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Local Initiation of Spermatogenesis in the Horse[1]

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ABSTRACT

Gross observation of testicular parenchyma of 1.5- to 2-yr-old horses reveals both light and dark regions. If this gross, differential shading reflects quantitative differences in the development of spermatogenesis and interstitial cell populations, the horse may prove to be a useful model for study of the paracrine relationships associated with initiation of spermatogenesis. The objective of this study was to characterize seminiferous tubules and interstitium of testes with gross, differential shading. Testes with both light and dark regions of parenchyma were obtained from horses 1.5–2 yr old and compared to parenchyma of fetal, 2-yr-old, or 5-yr-old horses. Stereology was used on tubular and interstitial components, and luminal development of seminiferous tubules was scored. Volume density of seminiferous tubules, percentage of tubules with large vacuoles or a complete lumen, and number of primary spermatocytes per gram were greater ($p < 0.05$) in light parenchyma than in dark parenchyma. The percentage of tubules with no lumen and the percentage of parenchyma occupied by interstitial space were greater ($p < 0.05$) in fetal and dark parenchyma than in light parenchyma. The number of Leydig cells per gram parenchyma was similar ($p > 0.05$) in both light parenchyma and dark parenchyma. A greater percentage ($p < 0.05$) of other (nonvascular, non-Leydig, nonmacrophage) cells was found in the dark parenchyma than in light parenchyma or in testes of 2- or 5-yr-old horses. The volume density of macrophages was notably greater ($p < 0.05$) in fetal and dark parenchyma than in light parenchyma or in testes from older horses. Variation in development of seminiferous tubules was not associated with the volume density of blood vessels. In conclusion, the gross, differential shading of equine testicular parenchyma with its corresponding differences in seminiferous tubular development is a clear example of the effect of local factors leading to the local initiation of spermatogenesis.

INTRODUCTION

The establishment of spermatogenesis in most species occurs randomly throughout the testis [1] with a corresponding uniformity of parenchymal coloration or shading. The horse testis is an exception in that a gross section taken from young horses (< 2) can have light testicular parenchyma in the center and dark parenchyma in the periphery [2–4]. The uniformly light testicular parenchyma is associated with completion of spermatogenesis throughout the testis in 2- to 3-yr-old horses, and subsequent darkening of the parenchyma in horses is associated with changes in interstitial cell populations [2, 3, 5]. Hence, the distinct pattern of light and dark regions in testes of young horses may result from quantitative differences in interstitial cell populations and may correlate directly with differences in tubular development. Initial development of seminiferous tubules in the center of the testis followed by tubular development in the periphery of the testis would imply a local control mechanism for the initiation of spermatogenesis [4].

Adult values for histological parameters (volume density of residual bodies and elongated spermatids) are reached by 2.5 yr of age whereas adult values for testicular weight, daily sperm production/horse, and Sertoli cell number/horse are reached by 4 yr of age [6]. The testicular composition of other interstitial components such as blood vessels, lymph vessels, and other cells, as well as seminiferous tubules, characterize age-related differences in horses aged 2–20 yr [5]. However, little attention has been directed toward quantitative characteristics of the developing interstitium as it may relate to local initiation of spermatogenesis. Likewise, relationships between the interstitium and seminiferous tubules leading to the initiation of spermatogenesis have not been evaluated in the horse.

If variation in differential shading correlates with the variation in spermatogenic development of seminiferous tubules, the horse would be a useful model for study of the initiation of spermatogenesis because regional sampling from the same testis would yield testicular parenchyma that has either initiated spermatogenesis or has not. The objective was to determine whether gross shading of testicular parenchyma and quantitative changes in interstitial cell or blood vessel density were associated with quantitative differences in seminiferous tubular development during initiation of spermatogenesis.

MATERIALS AND METHODS

Specimens

Testes from 258 horses aged 4 yr and younger were obtained from a slaughterhouse in connection with other studies [7]. Only horses from light-weight breeds, in good

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body condition, with a smooth hair coat were used. Tooth eruption and wear patterns were used to determine the age of horses [5]. Testes were removed and vascularly perfused with 2% glutaraldehyde in sodium cacodylate within 15 min of death. In the laboratory, testes were dissected from fascia and the epididymis, and weighed. Parenchymal weight is the difference between the whole weight and the weight of the tunica albuginea. Parenchymal volume is calculated by dividing the parenchymal weight by 1.05 g/ml (specific gravity; [5]). Slices of parenchyma weighing 1–3 g were placed in 2% glutaraldehyde in 0.1 M sodium cacodylate buffer and stored at 5°C. All testicular tissue was evaluated and scored on the basis of gross parenchymal shading (Fig. 1). Of the 258 horses evaluated, 16 horses 1.5–2 yr old exhibited gross, differential shading of parenchyma (both light and dark parenchyma in one or both testes). For stereologic evaluation, nine horses were randomly selected from the group of 16 horses with differential shading. Since tissues were obtained for other studies [7], the proportion of dark and light parenchyma was not obtained and could not be determined from the remaining tissues from each testis. Testes from three additional groups (fetal, 2-yr-old, or 5-yr-old), consisting of three horses for each group, were obtained and processed in the same manner. Five slices (2 × 3 × 4 mm) of testicular parenchyma (from each group as well as light and dark regions from horses with differential shading) were further fixed in 1% OsO_4 in sodium cacodylate buffer, dehydrated in a series of alcohols, and embedded in Epon [5].

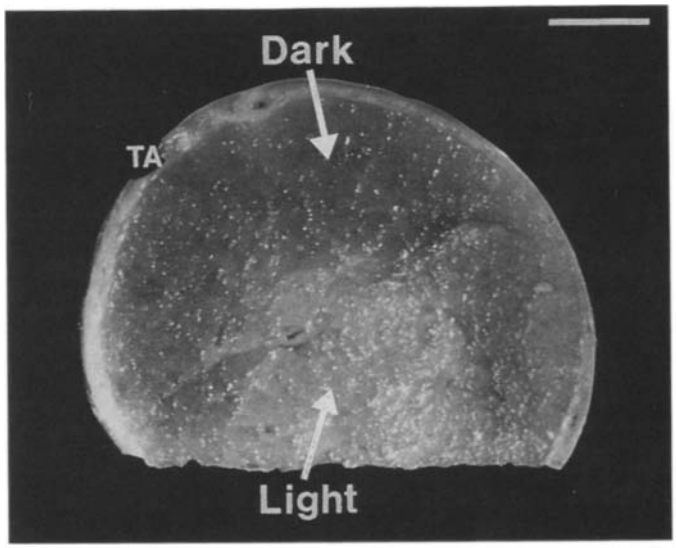

FIG. 1. Gross view of an equine testis revealing both dark and light, differential shading characteristics. Regions of dark parenchyma (dark) in periphery and light parenchyma (light) in center as well as tunica albuginea (TA) are indicated. This testis was from 2-yr-old horse and measured 2.6 cm in diameter. Bar = 5.0 mm.

