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Changes in circulating concentrations of testosterone and estrone sulfate after human chorionic gonadotropin administration and subsequent to castration of 2-year-old stallions

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ABSTRACT

Reproductive steroids testosterone (T) and estrone sulfate (E1S) are used as diagnostic markers for cryptorchidism in horses. The human chorionic gonadotropin (hCG) stimulation test is used as a diagnostic aid because administration of this hormone results in greater incremental differences in circulating steroid concentrations. Thoughts regarding optimal sampling times following hCG administration, however, are inconsistent. Additionally, determination of half-life of these steroids is important in postsurgical samples to confirm complete removal of testicular tissue. Objectives of this study, therefore, were to determine optimal sampling periods for peak T and E1S after hCG administration and half-life of these steroids after castration. Eight pony stallions were randomly assigned to control or treatment groups (5000 IU hCG). Blood samples were collected following hCG administration. Subsequently, stallions were castrated and blood samples were collected post-castration. The T concentrations were greatest at 72 h after hCG and were greater ($P < 0.02$) in samples from hCG-treated than control animals: 9,903.4 ± 384 and 784.0 ± 192 pg/mL, respectively (Mean ± SEM). The T concentrations were also greater at 1, 12, 24, 48 and 96 h. The E1S concentrations did not change after administration of hCG. The T response to hCG administration was biphasic with a maximal response between 48–96 h after administration. Half-lives of T and E1S were 1.1 and 0.7 h, respectively, and concentration of T and E1S was similar to that of geldings at 24 h post-castration, which, therefore, should be considered an optimal time to ensure complete castration has occurred.

1. Introduction

Testosterone (T) and estrone sulphate (E1S) are reproductive steroids produced by the Leydig cells of the testis in postpuberal horses. These steroids are in greater concentrations in peripheral circulation of stallions when compared to geldings (Eisenhauer et al., 1994; Roser, 2008; Almeida et al., 2011). Both hormones have been used as diagnostic markers in different aspects of stallion reproduction, including diagnoses of cryptorchidism, subfertility, lack of libido or testicular degeneration (Arighi et al., 1985; Cox

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et al., 1986; Roser, 1995; Palme et al., 1998; Parlevliet et al., 2011). For cryptorchidism, circulating concentrations of T and E1S have been used due to the marked differences in peripheral concentrations of these hormones among intact stallions, suspected cryptorchids, and gonadectomized male horses (Carneiro et al., 1998). Both steroids are nearly undetectable in castrated males due to the complete absence of Leydig cells and are greater in intact stallions and cryptorchid horses due to the presence of testicular tissue regardless of its location (Cox et al., 1986; Arighi and Bosu, 1989; Carneiro et al., 1998).

These differences are not apparent until puberty, when androgen and estrogen production markedly increase (Pigon et al., 1961; Naden et al., 1990). After sexual maturation, the concentrations of T and E1S in cryptorchids and intact stallions are ten and 100 fold greater, respectively, than in geldings (Arighi et al., 1985; Arighi and Bosu, 1989). Because the differences in these steroids are relatively large, there are some who advocate the use of a single determination of T or E1S concentration for the diagnosis of cryptorchidism. There, however, are still approximately 5 % of animals with incorrect diagnoses and 11 % that require further testing when only basal concentrations are used for diagnostic evaluations of cryptorchidism (Arighi and Bosu, 1989). In recent years, for the determination of serum anti-Mullerian hormone concentration there has been development of an accurate and reliable biomarker for the diagnosis of cryptorchidism (Claes et al., 2013a). The administration of hCG to stimulate the release of testicular hormones in horses is still a widely used method for diagnosis of cryptorchidism. The administration of hCG leads to markedly greater increments in the differences in circulating T and E1S between gonadectomized and suspected cryptorchid stallions (Arighi and Bosu, 1989; Zwain et al., 1989; Cox and Redhead, 1990; Tsunoda et al., 2007). Standard protocols for hCG stimulation have been utilized for sampling before hCG administration, followed by administration of an IV or IM dose of 2500 to 10,000 IU of hCG and a sample in the first 3 h after hCG administration (Samper et al., 2007; Youngquist and Threlfall, 2007; McKinnon et al., 2011). Results from several studies indicate T concentrations are greatest between 2 and 4 days after gonadotropin administration and that differences from pre-stimulation concentrations are much larger than those that have been reported to occur in the first 3 h after gonadotropin administrations (Roser, 1995; Tsunoda et al., 2007).

