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Comparative studies on testis, epididymis and serum hormone concentrations in foxes, and hybrids during the pre-breeding period

T.A. Yang, Y.H. Yang, X.C. Song, L.L. Liu, Y.F. Yang, X.M. Xing, F.H. Yang, Y.H. Peng*

State Key Laboratory for Molecular Biology of Special Economic Animals, Key Laboratory of Special Economic Animal Genetics and Breeding, Institute of Special Economic Animal and Plant Sciences, Chinese Academy of Agricultural Sciences, Changchun, PR China

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ABSTRACT

Silver fox and blue fox belong to different genera, and the hybrid males are reproductively sterile. In the present study, there was a comparison of testicular and epididymal morphology and serum hormone concentrations among silver foxes, blue foxes, and the hybrids during the pre-breeding period, using 20 male silver foxes, 20 male blue foxes, 15 male HSBs (silver fox male × blue fox female hybrids) and 15 male HBSs (blue fox male × silver fox female hybrids), respectively. Hybrids had a smaller diameter of seminiferous tubules than pure-species males, and testes of hybrid males did not differ in mean size and relative weight from pure-species males. There were many Sertoli cells and spermatogenic cells in silver foxes and blue foxes, while numbers of spermatogonia and primary spermatocytes were less with no secondary spermatocytes in the hybrids. Furthermore, mean serum testosterone and estradiol concentrations in the hybrids were less, and FSH, LH, and PRL were greater than that in silver foxes and blue foxes ($P < 0.05$), suggesting that lesser concentrations of testosterone and estradiol and greater concentrations of FSH, LH and prolactin can inhibit the completion of spermatogenesis during the pre-breeding period. The results indicate that fox hybrid sterility may result from failures at the early stages of spermatogenesis.

1. Introduction

Silver fox (*Vulpes fulvus*) and blue fox (*Alopex lagopus*) belong to different genera (Makinen and Gustavsson, 1982). The first hybrid generation of silver and blue fox has obvious heterosis characteristics with a greater fur economic value. The fox hybrids, however, are reproductively sterile, and the hybrids cannot produce normal sperm (Wipf and Shackleton, 1949). The molecular mechanism resulting in reproductive sterility of male hybrids is not well understood.

The testes are part of a select set of organs necessary for the continued propagation of a species that reproduces sexually (Shima et al., 2004). Spermatogenesis is a complex, multistep process, and is dependent on a highly controlled local environment which is the result of the cellular relationships that take place in the seminiferous tubules of the testis (Peri and Serio, 2000). The spermatogenic process is nearly the same in all mammals, and it occurs in successive mitotic, meiotic and post-meiotic phases (Eddy, 1998). The germ cell contributes to the proliferation and differentiation of many cell types, beginning with diploid spermatogonial stem cells and ending with haploid spermatozoa. In contrast, the somatic cell contributes to steroid biosynthesis by Leydig cells and the maturational function of Sertoli cells (Hamano et al., 2007). There, however, are characteristics related to the types and the number of spermatogonial generations and the morphological characteristics of germ cells present at the various stages of spermatogenesis that

* Corresponding author.
E-mail address: pengyinghua@caas.cn (Y.H. Peng).

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are specific to each species (De Rooij and Russell, 2000).

Results of previous studies indicate that during the breeding season mean testicular volume and the diameter of seminiferous tubules in silver and blue foxes were greater than that in the hybrids. Furthermore, there were many Sertoli cells and spermatogenic cells in silver and blue foxes, while spermatogenic cells were fewer in number with no fully developed sperm cells in the hybrids. In addition, mean serum LH and prolactin concentrations in silver and blue foxes were less and testosterone concentrations were greater than in the hybrids. The results indicate that germ cell meioses in the hybrids were arrested at the prophase stage of meiosis (Yang et al., 2016).

Testicular redevelopment begins some 5–6 months of age before the mating season (Smith et al., 1985). Because the developmental causes of hybrid male sterility are not fully understood, in the present study, analyses were conducted of morphological alteration of the testes and determination of serum hormone concentrations between hybrid and pure-species males during the pre-breeding period. Specific objectives were two-fold. There was elucidation during the pre-breeding period of when during development spermatogenesis failed in hybrids, because there are physiological differences between hybrid and pure-species males during the breeding season. Secondly, there was evaluation of the potential reasons as to why the fox hybrid males cannot reproduce.