Histological Evaluation

Sections were cut at 0.5 μm, stained with toluidine blue dye, and observed under brightfield microscopy and stereologically evaluated. Chalkley's point-counting method [8] was employed at 1000× to determine volume density of tubular or interstitial structures based on at least 3000 points/horse. These counts included seminiferous tubules, Leydig cells, macrophages, blood vessels, lymph vessels, other interstitial cells, and other nonvascular structures (Fig. 2). Nuclei of Leydig cells, Sertoli cells, round spermatids, primary spermatocytes, and spermatogonia/gonocytes were scored on a separate count using Chalkley's method of sampling with 10 000 points/horse at 1000×. Daily sperm production based on round spermatids or primary spermatocytes was calculated as described previously [9].

Since calculation of daily sperm production is based on the presence and number of germ cells that may not be present in immature testes, it was necessary to measure tubular development on the basis of tubule diameter, length, and luminal formation. Development of seminiferous tubules was observed at 100×, and tubules were scored (Fig. 3) on a scale of 1 (for fully closed lumen) through 5 (for complete lumen formation and full complement of germ cell types). A score of 3 was assigned when the tubules had vacuoles in the seminiferous epithelium and spermatogonia, and some primary spermatocytes were present. Tubular diameter, evaluated by 60 measurements/horse at 100×, also was used to establish tubular development and determine cross-sectional areas. The length of seminiferous tubules per gram parenchyma was calculated by using the formula for the volume of a cylinder. Length was calculated as the product of the volume density of tubules and parenchymal volume divided by the product of parenchymal weight and tubular cross-sectional areas [5].

To determine nuclear diameters, a subset of five of the nine horses with both light and dark regions of parenchyma was selected on the basis of parenchymal weights that best approximated the mean for all nine horses and compared with all horses in the other age groups. Epon sections cut at 20 μm, Nomarski optics, and a computerized digitizing unit were used to determine the maximum diameter of individual nuclei in Leydig cells, Sertoli cells, or germ cells totally embedded in the sections [5]. Given the diameters, the average volume of a single nucleus of germ cells was calculated by using the formula for the volume of a sphere. For Sertoli cell nuclei, which are not spherical, the height and width measurements were averaged before the formula for the volume of a sphere was used to approximate the nuclear volume. This approximated volume was corrected by an empirically derived correction factor of 0.73, which was applied to correct for the nonspherical shape of equine Sertoli cell nuclei [10]. The number of a given cell type was determined by dividing the product of nuclear volume density, parenchymal volume, and a histological correction factor for section thickness and nuclear diameter [11] by the volume of a single nucleus of that cell