Additionally, although most horses are post-puberty by 2 years of age, sexual maturity will not occur until 4–5 years of age (Johnson and Neaves, 1981; Johnson et al., 1991). During the first 2 years subsequent to birth, there is a steady increase in circulating concentrations of androgens and estrogens in stallions (Pigon et al., 1961; Khalil et al., 1998; Claes et al., 2013b) as well as in the relative abundances of the steroidogenic enzymes that regulated synthesis of the testicular steroids (Almeida et al., 2011). The circulating concentrations of androgens and estrogens are well characterized in sexually mature horses (Silberzahn et al., 1989; Zwain et al., 1989; Cox and Redhead, 1990; Roser, 1995; Tsunoda et al., 2007; Bollwein et al., 2008). Results from different studies, however, indicate that this might be different in horses less than 3 years of age (Cox et al., 1986; Arighi and Bosu, 1989). To the authors' knowledge there are no controlled studies on the effect of hCG stimulation on androgens and estrogens in postpuberal horses that are not yet sexually mature. Furthermore, although there are available data on the elimination rates of total androgens from the circulatory system (Ganjam and Kenney, 1975; Thompson et al., 1980; Martinez et al., 1991; Palme et al., 1998), the data on half-life of T vary considerably from 0.8 to 39 h (Thompson et al., 1980; Moeller et al., 2011) and data on the half-life of E1S are not available. The evaluation immediately post-surgery of pattern of decrease in circulating concentrations after induction of increases in these steroid hormones as a result of administration of gonadotropins, therefore, is important for determination if there are residual testicular tissues.

The objectives of this study, therefore, were: 1) to evaluate temporal changes in serum T and E1S concentrations in 2-year-old stallions after the administration of hCG, and 2) to determine the half-life of endogenous T and E1S after castration of 2-year-old stallions.

2. Materials and methods

Eight 2-year-old pony stallions of mixed breeding (2.24 ± 0.03 yr; means ± SEM) with a mean weight of 222.7 ± 9.5 kg that were maintained in paddocks and supplemented with hay, grain with water being provided *ad libitum* along with trace minerals were used for this study during April through May. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky (Protocol #: 2013-1088).

2.1. hCG stimulation tests

Eight pony stallions were randomly assigned to control ($n = 4$) or treatment ($n = 4$) groups. Treated stallions were administered an IV dose of 5000 IU of hCG (Chorulon, Intervet Inc, Millsboro, DE, USA), and ponies of the control group were administered an equivalent volume of sterile saline IV (5 mL). Blood samples were collected *via* a catheter placed in the jugular vein and flushed with heparinized saline. To avoid contamination or sample dilution, three 10 mL volumes of blood were collected into BD Vacutainer Red Top blood collection tubes at each collection period, and there was discarding the first tube collected at each period. The collections were initiated at 8:00 am before IV injections of hCG. Following hCG administration, samples were collected at 1, 2, 6, 12, 24, 48, 72, 96 h timepoints. Serum was separated by centrifugation (20 min; 1600×*g*) prior to storage at −20 °C.

2.2. Half-life determination of E1S and T

Ten days after the initial hCG administration, stallions were castrated using standard field castration techniques. Preanesthetic sedation was a combination of 1.1 mg/kg of xylazine (AnaSed®, Lloyd, Inc, Shenandoah, IA, USA) and 0.02 mg/kg of butorphanol (Torbugesic, Zoetis, Kalamazoo, MI, USA), and anesthesia was induced with 2.2 mg/kg of ketamine (Ketathesia, Henry Schein, Dublin,

OH, USA). Standard castrations were performed using Reimer emasculators. Blood samples to determine basal hormone concentration (time 0) were collected after induction of anesthesia and immediately after removal of both testes. Subsequent blood samples were collected at 0.5, 1, 2, 3, 4, 5, 6, 8, 10, 12, 24, 36, 48, 72, 96, 120, 240 and 360 h following castration. Serum samples were processed and stored as previously described in this manuscript.

2.3. Enzyme immunoassay (EIA)

The E1S concentrations were quantified (in triplicate) using a competitive Enzyme immunoassay (EIA) using an E1S antibody (522-2, UC Davis) (Stabenfeldt et al., 1991; Carneiro et al., 1998). The standard curve ranged from 0.05 ng/mL to 20 ng/mL, with a limit of detection of 0.05 ng/well. Intra- and inter-assay coefficients of variation (CV) were 10.2 % and 16.6 %, respectively.

