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## Histological, cytogenetic and endocrine evaluation in twenty-five unilateral cryptorchid horses

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### ABSTRACT

Data from 49 horses (25 unilateral cryptorchid and 24 as control group) are reported to determine macroscopic and microscopic characteristics of the testes, as well as to characterize their karyotype and hormonal levels. Histology showed that only Sertoli cells were found in the lumen of the seminiferous tubules of the cryptorchid testes, while spermatogenesis in the scrotal testes resulted normal. Cytogenetic evaluation showed that all cryptorchid horses were normal males ( $2n = 64, XY$ ). In addition, the lower testosterone production observed in cryptorchid horses was associated to the smaller size of the retained testicle as well as to the lower number of Leydig cells adjacent to the seminiferous tubules. This study confirmed that a simple determination of plasma testosterone levels was enough to differentiate between non-breeding stallions, cryptorchid and castrated animals, avoiding the administration of hCG.

### ARTICLE HISTORY

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### KEYWORDS

Horse; cryptorchidism; histology; testosterone; cytogenetic

## 1. Introduction

Cryptorchidism is a well-described congenital disease in horses (Cox et al. 1979; Coryn et al. 1981; Jann & Rains 1990) and its surgical treatment has been reported and widely discussed (Schebitz & Soller 1980; Fischer & Vachon 1998; Gabriele 1996; Hartman et al. 2015). However, even if histological evaluations of spermatogenesis in cryptorchid horses (Coryn et al. 1981; Arighi et al. 1987) and androgen secretion (Ganjam & Kenney 1975; Cox 1982; Cox et al. 1986; Arighi & Bosu 1989; Ras et al. 2010; Claes et al. 2013) have been thoroughly studied; only few studies have described clinical aspects, testicular histology, plasma testosterone levels and karyotype in cryptorchid horses (Blanchard et al. 1990; Constant et al. 1994).

The aims of this study were first to determine macroscopic and microscopic features of testes from 25 unilateral cryptorchid horses, and like so their karyotype; second, this study tried to assess the plasmatic testosterone levels in cryptorchid horses.

## 2. Materials and methods

### 2.1. Animals

Twenty-five cryptorchid crossbred horses were enrolled for this study. All patients, at physical examination, showed unilateral cryptorchidism. Horses aged  $5.4 \pm 1.14$  years (mean  $\pm$  SD) and weighed  $446.56 \pm 59.41$  kg. Palpation of inguinal area confirmed inguinal testis in 21 of 25 cryptorchid horses, whilst rectal exploration and ultrasonography confirmed the presence of abdominal retained testes in

remaining four horses. No alteration of sexual behaviour was observed. In all cases, bilateral orchiectomy under general anaesthesia was performed. The intra-abdominal testicle was extracted after a surgical incision in the projection area of the inguinal canal. The scrotal testicle was removed conventionally (Rijkenhuizen & van der Harst, 2017). In order to compare testosterone levels, 24 crossbred horses (8 breeding stallions, 8 non-breeding stallions and 8 geldings) were also enrolled for this study as control group. Ages of crossbred horses were  $6.1 \pm 1.2$ ,  $6.6 \pm 2.5$  and  $7.0 \pm 1.3$  years old (breeding stallions, non-breeding stallions and geldings, respectively) and the mean weight was similar to that observed in cryptorchid horses.

### 2.2. Endocrine evaluation

To determine plasmatic levels of testosterone, blood samples of cryptorchid horses were collected by left jugular into heparinized vacutainer<sup>®</sup> (BD, Madrid, Spain) tubes under standard protocol (Osman 2017). Two samples were taken (at 9.00–10.00 am and 4.00–6.00 pm) from each horse. Samples were collected daily starting the day before castration until the seventh day after surgery. In the control group, blood samples were also collected within the same timelapse. Samples were centrifuged ( $1500 \times g$  for 15 min) and plasma was harvested and frozen at  $-20^\circ\text{C}$ . The plasma concentrations of testosterone were determined using a solid-phase I125 radioimmunoassay (Coat-A-Count Testosterone<sup>®</sup>, Siemens, Camberley, UK). The assay sensitivity was of 0.05 ng/ml and the inter-assay coefficient of variation was 13.9%.

### 2.3. Cytogenetic analysis

To exclude a chromosome-related congenital anomaly, blood was sampled aseptically from the cryptorchid horses ( $n = 25$ ) also from jugular vein in heparinized tubes and submitted for leukocyte culture chromosome analysis. A leukocyte culture from 0.5 cc of blood, which had been incubated for 72 h at 37° C in Roswell Park Memorial Institute (RPMI) 1640 medium, was then performed. Metaphases were analysed with normal stain and G Typsin Giemsa (GTG) bands (Craig & Bickmore 1993).

### 2.4. Testicular processing

After orchietomy, testes were weighed without the epididymis and testicular length was measured using a handcaliber. Samples of testicular and epididymal tissues were fixed in 10% buffered neutral formalin, embedded in paraffin wax, sectioned at 4 µm and stained with haematoxylin and eosin and periodic acid-Schiff. Histologic sections were lastly examined.