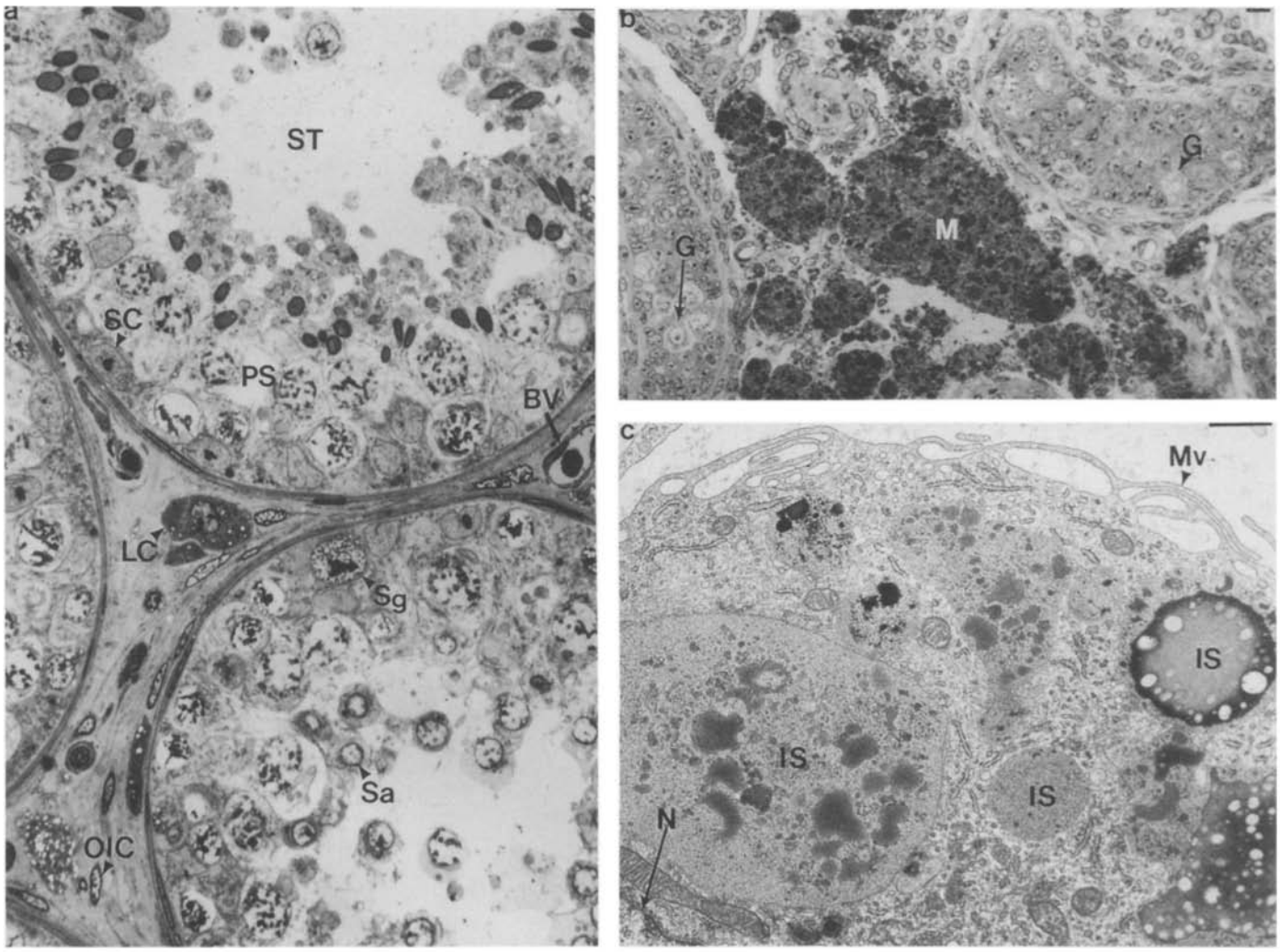

FIG. 2. Brightfield light micrographs and electron micrograph structures in **(a)** light and **(b, c)** dark testicular parenchyma. Seminiferous tubules (ST), Leydig cells (LC), macrophages (M), other interstitial cells (OIC), Sertoli cells (SC), gonocytes (G), spermatogonia (Sg), primary spermatocytes (PS), Sa spermatids (Sa) with spherical nuclei, and blood vessels (BV) are identified. **b)** When macrophages are in high density among interstitial components, often they are larger than tubular diameter (i.e., > 50 μm). **c)** Ultrastructural features of testicular macrophages include microvilli (Mv), a nonspherical nucleus (N), typical cellular organelles, and an abundance of ingested substance (IS) in various states of digestion. Bar = **(a, b)** 10 μm and **(c)** 1 μm.

type [5–7]. Electron microscopy was used to confirm the identity of macrophages [5].

Endocrine Analysis

Serum concentrations of FSH, LH, and testosterone (T) were determined by RIA as described previously [12–14]. A single blood sample, collected at the time of slaughter, was used as an estimate of daily concentrations for the nine horses with differential shading and three horses from each of the other age groups. Use of randomly selected blood samples obtained every 15 min for 12 h during the summer or winter has shown that the variation in serum LH or T concentrations among stallions was largely related to the temporal variation of an individual stallion's values around his daily mean [14]. These authors reported that the stallion has much less variation in pulsatile concentrations of T and gonadotropins than that reported for other domestic animals. Intratesticular testosterone (ITT) concentration was determined from testicular homogenates by RIA as described previously [15]. Specificity of the assay for T was confirmed as described previously [13]. The sensitivity, and intra- and interassay coefficients of variation were 0.05 ng, 9%, and 12%, respectively, for LH; 0.7 ng, 8%, and 10% for FSH, and 50 pg, 5%, and 8% for T.

Statistical Analysis

Data obtained from different age groups were analyzed by one-way analysis of variance with mean separation by the Student-Newman-Keul's test [16]. Data from horses with gross, differential shading of parenchyma were included as one group (i.e., parenchymal weight) or as two groups in the one-way analysis of variance for group comparisons.

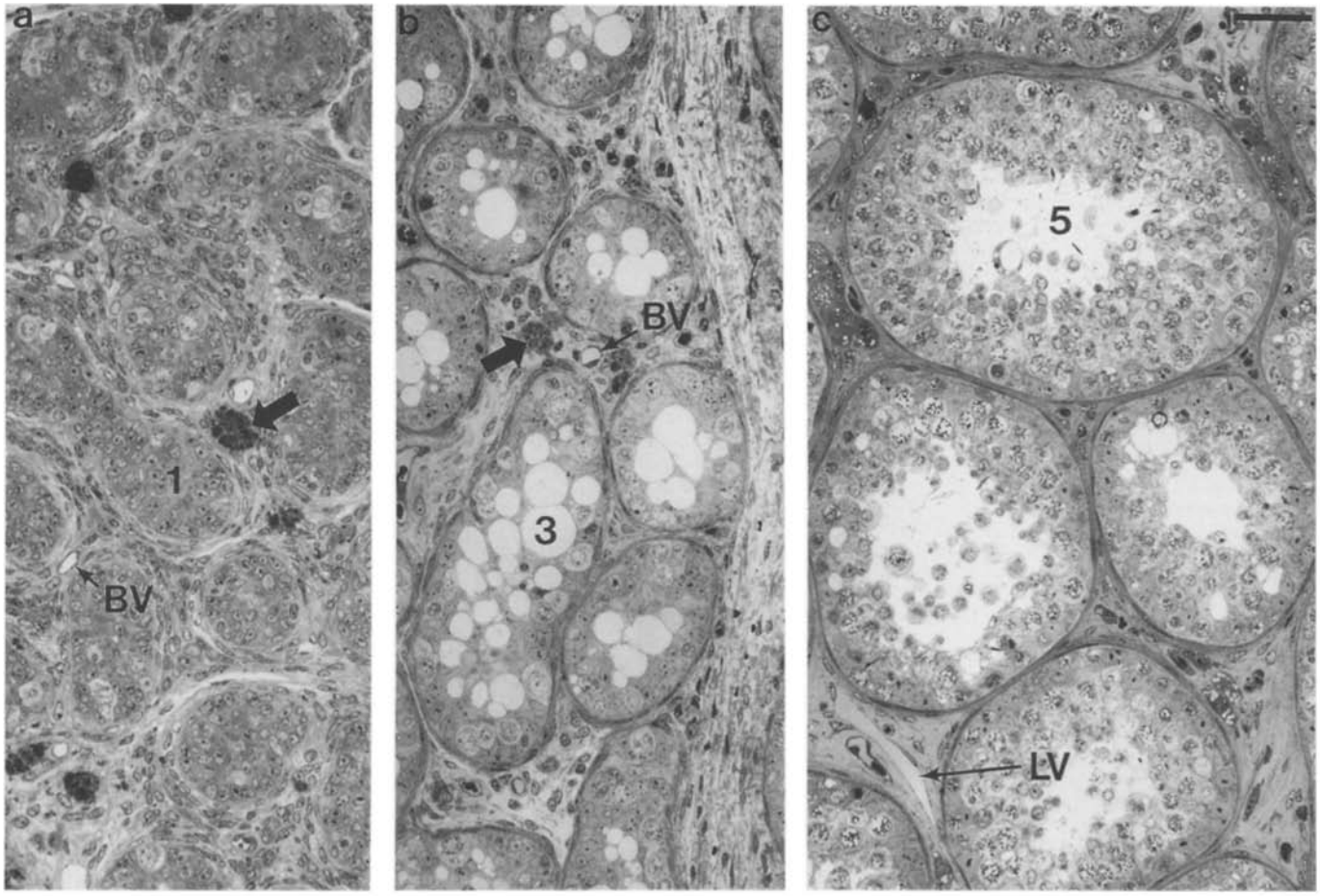

FIG. 3. Histologic view of seminiferous tubules in both **(a)** dark and **(b, c)** light parenchyma. Luminal scores for tubular development are **(a)** 1 (no lumen), **(b)** 3 (vacuoles between Sertoli cells), and **(c)** 5 (complete lumen). Macrophages (arrow), lymphatic vessels (LV), and blood vessels (BV) are indicated. Bar = 50 μm.