The T concentrations were quantified (in triplicate) with a competitive EIA (Illera et al., 1997, 2003). Serum samples were extracted using a standard diethyl ether extraction protocol. The assay was developed using a T antibody (R156/7, UC Davis) and T:HRP (UC Davis) and T (Steraloids Inc., Newport, RI, USA). The standard curve ranged from 0.02 to 10 ng/mL with a limit of detection of 0.021 ng/mL. Intra- and inter-assay CV were 11.9 % and 18.1 %, respectively. The assay was validated by comparing 60 samples against two other commercial assays: a radioimmunoassay Coat-a-count Total T RIA (PITKTT-8, Siemens Healthcare Inc, Alpharetta, GA, USA) with there being a correlation coefficient between values with use of this commercial assay and those with use of the the EIA of 0.921 ($P < 0.0001$) and the chemiluminescence Immulite Total T (PILKTW-12, Siemens Healthcare Inc, Alpharetta, GA, USA) of 0.962 ($P < 0.0001$; Fig. 1).

2.4. Statistical analysis

After evaluating data for normal distribution, a mixed model ANOVA was used for the hCG stimulation test results; group and time were used as fixed effects, and stallion was used as random effect. Tukey's HSD was used for *post-hoc* analysis. For determination of half-life of hormones, exponential one-phase decay analysis procedures were used. All analyses were performed using JMP software (JMP, Version 10. SAS Institute Inc., Cary, NC, USA) and GraphPad Prism (GraphPad Software, La Jolla, CA, USA). There were considered to be differences in mean values when there was a $P < 0.05$.

3. Results

Mean basal values for T prior to hCG administration were 723 pg/mL (min. 378-max. 1392 pg/mL). For E1S, mean basal concentrations were 227.9 ng/mL, ranging from 81.4 to 490.7 ng/mL. After hCG administration, circulating T concentrations were markedly different between groups at 1, 12, 24, 48, 72, and 96 h post-hCG administration ($P < 0.0001$), with the largest differences at 48–96 h. The T concentrations were not different between groups or from the basal T concentrations at 0, 2, or 6 h following hCG administration (Fig. 2). There were no differences in E1S concentrations between treatments or at any timepoint when samples were collected (Fig. 3).

Before castrations, mean basal T concentration was 1,365.5 ± 249.9 pg/mL (mean ± SEM) and mean T concentration 15 days after castration was 38.3 ± 7.1 pg/mL (Fig. 4). The estimated half-life for T was 1.1 h. The 95 % confidence interval at 15 days following

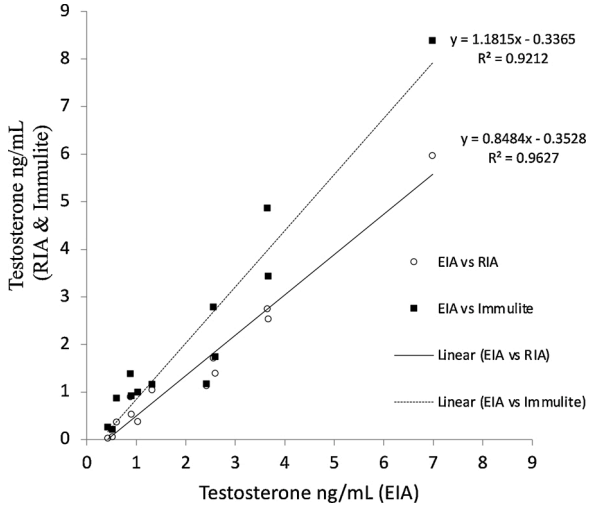

Fig. 1. Correlation between testosterone (T) concentrations determined with the different assays; EIA was validated against two other commercial assays: a radioimmunoassay (RIA) Coat-a-count Total T RIA (PITKTT-8, Siemens Healthcare Inc, USA) and chemiluminescence Immulite Total T (PILKTW-12, Siemens Healthcare Inc, USA); These assays had a correlation with the developed EIA of 0.921 compared with chemiluminescence method and 0.962 compared with RIA.

