The relationship of daily sperm production with number of Sertoli cells and testicular size in adult horses: role of primitive spermatogonia

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The number of Sertoli cells is important in spermatogenesis as noted by significant correlations between the number of Sertoli cells and the number of germ cells observed as early as type B₂ spermatogonia in the horse. However, the stage within spermatocytogenesis at which these relationships first occur is unclear. The relationships between the number of Sertoli cells and parenchymal weight and the number of germ cells during the mitosis of spermatogenesis were determined in 184 adult horses to identify the developmental stage (that is, the earliest germ cell) at which significant relationships are established. The total numbers of all types of A spermatogonia and of specific subtypes (A₁, A₂, A₃, B₁ or B₂) of spermatogonia were correlated with the number of Sertoli cells and with parenchymal weight. The number of each cell type was calculated using stereology. The number of Sertoli cells was correlated ($P < 0.01$) with parenchymal weight ($r = 0.85$) and with daily sperm production ($r = 0.83$), and parenchymal weight was correlated ($P < 0.01$) with daily sperm production ($r = 0.89$). The number of Sertoli cells was correlated ($P < 0.01$) with the number of type A ($r = 0.81$) and A₁ ($r = 0.74$) spermatogonia. Parenchymal weight was correlated with the number of type A ($r = 0.80$) spermatogonia and with the number of A₁ ($r = 0.67$) spermatogonia. These data are consistent with the hypotheses that the number of Sertoli cells is important in determining testicular size and daily sperm production and that the relationship of daily sperm production to the number of Sertoli cells or to parenchymal weight has already been established at the level of primitive spermatogonia.

Introduction

Recent studies of one- to five-year-old stallions (Johnson *et al.*, 1991b) showed significant ($P < 0.01$) correlations between daily sperm production per horse and number of Sertoli cells per horse ($r = 0.80$), between daily sperm production per horse and paired testicular parenchymal weight ($r = 0.94$), and between number of Sertoli cells per horse and paired parenchymal weight ($r = 0.85$). In horses of mixed ages (mostly ≤ 5 years old), Berndtson and Jones (1989) reported significant correlations between the relative number of Sertoli cells and numbers of various populations of germ cells. In adult horses (more than four years old), the number of Sertoli cells per testis was positively correlated significantly ($P < 0.01$) with the numbers per testis of round spermatids ($r = 0.65$), late primary spermatocytes ($r = 0.70$), early primary spermatocytes ($r = 0.67$), and type B₂ spermatogonia ($r = 0.63$) (Johnson and Tatum, 1989). In this study, testicular parenchymal weight was related to the

number of Sertoli cells ($r = 0.81$; $P < 0.01$) and the number of A spermatogonia ($r = 0.75$; $P < 0.01$). However, little attention has been directed to determining the earliest spermatogonial subtype in spermatogenesis, the number of which is significantly related to the number of Sertoli cells or to testicular size. Identification of the type of spermatogonial cell in spermatogenesis, the number of which first exhibits a significant relationship with the number of Sertoli cells or with parenchymal weight, will direct further research to enhance spermatogenesis and eliminate seasonality in horses to that developmental step in spermatogenesis.

To determine the cell type, the number of which first exhibits a significant relationship with number of Sertoli cells or testicular size, the total number of all types of A spermatogonia or the number of specific subtypes (A₁, A₂, A₃, B₁ or B₂) of spermatogonia was correlated with the number of Sertoli cells and paired parenchymal weight in a large number of adult horses. The seasonal variation in testicular weight, daily sperm production (Johnson and Neaves, 1981; Amann, 1989; Johnson, 1991b), and number of Sertoli cells (Johnson and Thompson,

1983; Johnson and Nguyen, 1986; Johnson, 1986; Johnson and Tatum, 1989; Johnson *et al.*, 1991a, b) allowed the relationship between Sertoli cells and daily sperm production, previously traced back to type B₂ spermatogonia (Johnson and Tatum, 1989) to be determined. It was found that the relationship was already established at the developmental step of the most primitive (A₁) spermatogonia.