Hence, when values for both dark or light regions of parenchyma were available (i.e., % seminiferous tubule), values for dark and values for light parenchyma constituted two independent groups for comparison with other age groups. Comparisons of data obtained on light or dark regions of the same testis also were tested by the paired *t*-tests [16]. Prior to analysis, percentage data were transformed to arc-sine. Linear and logarithmic correlations were tested for significance [16].

RESULTS

Gross regional differences in shading of testicular parenchyma (Fig. 1) within the same testis of horses aged 1.5–2 yr were characterized by histologically distinct differences (Fig. 3). Qualitative evaluation revealed inactive small seminiferous tubules composed mostly of gonocytes and Sertoli cells with no lumen in regions of dark parenchyma. Larger more active tubules, with evidence of luminal formation and all types of germ cells, characterized the light parenchyma. Stereologic evaluation revealed that dark parenchyma had a lower percentage (volume density) of seminiferous tubules than did light parenchyma (Table 1). The volume density of seminiferous tubules in dark parenchyma of testes with shading was intermediate in value between fetal parenchyma (which have only very dark parenchyma) and parenchyma from light regions or 2-yr-old horses (which have only light parenchyma). The interstitium of the fetal and dark parenchyma was occupied by a high density of Leydig cells, macrophages, and other (non-Leydig, nonmacrophage, nonvascular) interstitial cells (Table 1; Fig. 4). Some of the macrophages were extremely large, as shown by diameters exceeding the diameter of seminiferous tubules in the dark parenchyma (Fig. 2). While light microscopic observation revealed cellular characteristics of macrophages (Figs. 2 and 3), the identity was confirmed by the ultrastructure of macrophages shown by electron microscopy (Fig. 2c). Ultrastructural features of macrophages included extensive microvilli, a nonspherical euchromatic nucleus, and typical macrophage organelles. These included heterophagic vacuoles occupied by ingested sub-

TABLE 1. Effect of age and differential parenchymal shading on testicular characteristics.

	Fetal	Dark/Light			2-yr-old	5-yr-old
		Dark	Both	Light		
Number of horses	3	—	9	—	3	3
Age (yr)	243 ± 8 days*	—	1.7 ± 0.1	—	2 ± 0	5 ± 0
Testicular weight (g)						
Tunic weight	1.7 ± 0.9[a]	—	6.6 ± 0.5[b]	—	13.4 ± 0.7[c]	16.2 ± 1.9[c]
Parenchymal weight	20.0 ± 5.7[a]	—	23.1 ± 2.8[a]	—	90.0 ± 22.6[b]	121.1 ± 14.8[c]
Serum hormones						
FSH (ng/ml)	ND[†]	—	82.4 ± 4.5[a]	—	87.0 ± 12.9[a]	162.7 ± 77.4[b]
LH (ng/ml)	ND	—	3.7 ± 0.1[a]	—	5.2 ± 0.3[a]	41.9 ± 27.2[b]
Testosterone (pg/ml)	ND	—	123.4 ± 30.1[a]	—	404.0 ± 269.1[ab]	579.3 ± 249.6[b]
Intratesticular testosterone						
Per gram tissue	ND	—	4.2 ± 1.2	—	3.7 ± 2.3	6.4 ± 2.2
Volume density (%)						
Seminiferous tubule	1.9 ± 0.5[a]	47.4 ± 3.9[b]	—	71.1 ± 2.4[c]	76.4 ± 5.7[c]	72.8 ± 2.1[c]
Round spermatids	NA	0.07 ± 0.01[a]	—	0.4 ± 0.1[a]	1.2 ± 0.5[b]	2.4 ± 0.3[c]
Interstitium	98.1 ± 0.5[a]	52.6 ± 3.9[b]	—	28.9 ± 2.4[c]	23.6 ± 5.7[c]	27.2 ± 2.1[c]
Leydig cells	67.7 ± 4.1[a]	4.1 ± 0.8[b]	—	2.4 ± 0.6[b]	2.8 ± 0.9[b]	18.4 ± 2.5[c]
Nuclei only	4.7 ± 0.2[a]	0.27 ± 0.05[b]	—	0.20 ± 0.04[b]	0.18 ± 0.01[b]	0.9 ± 0.1[c]
Macrophages	2.9 ± 0.4[a]	3.2 ± 0.8[a]	—	0.5 ± 0.1[b]	0.2 ± 0.1[b]	0.03 ± 0.00[b]
Blood vessel	13.3 ± 1.9[a]	2.2 ± 0.3[b]	—	1.5 ± 0.2[b]	1.6 ± 0.8[b]	1.5 ± 0.3[b]
Lymph vessel	0.06 ± 0.04	0.81 ± 0.09	—	0.62 ± 0.06	0.42 ± 0.02	0.46 ± 0.06
Other interstitial cells	4.9 ± 0.8[a]	9.4 ± 1.0[b]	—	3.4 ± 0.3[a]	2.5 ± 1.1[a]	0.8 ± 0.2[c]
Other	9.3 ± 1.1[ab]	32.9 ± 2.8[c]	—	20.4 ± 2.0[d]	16.0 ± 4.0[ad]	6.0 ± 0.7[b]
Percentage of seminiferous tubules						
Tubule score 1–2	100 ± 0[a]	96.1 ± 2.0[a]	—	32.4 ± 5.1[b]	ND	ND
Tubule score 3–5	0 ± 0[a]	3.9 ± 2.0[a]	—	67.6 ± 5.1[b]	ND	ND
Potential daily sperm production/gram (10^6)						
Pachytene primary spermatocytes	NA[‡]	1.3 ± 0.9	—	18.4 ± 5.7	ND	ND
Round spermatids	NA	0.5 ± 0.1[a]	—	2.8 ± 1.1[a]	9.9 ± 4.0[b]	18.6 ± 2.1[c]
Leydig cell						
Nuclear diameter (μm)	9.41 ± 0.09[a]	6.92 ± 0.24[b]	—	7.27 ± 0.26[cb]	7.88 ± 0.18[c]	8.65 ± 0.26[d]
Number/gram parenchyma	96.7 ± 4.4[a]	13.9 ± 2.8[b]	—	8.5 ± 1.8[b]	6.2 ± 0.4[b]	24.5 ± 3.0[c]

[a,b,c,d]Means in rows with different superscripts differ ($p < 0.05$).
*Days of gestation.
[†]ND = Not determined.
[‡]NA = Not applicable as given cell type not present.