2. Materials and methods

The experiment was conducted at the fur animal farm of the Institute of Special Animals and Plants, Chinese Academy of Agricultural Sciences, Jilin, China, from the period of 20 November to 10 December 2017.

2.1. Animals and sample collection

The research with the animals used in the present study was approved by the Animal Welfare Committee of CAAS and was in accordance with The Code of Ethics of the World Medical Association.

There were 20 male silver foxes, 20 male blue foxes, 15 male hybrids of silver fox males × blue fox females (designated as HSB), and 15 male hybrids of silver fox females × blue fox males (designated as HBS) that were used during the pre-breeding period in this study. All hybrids were produced by artificial insemination.

The experiment was performed in silver foxes from 20 to 26 November, HSBs from 28 November to 5 December, HBSs from 23 to 27 November, and the blue foxes from 1 to 10 December.

2.2. Sampling

Samples of peripheral blood were collected at 0900 to 1100 every 5 days, and there were four other collections from each fox. Testes and epididymides were collected on the same day when blood was last collected.

Blood samples were collected using a 5-mL injector into 5-mL coagulation promoting tubes with separation gel. The tubes were kept chilled until centrifugation at 3000 *g* for 10 min. The serum was collected and stored at −20 °C.

Both testicles were collected after weighing every fox, excess fat was removed, and weighing the bilateral testes singly occurred using a micro-electronic scales (500 g/0.01 g), and the major axis, minor axis and thickness of the testis was measured. The testicular volume was calculated using the formula of ellipsoid volume: V = 4/3× π× major axis/2 × minor axis/2 × thickness/2 (Jallageas et al., 1994), and the relative weight of bilateral testis was calculated by the formula: relative weight of bilateral testis = weight of bilateral testis / body weight. The testes and epididymides were kept in 10% formaldehyde after the measurement.

2.3. Histological observations of testes and epididymides

The testes and epididymides were dehydrated in a series of ethanol, cleared in xylene and embedded in paraffin. Tissues were sectioned at 6 μm using a fully auto-mated rotary microtome (Leica, RM2255, Heidelberger, Germany). For histological observations, sections were stained with haematoxylin and eosin (H&E). There were ten testis slices and 15 cross-sections of seminiferous tubules in each slice chosen for morphometric analysis. An imaging system consisting of a Leica light microscope was used. Diameter of seminiferous tubules were measured using the "cross" method with a HMIAS image analysis system (LEICA).

2.4. Serum hormone assays

Serum hormone concentrations for FSH, LH, testosterone, estradiol and prolactin were measured by the ELISA (Enzyme-Linked Immunosorbent Assay) method, using an ELx800 Absorbance Reader (BioTek Instruments, Inc., Winooski, VT, USA), and using canine kits according to the manufacturer's guidelines.

The quantification using canine kits of FSH was (BW02730B, Wanjiang Biological technology Co., LTD, Shanghai, China), LH (BW02733B, Wanjiang Biological technology Co., LTD, Shanghai, China), testosterone (BW02729B, Wanjiang Biological technology Co., LTD, Shanghai, China), estradiol (BW02731B, Wanjiang Biological technology Co., LTD, Shanghai, China) and prolactin (BW02722B, Wanjiang Biological technology Co., LTD, Shanghai, China). All canine kits were used according to the manufacturer's guidelines.

As the present study using canine kits to test the fox serum hormone concentrations, to test for accuracy, standards in ELISA kits and the fox serum were simultaneously tested by comparing the slopes of the straight line in the standard curve. The standard curve

with standards in ELISA kits (testosterone: $r^2 = 0.994$, slope = 8.478; estradiol: $r^2 = 0.996$, slope = 86.212; FSH: $r^2 = 0.996$, slope = 7.469; LH: $r^2 = 0.996$, slope = 46.985; prolactin: $r^2 = 0.996$, slope = 337.14), exhibited a high degree of parallelism with the standard curve with the fox serum (testosterone: $r^2 = 0.999$, slope = 8.185; estradiol: $r^2 = 0.999$, slope = 88.734; FSH: $r^2 = 0.999$, slope = 7.369; LH: $r^2 = 0.999$, slope = 46.888; prolactin: $r^2 = 0.999$, slope = 328.86). The precision was assessed by five samples with the repeated assay of three. The results indicated that the use of these assays in foxes had non-specific interference.