Methods

Animals and Tissues

Testes from 184 adult horses (more than 4 years old) were obtained throughout one year from an abattoir and fixed by vascular perfusion with 2% glutaraldehyde (Sigma, St Louis, MO) in 0.1 mol sodium cacodylate buffer l⁻¹. Only horses of light-weight breed, in good body condition, and with a smooth hair coat were used. Ages were determined from eruption and wear of the incisors. Five randomly selected pieces of testicular tissue (10 mm × 5 mm × 2 mm) were further fixed in osmium tetroxide, embedded in Epon 812, sectioned at 0.5 µm, stained with toluidine blue, and observed by bright field microscopy. Stereological evaluation of nuclei of Sertoli cells, spermatogonia and spermatids was conducted as described for these same horses by Johnson and Tatum (1989) and Johnson (1991a). From a subset of adult horses (*n* = 9; 9.1 ± 1.0 years old; specimens obtained in December and *n* = 10; 11.7 ± 2.1 years old; specimens obtained in June), other large pieces of fixed testicular tissue (10 mm × 20 mm × 5 mm) were embedded in methacrylate, sectioned at 5.0 µm, stained with toluidine blue, and observed by bright field microscopy to determine the percentage of each subtype of spermatogonia. When distinction among the subtypes of spermatogonia was needed, as in the subset of horses above, it was necessary to use thicker sections (5.0 µm methacrylate), which revealed subtle differences in nuclear chromatin profiles that were not seen in thinner Epon sections. Details of nuclear profiles for these five spermatogonial subtypes in the horse and their appearance in seminiferous epithelium in different spermatogenic stages were described by Johnson (1991a). The subset was limited to 19 horses owing to labour intensity of these measurements.

Stereology and calculation of number of germ cells

The total number of A plus B₁ spermatogonia (A₁, A₂, A₃ and B₁ spermatogonia combined), B₂ spermatogonia, or early (preleptotene, leptotene and zygotene) primary spermatocytes were determined by stereology of Epon sections on the basis of nuclear volume density, parenchymal volume and volume of a single nucleus for each cell type (Johnson, 1985; Johnson and Tatum, 1989). Shrinkage of equine tissues fixed with glutaraldehyde and then with osmium tetroxide before subsequent Epon embedding was determined by Johnson and Neaves (1981) to be negligible. Stereologic procedures included the point-counting method of Chalkley (1943) of 0.5 µm sections to determine the nuclear volume density (percentage of the parenchyma occupied by nuclei of each type of cell). Nuclear volume of individual cells was determined in 20 µm Epon

sections by the measurement of the maximum nuclear diameter and the formula for the volume of a sphere (Johnson and Neaves, 1981). Since season does not alter (*P* > 0.05) the nuclear diameter of germ cells with spherical nuclei (A + B₁ spermatogonia (6.8 ± 0.1 versus 6.9 ± 0.1 µm) in winter and summer, respectively), B₂ spermatogonia (6.5 ± 0.1 versus 6.4 ± 0.1 µm), and early primary spermatocytes (8.8 ± 0.1 versus 8.0 ± 1 µm) in the stallion, these nuclear volumes were not influenced by season (Johnson, 1985). Likewise, the volume of a single Sertoli cell nucleus in the winter (678 ± 71 fl) was similar (*P* > 0.05) to that in the summer (693 ± 38 fl; Johnson and Nguyen, 1986; Johnson and Thompson, 1986). The number of a given cell type was determined by dividing the product of the nuclear volume density, parenchymal volume and a histological correction factor for section thickness and nuclear diameter (Weibel and Paumgartner, 1978) by the volume of a single nucleus of that cell type (Johnson and Neaves, 1981).

At each stage of the spermatogenic cycle (Swierstra *et al.*, 1974), the number of each subtype of spermatogonia (A₁, A₂, A₃ or B₁) was counted per 100 Sertoli cells in methacrylate sections to determine the percentage of each subtype in the total population of A and B₁ spermatogonia for each of the 19 horses in the subset (Johnson, 1991a). The number of a given spermatogonial subtype was calculated by multiplying the percentage of that subtype of spermatogonia (for a given horse) by 100 and by the total number of A plus B₁ spermatogonia for that horse.

Statistical analysis

The relationship between paired dependent variables was determined by simple regression analysis (Sokal and Rohlf, 1969). Correlation coefficients between paired variables were tested for level of significance (Sokal and Rohlf, 1969).

Results

The number of Sertoli cells per horse and paired parenchymal weight were highly correlated with daily sperm production per horse (Fig. 1a, b). Likewise, paired parenchymal weight and number of Sertoli cells per horse were significantly correlated with the number of A plus B₁ spermatogonia per horse (Fig. 1c, d). The number of Sertoli cells per horse was correlated (*r* = 0.85; *P* < 0.01) with testicular size as measured by paired parenchymal weight.