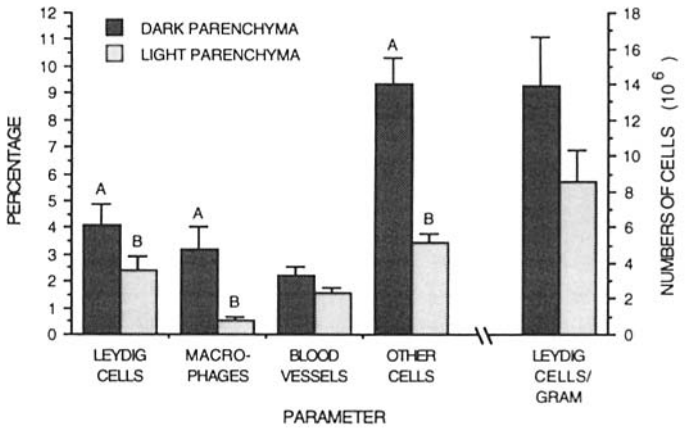

FIG. 4. Effect of gross, differential shading of parenchyma on percentages of Leydig cells, macrophages, other (non-Leydig, nonmacrophage, nonvascular) cells, and blood vessels as well as number of Leydig cells. Dark parenchyma had higher percentage of Leydig cells, macrophages, and other interstitial cells, but percentage of blood vessels was similar to that of light parenchyma. Number of Leydig cells/g parenchyma was higher in dark parenchyma, but value was not statistically different from that of light parenchyma. Paired means with different superscripts (A,B) are different ($p < 0.05$).

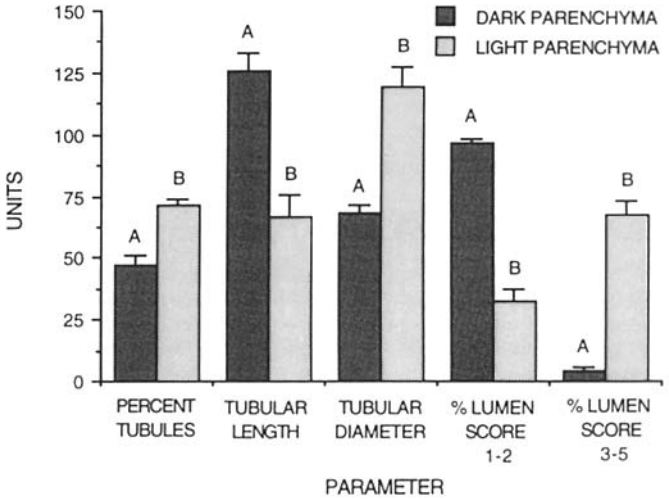

FIG. 5. Effect of gross, differential shading of parenchyma on characteristics of seminiferous tubules. Light parenchyma had more developed seminiferous tubules as noted by higher percentage of tubules, greater tubular diameter, and higher percentage of tubules with luminal score of 3–5. Dark parenchyma had longer tubules/g parenchyma and higher percentage of tubules with luminal score of 1 or 2. Units equal 1%, 1 m, 1 μm, 1%, and 1% for tubular parameters, respectively. Paired means with different superscripts (A, B) are different ($p < 0.05$).

stances in varied states of digestion. The volume density of macrophages in the dark parenchyma was similar to that of fetal parenchyma, whereas the density of macrophages in the light parenchyma and 2-yr-old and 5-yr-old horses was almost inconsequential (Table 1). The densities of blood vessels and lymph vessels and the number of Leydig cells/g were not significantly different for the light and dark parenchyma in the same testis (Fig. 4). Fetal testicular parenchyma had a much higher density of Leydig cells than did light parenchyma. Fetal testicular parenchyma had a very high density of blood vessels and Leydig cells (Table 1).

Whereas the dark parenchyma was composed mostly of interstitium, a higher percentage of light parenchyma was occupied by seminiferous tubules (Table 1; Fig. 3). Seminiferous tubules in the light parenchyma had larger diameters but a shorter tubule length per g parenchyma (Fig. 5). Likewise, the percentage of tubules with a tubular development score of 3–5 was also higher in the light parenchyma than in the dark parenchyma (Table 1; Fig. 5).

On the basis of volume density of tubules and/or tubular development, the peripheral regions of dark parenchyma were similar to fetal parenchyma, and light regions of parenchyma were similar to parenchyma of post-pubertal horses. The percentage of parenchyma occupied by seminiferous tubules in the light parenchyma was similar to percentages in 2-yr-old and 5-yr-old horses (Table 1). Dark parenchyma was similar to fetal tissue in tubular development (higher density of tubules with category 1–2 lumen) along with smaller tubular diameter and longer tubular length. All these factors contribute to a darker, more developmentally quiescent region.

Cellular components within the tubules followed the same trend as that based on the tubular development score. Advanced cell types were present in higher densities in the light parenchyma (Table 1). The numbers per gram parenchyma of primary spermatocytes and round spermatids were greater in the light parenchyma than in the dark parenchyma (Fig. 6). However, this difference in volume density of round spermatids was not statistically significant because too few tubules in the light parenchyma had developed a full complement of these germ cells. In contrast, the numbers of more immature germ cells (spermatogonia) were greater in the dark parenchyma. Fewer Sertoli cells per gram in the light parenchyma reflect expansion of the tubular lumen and the increase in numbers of germ cells per gram (Fig. 6) as well as shortening of the tubular length per gram (Fig. 5). Hence, according to the larger density of Sertoli cells and immature germ cells, dark parenchyma had less-developed tubules.

Correlations between interstitial characteristics and measures of development of seminiferous tubules indicate significant relationships between these two compartments of the testis. Diameters of seminiferous tubules (Fig. 7) and percentages of tubules with a development score of 3–5 (Fig. 8) were significantly related to volume densities of

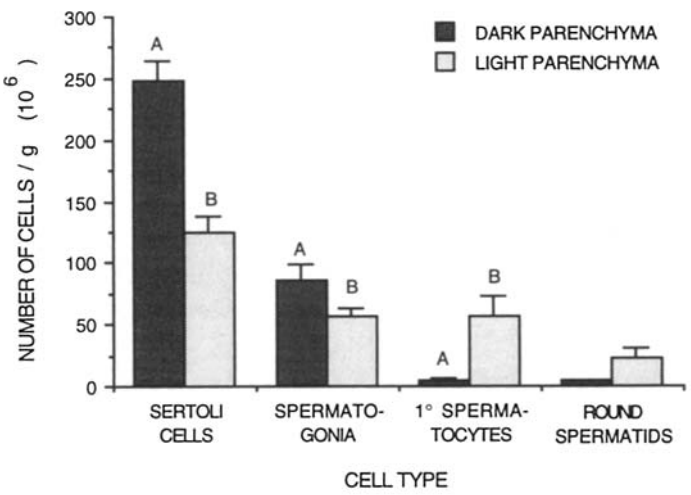

FIG. 6. Effect of shade of parenchyma on number of Sertoli cells, spermatogonia (gonocytes), primary spermatocytes, and spermatids with spherical nuclei (round). Light parenchyma had greater number/g parenchyma of primary spermatocytes. Dark parenchyma had larger number/g of Sertoli cells and of spermatogonia (gonocytes). Paired means with different superscripts (A, B) are different ($p < 0.05$).