In each section of the cryptorchid and scrotal testes, the outer diameters of the seminiferous tubules were measured using a calibrated micrometer (Axioskop® 40 Zeiss, NY, USA), and a dedicated software (SPOT® Advanced Version 4.0.5, Diagnostic instruments, Michigan, USA). A total number of 20 seminiferous round tubules selected at random from each testis were measured and the mean diameters were calculated. The number of Sertoli cells in each section of the seminiferous tubules, and the number of Leydig cells adjacent to the seminiferous tubules were also determined.

The results are presented as mean ± standard error. Data on the number of cells (Sertoli and Leydig) by seminiferous tubule and the diameter of the seminiferous tubules were analysed using the general linear model procedure (ANOVA) available in SPSS 13.0 (SPSS Inc., Chicago, IL, USA). The Spearman correlation was used to correlate the histological parameters with testicular weight. In addition, the two-way repeated measures ANOVA was used to analyse the testosterone concentrations between the experimental groups. Comparisons were considered to be statistically significant when  $p < .05$ .

## 3. Results and discussion

Plasmatic testosterone levels are shown (Tables 1 and 2). Before surgery, testosterone levels of cryptorchid horses were not significantly different to those obtained in non-breeding stallions (0.72 vs 0.84 ng/ml, respectively), but higher than those observed in geldings (0.1–0.2 ng/ml). Plasmatic levels of testosterone were significantly higher ( $p < .01$ ) in breeding stallions than in other horses. Within the assessed groups, no differences

**Table 1.** Plasmatic levels (mean ± SD) of testosterone (ng/ml) in cryptorchid horses, before and after surgery.

	Sampling time			
	Before surgery	2 days after surgery	4 days after surgery	7 days after surgery
09.00–10.00 am	0.72 ± 0.10 <sup>a</sup>	0.58 ± 0.06 <sup>ab</sup>	0.30 ± 0.12 <sup>b</sup>	0.22 ± 0.09 <sup>b</sup>
04.00–06.00 pm	0.73 ± 0.05 <sup>a</sup>	0.61 ± 0.15 <sup>a</sup>	0.29 ± 0.09 <sup>b</sup>	0.19 ± 0.1 <sup>b</sup>

Note: Different letters (in superscripts) within the same file denote significant differences.

<sup>a,b</sup> $p < .05$ .

( $p < .05$ ) in testosterone levels measured between the morning and afternoon were observed. After surgery, testosterone levels progressively declined in cryptorchid horses, reaching similar plasmatic levels to geldings between 3 and 5 days after neutering. For all cryptorchid animals, a chromosomal number of  $2n = 64, XY$  was observed.

Testicular weight and microscopic findings were summarized in Table 3. As can be seen, testicular weight was significantly higher ( $p < .01$ ) in scrotal than in retained testis and the firmness of retained testes was obviously lower compared with scrotal testes. No microscopic lesions compatible with testicular neoplasia were observed in scrotal or retained testes. The seminiferous tubules, interstitial tissue and efferent ducts of the retained testes showed a loss in their normal configuration with a variation in their structural homogeneity (Figure 1). Spermatogenesis was completely blocked in the retained testes and no germinal cells were found in the seminiferous tubules, and these were significantly smaller ( $p < .01$ ) in comparison to those assessed in the scrotal testes. The number of Leydig cells adjacent to the seminiferous tubules was higher in the scrotal testes than in the retained testes ( $p < .01$ ). Similarly, the mean number of Sertoli cells was significantly higher ( $p < .05$ ) in the scrotal testis. Spermatogenesis in the scrotal testes appeared normal, with a suitable number of spermatozoa being found in the efferent ducts, and the interstitial tissue and seminiferous tubules showed a normal histological aspect.

A direct relationship has been established between the scrotal circumference, the seminal production and the plasmatic levels of testosterone (Sajjad et al. 2007) in some species. In our study, the lower size of retained testes occurred because of the degeneration of seminiferous tubules; these results agree with previous findings in cryptorchid horses (Coryn et al. 1981; Arighi et al. 1987), in which a similar reduction in spermatogenesis and an apparent normal aspect of Leydig cells both in scrotal and retained testicles was observed. Hence, it could be assumed that these cells would maintain their normal production of testosterone.

**Table 2.** Plasmatic levels (mean ± SD) of testosterone (ng/ml) in breeding stallions, non-breeding stallions and gelding horses.

	Type of horses		
	Breeding stallions	Non-breeding stallions	Geldings
09.00–10.00 am	2.66 ± 0.21 <sup>a</sup>	0.83 ± 0.22 <sup>b</sup>	0.17 ± 0.09 <sup>c</sup>
04.00–06.00 pm	2.41 ± 0.32 <sup>a</sup>	0.88 ± 0.14 <sup>b</sup>	0.14 ± 0.12 <sup>c</sup>

Note: Different letters (in superscripts) within the same file denote significant differences.