In a subset of 19 horses representing both winter and summer seasons, a similar relationship was found; however, the relationship was traced back further in spermatogenesis to primitive spermatogonia. The number of Sertoli cells per horse was significantly correlated with daily sperm production per horse, number of A plus B₁ spermatogonia, and number of each subtype of spermatogonia and early primary spermatocytes (Fig. 2). In the same subset of horses, paired parenchymal weight was significantly (*P* < 0.01) correlated with daily sperm production, number of A plus B₁ spermatogonia, and each subtype of spermatogonia (except A₂ spermatogonia) and young primary spermatocytes (Fig. 3). For correlation of the number of A₂ spermatogonia with either number of Sertoli cells

Fig. 1. The effect of the number of Sertoli cells and parenchymal weight on daily sperm production and number of the combination of all A plus B₁ spermatogonia in 184 adult horses (more than 4 years old) whose testes were obtained throughout the year. All correlation coefficients ((a) $r = 0.827$, (b) $r = 0.887$, (c) $r = 0.806$, and (d) $r = 0.802$) are significant ($P < 0.01$). (a) Variation in the number of Sertoli cells per horse accounts for 68% of the variation in daily sperm production per horse and (c) 65% of the variation in number of A plus B₁ spermatogonia per horse. Variation in parenchymal weight per horse accounts for (b) 79% of the variation in daily sperm production and (d) 64% of the variation in number of A plus B₁ spermatogonia.

or paired parenchymal weight, the correlation coefficient was lower ($P < 0.05$ or nonsignificant, respectively) than for all other types of germ cell ($P < 0.01$).

Discussion

Two notes of caution are considered important with our findings. First, correlation tends to be higher when common factors are involved. Parenchymal weight for a given horse was a common factor in estimates of all cell populations, including germ cells and Sertoli cells. However, parenchymal weight (126 ± 6 g in the non-breeding versus 163 ± 7 g in the breeding season; $P < 0.01$) is a major factor in seasonal variation in the number of testicular cells (Johnson, 1991a). Second, the underlying cause for the relationship between cell population sizes cannot be determined from correlations. Indeed, the underlying cause may be factors (for example hormones, growth factors or mitogens; Bardin, 1988) that stimulate both spermatogonia and the number of Sertoli cells or parenchymal weight to keep their population size parallel even when season effects for each of these variables are known (Johnson, 1991a, b). However, Sertoli cell mitogens (Feig *et al.*, 1980) may be important in a paracrine or autocrine fashion.

Seasonally, stallions modulate or regulate spermatogenesis by altering both the number of primitive spermatogonia and the yield of early spermatogonial subtypes (Johnson, 1991a),

but they continue to produce spermatozoa throughout the year (Thompson *et al.*, 1977; Johnson and Thompson, 1983). The effect of season on spermatogenesis is a function of germ cell degeneration during meiosis and seasonal modulation of the number of A plus B₁ spermatogonia (Johnson, 1991b). The number of A plus B₁ spermatogonia in the breeding season was twice that in the nonbreeding season (Johnson, 1985).

Although previous study of these horses revealed seasonal variation in the numbers of different subtypes of spermatogonia (Johnson, 1991a), the current study reveals that seasonal changes in the number of these spermatogonial subtypes parallel changes in the number of Sertoli cells per horse and in testicular size (paired parenchymal weight). On the basis of a subset of 19 horses representing both seasons and in which the number of each subtype of spermatogonia was determined, significant correlations were found between the number of Sertoli cells and each subtype of spermatogonia including the most primitive, A₁ spermatogonia. The variation in number of Sertoli cells in these adult horses in both seasons that could be explained by variation in number of each subtype of spermatogonia ranged from 25 to 60%. Paired parenchymal weight was significantly correlated with number of each subtype of spermatogonia except A₂. Variation in the number of each subtype of spermatogonia accounted for 18–71% of the variation in paired parenchymal weight. Variation in number of the most primitive (type A₁) spermatogonia accounted for 55 and 46% of variation in number of Sertoli cells and

Fig. 2. The effect of number of Sertoli cells per horse in a subset of 19 horses representing both the breeding (June) and nonbreeding (December) seasons on (a) daily sperm production per horse and on the number per horse of (b) all A plus B₁ spermatogonia, (c) A₁ spermatogonia, (d) A₂ spermatogonia, (e) A₃ spermatogonia, (f) B₁ spermatogonia, (g) B₂ spermatogonia and (h) young primary spermatocytes. The R^2 value on each graph reveals the percentage of the variation in number of Sertoli cells that is attributed to variation in daily sperm production or number of each germ cell. Correlation coefficients: ((a) $r = 0.856$, (b) $r = 0.834$, (c) $r = 0.744$, (e) $r = 0.748$, (f) $r = 0.776$, (g) $r = 0.763$ and (h) $r = 0.845$) are significant ($P < 0.01$) and the correlation coefficient between (d) number of Sertoli cells and number of A₂ spermatogonia is significant ($P < 0.05$).