interstitium, macrophages, or other interstitial cells (non-Leydig, nonvascular, and nonmacrophage). As diameters of tubules increased (Fig. 7) with luminal development (Fig. 8), the percentage of the parenchyma occupied by interstitium reduced in a reciprocal, linear relationship. However, the relationship between volume densities of macrophages or other interstitial cells and tubular development was logarithmic, indicative of a threshold relationship. If volume densities of macrophages exceeded 1.5% or of other interstitial cells exceeded 6%, there was minimal tubular development as judged by tubular diameter (Fig. 7) and percentage of tubules with luminal scores of 3–5 (Fig. 8).

The number of Leydig cells did not appear to relate to tubular development. The volume density of Leydig cells in the parenchyma was not related to tubular development (Figs. 7 and 8). Further, on the basis of analysis of three pieces of tissue from light or dark parenchyma, the light parenchyma (27.5 ± 10.5 ng/ml) did not produce more T in response to hCG stimulation for 2 h than did the dark parenchyma (23.0 ± 5.2 ng/ml). Unfortunately, ITT concentrations were not determined separately for light and dark regions of parenchyma. However, ITT content, measured largely in the light regions of horses with light and dark parenchyma, was similar to that in 2-yr-old and 5-yr-old horses (Table 1). Serum concentrations of FSH, LH, and T were similar ($p > 0.05$) in horses with both light and dark regions of the same testis and 2-yr-old horses.

DISCUSSION

Gross differences in coloration within the parenchyma of the same testis of prepubertal horses is brought about by focal growth initiated in the central region (Figs. 1 and

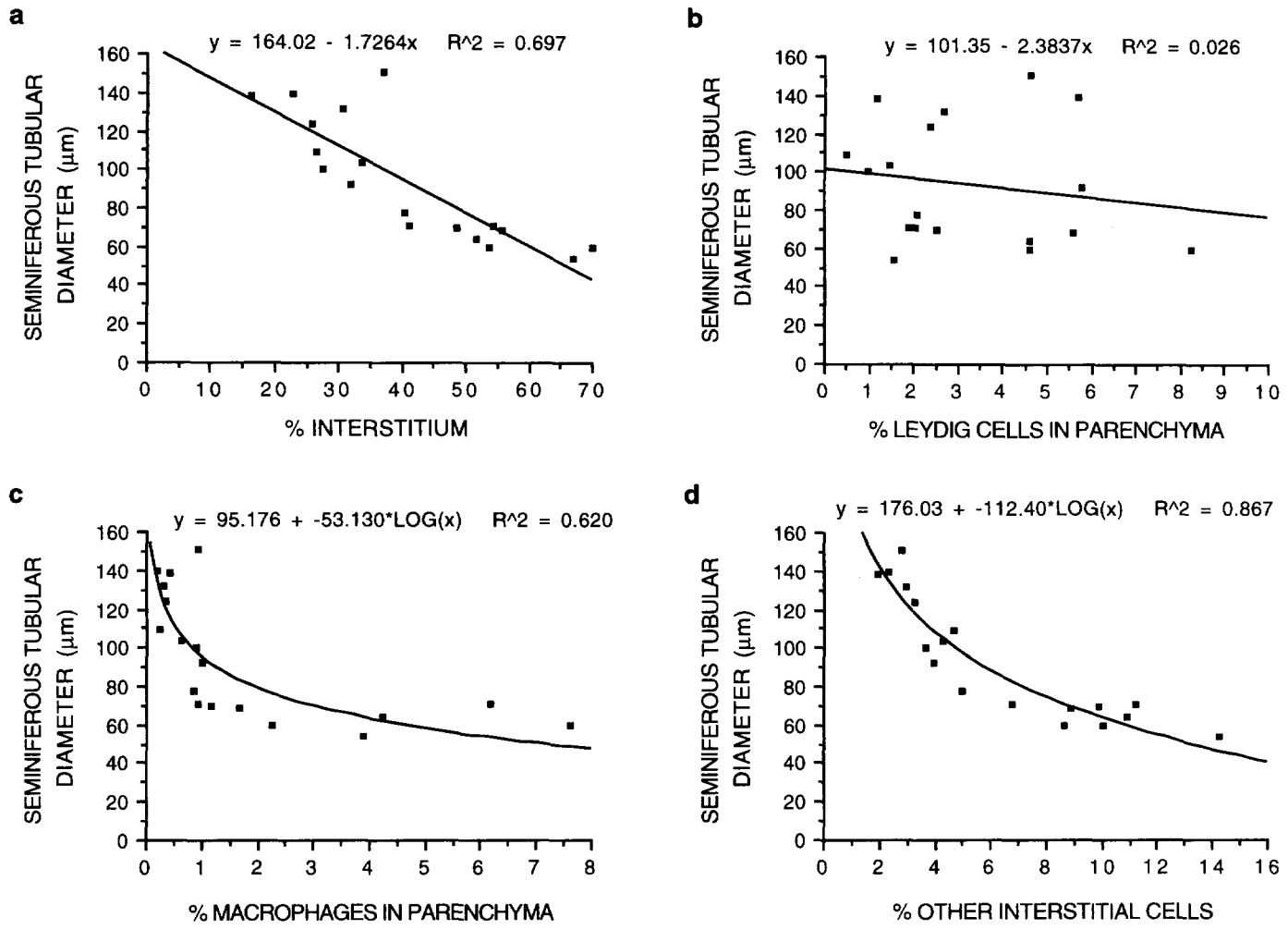

FIG. 7. Relationships between diameter of seminiferous tubules and interstitial characteristics. Percentages of interstitium (linear), macrophages (logarithmic), and other interstitial cells (logarithmic) are significantly related to this measure of tubular development. Tubular development is minimal when macrophages exceed 1.5% of parenchyma or when other interstitial cells exceed 6%. However, percentage of Leydig cells is not related ($p > 0.05$) to tubular diameter.

