mediating the formation of interendothelial cell gaps since these gaps are not observed in hCG-treated leukopenic animals (Widmark *et al.*, 1987).

In normal rats, LH is released episodically from the pituitary. These LH pulses are generally followed by a 2–5-fold increase in plasma testosterone concentration (Ellis & Desjardins, 1982). Stimulation of adult rats with 2·5 μg ovine LH results in approximately 2-fold increases in plasma and testicular interstitial fluid testosterone concentrations (Widmark *et al.*, 1989), whereas maximal LH stimulation of the testis causes approximately a 10-fold increase (Hodgson & de Kretser, 1982). These results suggest that administration of 2·5 μg ovine LH may mimic physiological increases in serum LH. Leucocyte accumulation (3·5-fold increase) and carbon leakage in post-capillary venules were induced after treatment with this low LH dose. Mating of sexually experienced adult male rats results in a rapid 2–3-fold increase in plasma LH followed by a similar increase in testosterone (Kamel *et al.*, 1977), but other hormones such as prolactin (Kamel *et al.*, 1977), oxytocin and arginin vasopressin (Murphy *et al.*, 1988) may also be increased. In our present experiments mating also resulted in an increase in intravascular leucocyte concentration (a 2·8-fold increase which is not observed in muscle, indicating an organ-specific response) and in the formation of carbon leakage sites in post-capillary venules. The magnitude of the response was similar to that after 2·5 μg LH, suggesting that the effect of mating may be caused principally by endogenous LH release. The LH release induced by mating or LHRH treatment is followed by leucocyte accumulation and an inflammation-like increase in vascular permeability around pre-ovulatory follicles (Espey, 1980; Okuda *et al.*, 1980; Cavender & Murdoch, 1988; Bergh & Cajander, 1989), suggesting that LH induces similar effects on the microcirculation in the gonads in both sexes.

What is the physiological role of LH-induced vascular changes in the testis? The magnitude of the vascular response in the testis after physiological LH release is apparently discrete and considerably smaller than after maximal hCG stimulation. Superficially, this could suggest that these changes are only of importance when trying to understand the pathophysiology of testis damage (van Vliet *et al.*, 1988; Kerr & Sharpe, 1989) induced by supramaximal hormone stimulation. This in itself is, however, an important topic since cryptorchid infants and infertile men are currently treated with comparable doses of hCG (see Bergh, 1989). Experimentally cryptorchid testes appear to be particularly susceptible to hCG since in such testes vascular permeability is increased so much that intratesticular pressure is increased up to 40 mmHg (Hjertkvist *et al.*, 1988). In this study we

observed roughly one leakage site per 10 cross-sectioned microvessels (section thickness 1 μm) after treatment with hCG in high doses, suggesting approximately one leakage site per 10 μm microvessel length. This figure is apparently not very different from that in the rat mesenterium after administration of histamine in high doses (Fox *et al.*, 1980; Table 3), demonstrating that hCG in high doses really induces major, perhaps adverse, effects on the testicular microcirculation.

The present observation that low doses of LH also influence the testicular microcirculation indicate that the LH-induced mechanisms causing leucocyte accumulation and vascular leakage may also be of importance for the physiology of the testis. We observed carbon leakage sites (clearly larger than 250 nm which is the resolving power) with the light microscope in about 1% of cross-sectioned microvessels from the mated or LH-treated animals. This could indicate that one major leakage site for macromolecules could be present per 100 μm of microvessel length, leading to a large number of leakage sites per testis. The smallest leakage site that can be detected in the light microscope is about 100 times larger than an albumin molecule, and carbon leakage in general has previously been assumed to occur only in pathological conditions since it requires a dramatic increase in the size of the intercellular cleft (Cotran *et al.*, 1967). It therefore appears that, although the number of carbon-labelled blood vessels is very low after mating or treatment with a low dose of LH, this increase in permeability could be of importance for testicular function, although the physiological effects, if any, of such a change in vascular permeability are unknown. It is obvious that studies using more sophisticated methods than carbon labelling are needed to answer this question. The present observation that the morphology of the interendothelial cell clefts may be dramatically changed in the testis as a result of a physiological stimulus is also of importance for understanding of the regulation of vascular permeability in general. Vascular permeability in organs with continuous non-fenestrated endothelium such as the testis was previously assumed to be controlled by the presence of small and large endothelial pores of fixed size located in the capillaries, but more recent studies indicate that vascular permeability for macromolecules is controlled by rapidly occurring endothelial cell contractions changing the size of the interendothelial cell clefts principally in small post-capillary venules (Crone, 1987). Our observations suggest that this mechanism may be very active in the testis and that mechanisms similar to those that increase vascular permeability in inflammation could be used by the organism to influence vascular permeability in some organs (testis, ovary) under physiological conditions. In summary, we suggest that the ability of LH to induce changes in testicular vascular permeability may be of importance both for testicular physiology and pathophysiology.

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