parenchymal weight, respectively. As a general rule, the correlation coefficients were lower as the germ cell types occurred earlier in spermatocytogenesis. The percentage of

variation in the correlation that is not explained is sufficient to account for the seasonal variation in germ cell ratios during spermatocytogenesis reported by Johnson (1991a).

Fig. 3. The effect of parenchymal weight per horse in a subset of 19 horses representing both the breeding and nonbreeding seasons on (a) daily sperm production per horse and on the number per horse of (b) all A plus B₁ spermatogonia, (c) A₁ spermatogonia, (d) A₂ spermatogonia, (e) A₃ spermatogonia, (f) B₁ spermatogonia, (g) B₂ spermatogonia, and (h) young primary spermatocytes. The R^2 value on each graph reveals the percentage of the variation in parenchymal weight that is attributed to variation in daily sperm production or number of each germ cell. Correlation coefficients ((a) $r = 0.902$, (b) $r = 0.884$, (c) $r = 0.675$, (e) $r = 0.840$, (f) $r = 0.829$, (g) $r = 0.767$ and (h) $r = 0.952$) are significant ($P < 0.01$). The correlation coefficient between (d) parenchymal weight and number of A₂ spermatogonia is not significant ($P > 0.05$).

In horses, it is difficult to calculate precisely the amount of loss that occurs from degeneration during spermatocytogenesis; however, seasonal differences have been detected in the

different spermatogonial subtypes that degenerated with a greater yield early and reduced yield late in spermatocytogenesis in the breeding season (Johnson, 1991a). Losses in

potential sperm production have been estimated at 25% in mice (Oakberg, 1956), 11% in Sherman rats (Clermont, 1962), and 75% in adult Sprague-Dawley rats (Huckins, 1978). Ortavant (1958) found greater degeneration of spermatogonia in rams following long-day illumination characteristic of the nonbreeding season in that species. While the absolute amount of degeneration during spermatocytogenesis is unknown in humans, the amount of degeneration and specific type of spermatogonia that degenerates are not influenced by age (Johnson, 1986).

Bardin (1988) summarized reports noting that the proliferation rate of Sertoli cells in neonatal rats was important in determining testicular size and number of germinal elements in adults. The relationship between number of Sertoli cells and the number of spermatogonia is consistent with our previous findings in younger horses (Johnson *et al.*, 1991b) and are consistent with the findings that the relationship between number of Sertoli cells and daily sperm production could be traced to the number of type A spermatogonia (Johnson, 1985). Although correlations have not been reported in the context of identifying the origin (within spermatocytogenesis) of the relationship between sperm production rates and numbers of Sertoli cells in horses, seasonal variation in the number of spermatogonia has been found (Johnson, 1991a). Seasonal variation in the number of renewing stem cells has been found in rams and red deer stags, and there is a relationship between the number of A₁ stem cell spermatogonia and the number of Sertoli cells in the ram (Hochereau-de-Reviere, 1981). Studies reporting ratios of germ cells per Sertoli cell in the horse (Johnson and Thompson, 1983; Johnson, 1986; Berndtson and Jones, 1989) or other studies in which the number of Sertoli cells in the horse were determined (Johnson and Nguyen, 1986; Jones and Berndtson, 1986; Russell *et al.*, 1990) have not quantified specific subtypes of spermatogonia for comparison with number of Sertoli cells. Sertoli cells are generally assumed to have a stable population size in adults (Hochereau-de-Reviere and Courrot, 1978; Jones and Berndtson, 1986; Sinha Hikim *et al.*, 1988; Russell *et al.*, 1990) because evidence of Sertoli cell division and Sertoli cell death is not obvious in adults. However, the age-related loss of Sertoli cells in humans (Johnson *et al.*, 1984) was not accompanied by obvious degeneration of Sertoli cells, and mitotic activity at the base of seminiferous epithelium is generally considered only spermatogonial in nature.