3). The present quantitative study confirms much earlier qualitative studies by Bouin and Ancel in 1905 [2] and Nishikawa and Horie in 1955 [3], who reported that gross coloration of equine testicular parenchyma parallels tubular development and testicular size in prepubertal horses. Further, Nishikawa and Horie showed that "spermatozoa" (presumably mature spermatids) were first seen in the centrally located tubules followed by a uniform presence of these spermatids in most tubules throughout the testis [3].

Light regions of testicular tissue (Fig. 1) result from an increased tubular volume density (Fig. 5) and tubular luminal development (Figs. 2 and 3) coupled with a reduced number of pigmented cells (macrophages and Leydig cells) in the interstitium (Figs. 4 and 6). With increased tubular volume density, the interstitial region is diluted, and the parenchyma becomes lighter. Further, the tubules themselves become lighter as the lumen forms (Fig. 5), and the density of Sertoli cells and spermatogonia is reduced (Fig. 6). Like spermatozoa, mature seminiferous tubules that produce spermatozoa are usually not pigmented. The exception is residual body formation and phagocytosis, which provide the basis for identifying seminiferous tubules by transillumination in rodents [17–19] and in horses [20]. With increased pigmentation of the interstitium as the Leydig cell population increases in horses 4 yr old and older, the lack of pigment in seminiferous tubules makes tubules visible from the interstitium when viewed by a dissecting microscope [5].

The interstitium itself becomes lighter with development from dark parenchyma to light parenchyma. The reduction in density of macrophages (Figs. 4, 7, and 8) and the reduction in percentage of Leydig cells (Fig. 4) were responsible for a lighter interstitium. As mentioned, large clusters of Leydig cells themselves cause darkness in testicular parenchyma [5]. Bouin and Ancel [2] reported that dark regions of prepubertal equine testicular parenchyma were characterized by the removal of degenerating fetal interstitial cells by abundant, large macrophages. Gradual light-

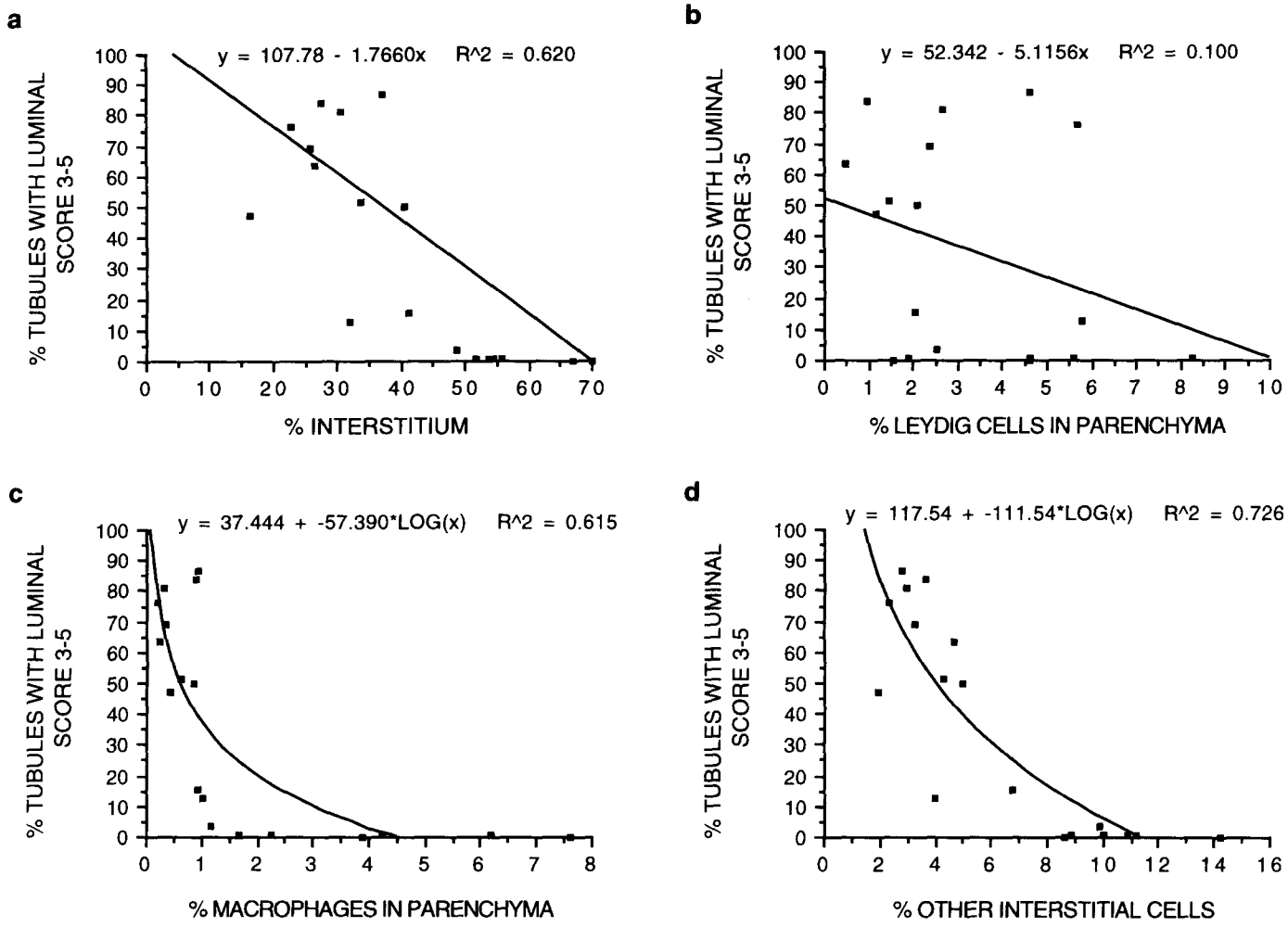

FIG. 8. Relationships between percentage of tubules with luminal score of 3–5 and interstitial characteristics. Percentages of interstitium (linear), macrophage (logarithmic), and other interstitial cells (logarithmic) are significantly related to this measure of tubular development. Tubular development is minimal when macrophages exceed 1.5% of parenchyma or other interstitial cells exceed 6%. However, percentage of Leydig cells is not related ($p > 0.05$) to tubular diameter.

ening of testicular parenchyma during pubertal development has been attributed to regression of fetal Leydig cells [3]. Coloration of the parenchyma in horses has been shown to provide an index denoting testicular size [3], interstitial development [5], and age of the horse [3, 5].