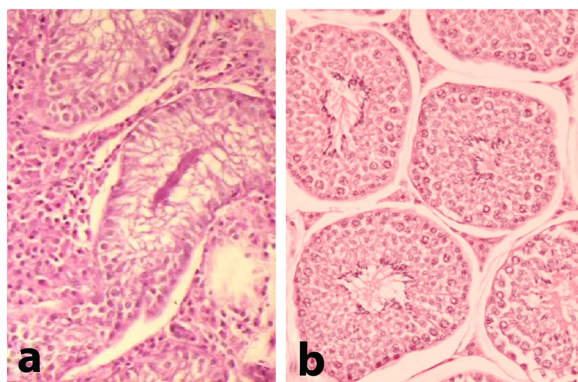
<sup>a,b</sup> $p < .01$ ; <sup>b,c</sup> $p < .05$ .

**Table 3.** Testicular weight and microscopic characteristics of the retained and scrotal testes (mean ± SD).

	Testes characteristics			
	Weight	Seminiferous tubules diameter (µm)	Sertoli cells <sup>1</sup>	Leydig cells <sup>2</sup>
Scrotal testes	212.0 <sup>a</sup> ± 23.2	222.0 <sup>a</sup> ± 9.0	18.9 <sup>a</sup> ± 2.2	22.3 <sup>a</sup> ± 1.8
Retained testes	81.9 <sup>b</sup> ± 6.2	112.4 <sup>b</sup> ± 5.5	8.3 <sup>b</sup> ± 1.1	7.8 <sup>b</sup> ± 1.3

Note: Different superscript letters within the same row denote significant differences.

<sup>a,b</sup> $p < .01$ .



**Figure 1.** Histological section of a retained (a) and scrotal and (b) testes. Absence of spermatogenic cells can be observed in the retained testes. Hematoxylin-eosin.  $\times 20$ .

A recent study (Murase et al. 2015) has confirmed the usefulness of the measurement of AMH (Anti-Müllerian hormone) concentrations for diagnosis of equine cryptorchidism. However, determination of the plasma levels of testosterone has been shown to be a valid technique for the diagnosis of cryptorchidism in horses (Cox 1982; Cox et al. 1986; Arighi & Bosu 1989; Claes et al. 2013). Moreover, hCG administration to stimulate androgenic production does not significantly modify testosterone concentrations (Arighi & Bosu 1989), making it hard to distinguish between breeding stallion and non-breeding stallions. In our case, we were able to differentiate easily between cryptorchid and geldings horses, without hCG administration. It is widely accepted that plasma levels of testosterone in breeding stallions are higher than in cryptorchid horses (Arighi & Bosu 1989). However, Coryn et al. (1981) did not find differences in the plasma levels of testosterone between breeding stallions and unilateral cryptorchid horses. Different results obtained in these studies could be related with different factors. Claes et al. (2013) confirmed that age and season influenced the plasma testosterone concentrations, being higher during the breeding season, and lower concentrations of testosterone were detected in cryptorchid horses younger than two years of age. In our study, all cryptorchid horses aged between 2 and 7 years, and no apparent influence of age was observed. In addition, more than 80% of surgeries and, therefore, testosterone determinations were performed during the reproductive season (March–September) and the low number of animals evaluated outside this period does not allow comparing our results with the study of Claes et al. (2013). In our study, scrotal testicles showed a higher number of Leydig cells than in retained testicles. However, the higher number of Leydig cells observed in scrotal testicles may explain the higher level of testosterone production in breeding stallions than in cryptorchid horses. This hypothesis is aligned with our results, in which we found the highest levels in breeding stallions, followed by non-breeding stallions and, lastly, by cryptorchid horses.

All the analysed metaphases in cryptorchid testes revealed a chromosomal number of  $2n = 64, XY$ . Equine cryptorchidism has been occasionally associated with alterations in karyotype (Dunn et al. 1974; Constant et al. 1994), while in other studies it has not been possible to confirm alterations in number or

structure (Cox et al. 1986). Cytogenetic study showed that our horses were normal male horses, and no chromosomal alteration in number or structure was observed. In horses, where alterations in karyotype have been described, histological findings and hormonal levels (Dunn et al. 1974; Kubien et al. 1993) were similar to those observed in our study.

Few studies have confirmed the production of testosterone in cryptorchid horses (Coryn et al. 1981; Arighi & Bosu 1989; Claes et al. 2013), in line with our findings. In our opinion, lower testosterone production in cryptorchid horses (in comparison with breeding stallions) can be due to the smaller size of the retained testicle, as well as to the lower number of Leydig cells adjacent to the seminiferous tubules of the retained testicles. Moreover, as reported in our study, plasma testosterone determination without hCG stimulation is a faster and more economic diagnostic procedure for the clinician.

#### 4. Conclusion

Results clearly confirmed the direct relationship between hormonal levels of androgens and the number of Leydig cells; therefore, a simple determination of plasma testosterone levels was enough to differentiate between breeding stallions, cryptorchid and castrated animals, without hCG administration.

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#### Disclosure statement

No potential conflict of interest was reported by the authors.

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