On the basis of four different groups of horses, we conclude that the number of Sertoli cells are not stable in adult horses. In the first group of 20 horses, aged 4–5 years, it was found that the Sertoli cell population was 48% ($P < 0.05$) greater in the middle of the breeding season than at the onset of the breeding season (Johnson and Thompson, 1983). In the same horses, the length of seminiferous tubules increased from 1.9 ± 1.0 Km at the onset to 2.9 ± 0.3 Km ($P < 0.01$) in the middle of the breeding season, yet tubular diameter, size of Sertoli cell nuclei and the number of Sertoli cells per tubular cross-section were similar during the same period. In the second group of 201 horses aged 6 months to 20 years, number of Sertoli cells per horse increased by 36% in the breeding season (summer) over the nonbreeding season (winter; Johnson and Thompson, 1983). The third group of 184 different horses has been used to reveal trimonthly (Johnson and Nguyen, 1986) or monthly

(Johnson and Tatum, 1989) differences in numbers of Sertoli cells throughout the year. In the fourth group composed of 123 horses aged 1–5 years, it was found that the number of Sertoli cells was greater ($P < 0.05$) in the breeding season even in these young horses (Johnson *et al.*, 1991b).

The obvious lack of a stable population of Sertoli cells in horses may have implications for other species. To confirm that the number of Sertoli cells per testis was not stable in adult horses, and to circumvent assumptions made for a given stereological approach to quantify Sertoli cells, we used five different stereological approaches to determine the number of Sertoli cells in the nonbreeding and breeding seasons. These were discussed in detail by Johnson *et al.* (1991b). Although others have quantified Sertoli cells in other seasonal breeders (Hochereau-de-Reviere and Courrot, 1978; Sinha Hikim *et al.*, 1988) or in horses in the breeding season (Jones and Berndtson, 1986; Berndtson and Jones, 1989; Russell *et al.*, 1990), to our knowledge, no other investigators have evaluated the effect of season on the number of Sertoli cells in horses. Indeed, the age-related reduction in numbers of Sertoli cells in humans (Johnson *et al.*, 1984) may be a net loss in the turnover of a larger percentage of cells.

Evaluation of the impact of stem cell spermatogonia on seasonal differences in spermatogenesis or the relationship of these cells to other testicular cell populations is complicated by our failure to understand the relationship between histologically distinguishable spermatogonia and different generations of spermatogonial subtypes, as well as our lack of knowledge of the lifespans of the given subtypes in horses (Johnson, 1991a). Further, A₁ or A₂ spermatogonia potentially present more than one generation of spermatogonia. Although A₁ possibly represents the stem cell, it probably represents the committed aligned spermatogonia (Amann, 1989; Johnson, 1987, 1991b) described by Huckins (1971) in rats. Intercellular abridges between adjacent A₁ spermatogonia connect as many as six cells (Johnson, 1991a). Confirmation of any stem cell renewal model is difficult, if not impossible. Even in rats, in which spermatocytogenesis has been studied most thoroughly, the stem cell has been attributed to every subtype of spermatogonia (A₁, A₂, A₃, A₄ or A₅) other than B spermatogonia (Huckins, 1971).

Seasonal modulation of the number of A spermatogonia in a species results from proliferation and yield of renewing stem cells (Hochereau-de-Reviere, 1981), and both number of A₁ spermatogonia and their yield are important in regulating spermatogenesis in horses (Johnson, 1985, 1991a). Stem cell spermatogonia are the earliest (most primitive) form of germ cells, which may be dormant (reserve) in testes active in spermatogenesis (A₀ stem cell; Clermont and Bustos-Obregon, 1968) or actively involved in the production of other stem cells or proliferating spermatogonia (A_s stem cell; Huckins, 1971). In any event, these cells carry on the lineage throughout the life of adult males. It is not clear whether A₁ spermatogonia are the equine stem cells; however, their numbers are very important in the relationship between Sertoli cell number or parenchymal weight and daily sperm production in horses. This finding is a first step to direct further studies to the causative factors that enhance the numbers of A₁ spermatogonia and Sertoli cells to eliminate seasonality in horses and enhance spermatogenesis in general.

Special thanks are extended to Beltex Corporation, 3801 North Grove, Fort Worth, Texas, for kindly providing horse tissues, to H. B. Nguyen and M. Barletta for providing excellent technical assistance, and to V. Marsh for typing and providing editorial assistance. This work was supported in part by NIH Grant HD-16773 and a Research Enhancement Grant from the Institute for Equine Science and Technology at Texas A&M University and Texas Agricultural Experiment Station.

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