The temporal relationship between the reduction in volume density of macrophages or other interstitial cells and development of seminiferous tubules (Figs. 7 and 8) is consistent with a negative influence of these cells on tubular development. In dark parenchyma, macrophages could be very large, > 50 μm (Fig. 2), and could have a high volume density, > 7% of the parenchyma. A large population of macrophages within the testis is found in the rat [21–25]. In rats, the interstitial macrophage is closely associated with Leydig cells and is functionally related to these interstitial cells [26]. Additionally, macrophages have been found to be steroidogenic, sharing a surface antigen with the Leydig cell [26]. Although they exhibit typical macrophage morphology, the testicular macrophages seem to be involved in roles beyond traditional immunological mechanisms [27]. Leydig cells can form membrane digitations with macrophages [28]. This occurs just prior to the major steroidogenic activity of Leydig cells associated with puberty. In rats, as well as in horses (Figs. 7 and 8), the macrophages emerge in a transient manner during the period of rapid testicular growth and maturation [29]. In the horse, macrophages occupied a large volume of the testis in dark parenchyma but were almost nonexistent within the light area of the same testis (Table 1). The macrophages, as well as other interstitial cells, may play a role in preventing the initiation of spermatogenesis in the dark parenchyma when a threshold percentage of 1.5% is surpassed (Figs. 7 and 8).

Macrophages and Leydig cells seem to be functionally related since the macrophages release factor(s) in culture, primarily tumor necrosis factor alpha, which alters the secretion of T by Leydig cells [30]. The physiological significance of this factor has yet to be established; however, Leydig cells do respond to it, which further supports the

hypotheses that macrophages play a role in Leydig cell regulation [27]. This link between two main interstitial cells illustrates a relationship that indirectly may effect the development of the seminiferous tubule and the onset of spermatogenesis. However, the gonadotropins released systemically at puberty are required for testicular function and maturation, although responsiveness of the testicular cells to these hormones is regulated by factors produced and acting within the testis [31].

Leydig cell function and systemic hormonal concentrations do not appear to work independently of local factors in initiation of spermatogenesis in the horse. Many experimental results have indicated that Leydig cell function is modulated by seminiferous tubule secretory products, particularly the trophic effect of Sertoli cells via diffusible factors, as well as testicular interstitial fluid [31]. However, the volume density or numbers of Leydig cells were not correlated to measurements of seminiferous tubule development in this study (Figs. 7 and 8). A possible explanation for the lack of a significant relationship between Leydig cells and tubular development is that it was not possible to distinguish residual fetal Leydig cells from new ones being added with pubertal development. However, Leydig cell function (based on HCG-stimulated production of T) was not different between light and dark regions of parenchyma. Therefore, T production by Leydig cells did not appear to be the causative factor in the local initiation of spermatogenesis in the horse.

In order to further study this transient developmental stage in the equine testis, an age range, a range of testicular weight, or a combination of the two is needed. Because of the transient nature of shading, only 12% of the 1.5- to 2-yr-old horses in this study had gross, differential shading of the parenchyma. Also, the weight of testes with differential shading averaged 23 g (Table 1). Nishikawa and Horie [3] noted that testicular weight was more important than age in predicting the development of tubules in testicular parenchyma. They noted that maturation was directly related to testicular weight, and that the size with differential shading (coloration) was between 10 g and 70 g. Others [6] described the stage of light and dark within the same testis in testes weighing 15–20 g, a value correlating with the findings of our study. However, the fact that puberty occurs at approximately 2 yr of age, with the horse having adult values by 3 yr of age [3], the age group of the animal must also be considered. Hence, given the age group (1.5–2 yr) to be examined and palpation prior to castration to determine testicular size, testes with differential shading should be obtained routinely from clinical castrations without the cost of animals and without the need of using animals for research purposes only.

Although season did not seem to be a factor in the present study, the season or length of photoperiod is known to influence testicular size and spermatogenesis in many mammals, including horses [5, 6, 12]. A number of factors, both internal and external, influence central nervous system modulation of the endocrine system [32]. Since these factors can influence the chronological age at which a given animal reaches puberty, they will affect the specific age (within the age group) at which a given horse will experience this transient phase. Energy intake, breed, and birth season are also factors that may be important in determining the optimal time to sample the testis to obtain maximum size of light and dark regions for further experimentation. However, size of the testis (i.e., between 10 g and 70 g [3]) appears to be the most important factor in obtaining testicular tissue with gross shading since these are the weights at which the testis is undergoing differential growth. Since all intact adult horses experienced testicular growth between 10 g and 70 g, they must have gone through the transient phase of testicular shading.

Focal growth within the equine testis (Figs. 1–3) confirms that local factors regulate the initiation of spermatogenesis. Given that serum concentrations of LH and FSH were similar in horses with differential shading and 2-yr-old horses with uniform light parenchyma (Table 1) and that the volume density of blood vessels was similar between light and dark regions of the same testis (Fig. 4), there must be a local mechanism that initiates spermatogenesis. Although blood flow in light and dark regions was not measured in the present study, similar volume densities for blood vessels in both regions indicate that the blood vasculature was similar in light and dark regions. Therefore, local initiation probably cannot be explained on the basis of differential availability of gonadotropins via blood flow differences. Though not concentrated to an extent that gross shading of the parenchyma was noted, a small focal pattern of development is seen in the bull during initiation of spermatogenesis [33]. Indeed, local growth/regulatory factors may be involved in initiation and/or regulation of spermatogenesis in general [34–38]. In recent years, at least four classes of molecules have been reported to exert a local action on the testis. These include regulatory peptides, growth factors, extracellular matrix components, and arachidonic acid metabolites [35].

Gross, differential shading of the fresh equine testis and its corresponding local initiation of spermatogenesis make possible several biochemical and cell culture experiments. Differential polymerase chain reaction of light and dark parenchyma may facilitate production and identification of unique DNA probes associated with initiation of spermatogenesis. In situ hybridization using these unique DNA probes can identify the cells that are major players in the initiation of spermatogenesis. Since the seminiferous tubules in dark regions should be primed for Sertoli cell differentiation and spermatogonial proliferation, culture of these tubules isolated [20] from dark parenchyma may be a useful bioassay to identify the role of known (or new) growth factors in spermatogenesis.

In summary, the transient state of differential shading in the horse testis is a remarkable example of the effect of local factors on the development of seminiferous tubules. It allows study of testicular parenchyma in which some seminiferous tubules have initiated spermatogenesis and those that have not, even though both regions presumably have been exposed to the same systemic endocrine environment. Hence, the transient state of differential development of equine tubules appears to be a useful model for studying the paracrine regulation of the initiation of spermatogenesis in mammals.

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