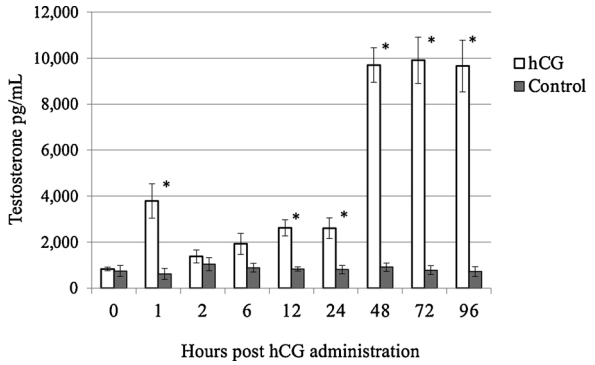

Fig. 2. Serum testosterone (T) concentrations following human chorionic gonadotropin (hCG) administration to 2-year-old pony stallions; T concentrations in control group ($n = 4$) did not change overtime; In the hCG-treated group ($n = 4$), there was an increase 1 h post-stimulation, followed by a decrease and a second peak that started 12 h following hCG administration with concentrations plateauing from 48 until 96 h when there were the largest incremental differences between treated and the control group were detected; * T concentrations differ from control or T0 ($P < 0.0001$); Data are expressed as mean ± SEM.

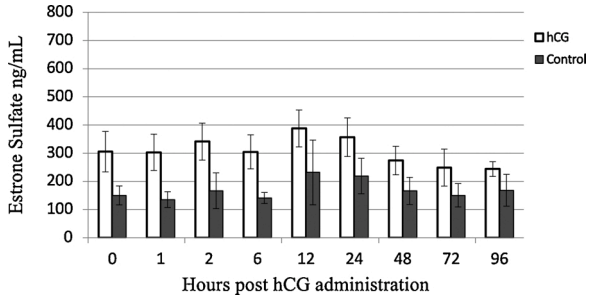

Fig. 3. Serum estrone sulfate (E1S) concentrations following hCG administration to 2-year-old pony stallions; There were no differences in the hCG-treated ($n = 4$; $P > 0.05$) compared with the control (n=4) group; T concentrations in the control group and hCG-treated group did not change overtime; Data are expressed as mean ± SEM.

castration was 38.3 ± 40.4 pg/mL (means ± 2SD) with a range of 0–78.7 pg/mL. All samples collected subsequent to 24 h of castration, including at this timepoint (*i.e.*, 24 h), were within this range (Fig. 4).

For E1S, the mean basal concentration was 131.14 ± 58.9 ng/mL and the mean E1S concentration at 15 days following castration was 0.6 ± 0.2 ng/mL. The half-life of E1S was estimated to be 0.7 h. The 95 % confidence interval at 15 d following castration was 0.6 ± 1.7 ng/mL with a range of 0–2.31 ng/mL. As for T, all samples collected 24 h subsequent to the time of castration, including this

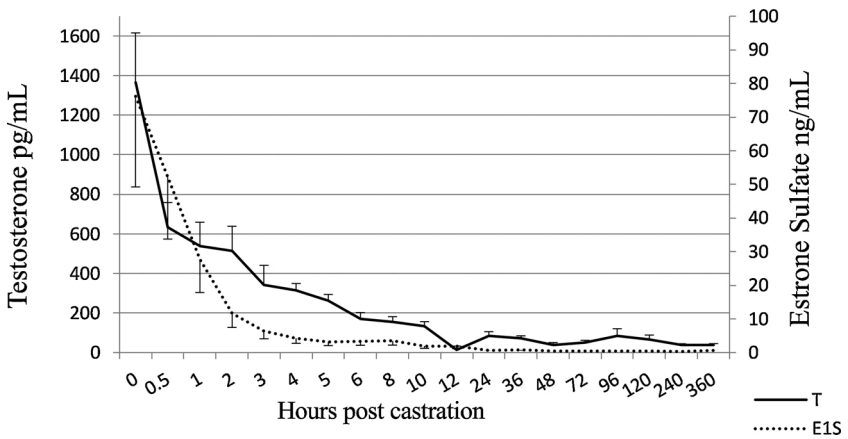

Fig. 4. Decease of serum testosterone (T) and estrone sulfate (E1S) concentrations after castration of 2-year-old pony stallions ($n = 8$); T half-life was 1.07 and T was 0.72 h; The rate of decrease in concentrations of these hormones plateaued between 12 and 24 h following castration, indicating that concentrations after 24 h from the time of hCG administration were not different from final concentrations; Data are expressed as mean ± SEM.

timepoint (*i.e.*, 24 h), were within this range for E1S.

4. Discussion

In the present study, serum T concentrations were the greatest between 2 and 4 days following hCG administration, although there was an increase in serum T 1 h following hCG administration. Results when there are uses of standard clinical protocols indicate T concentration should be quantified within the first 3 h after hCG administration (Samper et al., 2007; Youngquist and Threlfall, 2007; McKinnon et al., 2011). At 1 h following hCG administration, there was a fourfold increase in T compared to concentrations of animals in the control group at 1 h following hCG administration; however, there was no difference in T concentrations between the two groups when there was comparisons to samples collected at 2 or 6 h following hCG administration. Following this decrease in T concentration to basal values at 2 and 6 h, there is a second increase in T concentration at 12 h following hCG administration with maximal T concentration occurring at 72 h. The difference in the duration of the initial release of T peak after hCG administration might be related to the younger age of the stallions in the present as compared with previous experiments. While these stallions were 2 years of age (2.24 $\pm$ 0.03 year) and in all stallions that were administered hCG there was an increase in T, these stallions were not yet sexually mature. Results for a number of studies indicate values for testicular variables including weight, daily sperm output, Leydig cell numbers, abundance of steroidogenic enzymes, circulating estrogen and androgen concentrations continue to increase until stallions are sexually mature (Pigon et al., 1961; Johnson and Neaves, 1981; Khalil et al., 1998; Stewart and Roser, 1998; Almeida et al., 2011). In contrast to what occurred in these relatively younger stallions in the present study, mature stallions had greater T concentrations than those that were basal for the first 3 h following administration of gonadotropin (Arighi and Bosu, 1989; Cox and Redhead, 1990; Parlevliet et al., 2011). If the increase in T is of the same pattern in cryptorchids, sampling during the period when there is the initial increase in T could result in failure to identify cryptorchidism in younger stallions due to a smaller increase in testosterone concentrations during this initial time period subsequent to gonadotropin administration. Sampling 24–96 h following administration of a gonadotropin, therefore, will result in a greater percentage of conclusive diagnoses due to the sustained and large response in T release. Similarly, in a previous study the testicular response in T release to hCG administration was biphasic indicating that results were more reliable when there was assessment of T concentrations 24 h following administration of hCG compared to evaluations at 1 h following this administration in horses with testicular tissue including cryptorchids, hemicastrates and intact stallions (Arighi and Bosu, 1989). The biphasic response in T release to hCG administration has been reported in a number of different species (Okuda et al., 1991). The mechanism(s) resulting in this response, however, is not well understood. There has been speculation that this biphasic response could be due to the longer half-life of hCG compared to endogenous gonadotropins and additionally, that there might be a priming effect at the Leydig cells during the first response to hCG administration resulting in the biphasic response pattern in T after hCG administration (Padron et al., 1980; Ulloa-Aguirre et al., 1985).

Results from other studies have indicated there are different responses to a single injection of hCG in stallions. As compared with results in the present study, results in some other studies indicated there was an initial sustained response in T release for several hours after administration of a gonadotropin (Arighi and Bosu, 1989; Cox and Redhead, 1990; Parlevliet et al., 2011). In other studies, results indicated there was an initial response during the first 12 h after gonadotropin administration, however, there was a gradual and sustained increase until 36 h following administration (Zwain et al., 1989). There have been many reports of a large increase in T occurring 2–5 days after the administration of hCG (Arighi and Bosu, 1989; Silberzahn et al., 1989; Zwain et al., 1989; Cox and Redhead, 1990; Tsunoda et al., 2007). These differences in results among studies can be attributed to biological variations in the animals evaluated such as the age of the stallions, and furthermore in some studies to variations in season of the year and reproductive status of the horse or to technical differences in experimental design, hCG dosage or sampling strategies. There may also be certain conditions that contribute to variations in the response to gonadotropin administration such as when there is cryptorchidism the biological response may differ among animals depending on the location of the cryptorchid testis, if the condition is bilateral or unilateral, and if the stallions are hemicastrates. If the response in T concentration to hCG administration is of the same pattern in cryptorchid stallions, sampling during the time with a larger and sustained increment in response should potentially provide for more reliable and conclusive results when there is use of this test. In the present and previous studies (Arighi and Bosu, 1989; Silberzahn et al., 1989; Zwain et al., 1989; Cox and Redhead, 1990; Tsunoda et al., 2007) results indicate there is a period of time between 48–72 h following hCG administration that is optimal for reliable assessments to occur.

Results from several studies indicate there are increases in circulating concentrations of estrogen following hCG administration to stallions. While in one study, it was reported there were increases in circulating ES, (Arighi and Bosu, 1989) results from other studies indicate that there were increases in circulating concentrations of estradiol or estrone following hCG administration (Zwain et al., 1989; Tsunoda et al., 2007). In contrast to results in these previous studies, results from another study indicated one stallion responding to hCG with increasing circulating E1S while there was no such response in another stallion (Cox and Redhead, 1990). Results from all these previous studies indicated there was an increase in estrogen concentrations after hCG administration to stallions that were at least 4 years of age (Silberzahn et al., 1989; Zwain et al., 1989; Cox and Redhead, 1990; Tsunoda et al., 2007). The only exception to these findings is that from the study of Arighi and Bosu (1989). In this previous study, horses ranging from 1 to 15 years of age were subsequently assigned by age (less than 3 and greater than 3 years of age) and there were lesser E1S concentrations in the younger as compared with the older stallions. Results from the present study indicate that there was no response in circulating concentrations of E1S after hCG administration in 2-year-old stallions. Age, therefore, might be the determining factor largely responsible for these different results because stallions continue to reproductively mature after 2 years of age (Pigon et al., 1961; Johnson and Neaves, 1981; Khalil et al., 1998; Stewart and Roser, 1998; Almeida et al., 2011) and even though the 2-year-old stallions in the present study were postpuberal, they were not sexually mature. In addition to age, other factors that might contribute to the

differences in the response of E1S to hCG might be related to the stallions used in the present study being mixed breed ponies. In addition to age, other factors such as breed might have contributed to the differences in E1S concentrations. In the present study, there was use of mixed breed ponies, and breed has been reported to affect estrogens concentrations during pregnancy in horses (Palme et al., 2001), or for example there are relatively lesser concentrations of estrogens in donkeys than horses (Schuler et al., 2019).

The half-life of T in the present study (1.1 h) was similar to that reported previously (0.8 h) (Thompson et al., 1980), however, very different from the 33 to 39 h after intramuscular administration of an aqueous suspension of T (Moeller et al., 2011). To the authors' knowledge, there are no exact data available in the literature for the half-life of ES. In humans, the half-life of E1S is long (10−12 h), and it has been suggested that it could be similar in the horse (Lemazurier and Seralini, 2002). Results from the present study, however, indicate that half-life of E1S in the horse was 0.7 h, therefore, there were rapid decreases in circulating concentrations after castration. Results from the present study are consistent with those in a previous study in horses that indicated there was a shorter half-life of E1S (Palme et al., 1998). Both T and E1S were at basal concentrations within 24 h following castration and these values were not different from concentrations 15 days after castration. These results indicate that to confirm complete removal of testicular tissue, circulating concentrations of T or E1S could be quantified 24 h after surgery for reliable evaluations to occur, as previously reported (Arighi and Bosu, 1989; Palme et al., 1998).

5. Conclusion

In summary, the results from the present study indicate in 2-year-old pony stallions, the optimal times for blood sampling after hCG administration are 1, 12, 24, 48, 72 and 96 h, with the largest incremental increase in serum T occurring at the latter three timepoints. The E1S concentration did not change after hCG administration in this group of 2- year-old pony stallions. The concentrations of E1S and T after castration of stallions indicated that when complete removal of testicular tissue is questionable, the animal could be evaluated for either T or E1S at 24 h after surgery. When all testicular tissue was removed, T or E1S concentrations were not different at 24 h following castration from concentrations at 15 days following castration.

Author's contributions

A. Esteller-Vico: Participated in the experimental design, animal and laboratory work, data analysis, manuscript writing and revisions.

B.A. Ball: Experimental design, funding, data analysis and manuscript revision.

J.W. Bridges: animal and laboratory work.

S.E. Hughes: Animal work.

E.L. Squires: Experimental design and manuscript revision.

M.H.T. Troedsson: Experimental design and manuscript revision.

Declaration of Competing Interest

The authors report no declarations of interest.

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