The sensitivity of the assays was defined as the least standard difference from zero ($P < 0.05$), using an unpaired t-test. The sensitivity was ≤0.1 mIU/mL serum for the FSH assay and ≤1.0 mIU/mL, ≤1.0 pg/mL, ≤1.0 pg/mL and ≤1.0 mIU/L for the LH, estradiol, testosterone and prolactin assays, respectively. The intra-assay and inter-assay coefficients of variation with FSH, LH, estradiol, testosterone and prolactin were less than 15%, respectively.

2.5. Statistical analysis

The data on morphological variables of testis and serum hormone concentrations are presented as means ± SEM. Analysis of variance and group comparisons of significance were conducted using Duncan's program in SAS (SAS Institute Inc., Cary, NC, USA). Values were considered to be significant if the probability was $P < 0.05$.

3. Results

3.1. Histological observations

3.1.1. Histological observations of testis

During the pre-breeding period, the histological structure of the fox testis is shown in Fig. 1. There were many spermatogonia, primary spermatocytes, and secondary spermatocytes in testicular seminiferous tubules of the fertile blue and silver fox (Fig. 1A and B). The testicular structure of the HSB and HBS was quite different with only a small number of spermatogonia, and primary spermatocytes, and no secondary spermatocytes being present (Fig. 1C and D).

3.1.2. Histological observations of epididymidis

The histological organization of the fox epididymidis is shown in Fig. 1. There were no sperm in the lumen of epididymal duct among silver, blue, HBS, and HSB foxes.

3.2. Morphological variables of testis and serum hormone concentrations

During the pre-breeding period, there was no difference of the mean testicular volume and relative weight of bilateral testis among silver, blue, HBS, and HSB foxes ($P > 0.05$). The diameter of seminiferous tubules, mean serum testosterone and estradiol concentrations in HBSs and HSBs were less than that in silver and blue foxes ($P < 0.05$). The mean serum FSH, LH, and PRL concentrations in HBSs and HSBs were greater than that in silver foxes and blue foxes ($P < 0.05$; Table 1).

4. Discussion

Foxes are seasonal long-day breeders, with reproductive functions and behaviors only occurring from February to May (Mondain-Monval et al., 1985). Foxes are monoestrous, having only one oestrus per breeding season. With the absence of pregnancy the luteal phase is on average 3 months, followed by a 9-month anoestrus until the next breeding season (Farstad, 1998). The blue fox initially expresses oestrus beginning in March, and most foxes come into oestrus in mid-March to mid-April. The silver fox expresses oestrus from the end of January to the beginning of March in commercial pelt production enterprises (Farstad, 1998).

Two months prior to oestrus (typically December), the reproductive organs of the silver fox males change shape and size. Sperm production in males begins in August-September, with the testicles there are of greatest weight in December-February (Harris et al., 2008). When the blue fox is 9–10 months of age there is attainment of sexual maturity. When these foxes breed, it is between the months of March to April, and there is on average a 52 day gestation period.

Previous studies were conducted from the period 10 January to 6 April during the breeding season when the silver and blue fox testes are fully mature (Yang et al., 2016). The present study, however, was conducted from the period of 20 November to 10 December during the pre-breeding period when testes of the silver, blue, and hybrid foxes begin to develop, but are not yet fully mature.

In the present study, during the pre-breeding period, testes of reproductively sterile hybrids were similar in size as the testes of pure-species males, but the diameter of seminiferous tubules was smaller. Results of previous studies indicate that during the breeding season the diameters of seminiferous tubules in silver foxes (246.30 ± 2.5 μm) and blue foxes (223.09 ± 2.16 μm) were greater than in HBSs (169.55 ± 1.85 μm) and HSBs (149.77 ± 2.12 μm) (Yang et al., 2016). While, results of the present study indicate that during the pre-breeding period, the diameters of the seminiferous tubules were 202.86 ± 1.69 μm in silver foxes, 187.82 ± 2.10 μm in blue foxes, 149.62 ± 1.58 μm in HBSs and 176.26 ± 1.90 μm in HSBs. Obviously, from the pre-breeding period to the breeding season, the diameter of seminiferous tubules in silver and blue foxes increased significantly, however, in the HSBs and HBSs foxes this increase in diameter did not occur. These results indicated that the development of seminiferous tubules in the reproductively sterile fox hybrids was disrupted. Results of the present study indicate that there is not a smaller testes size in the

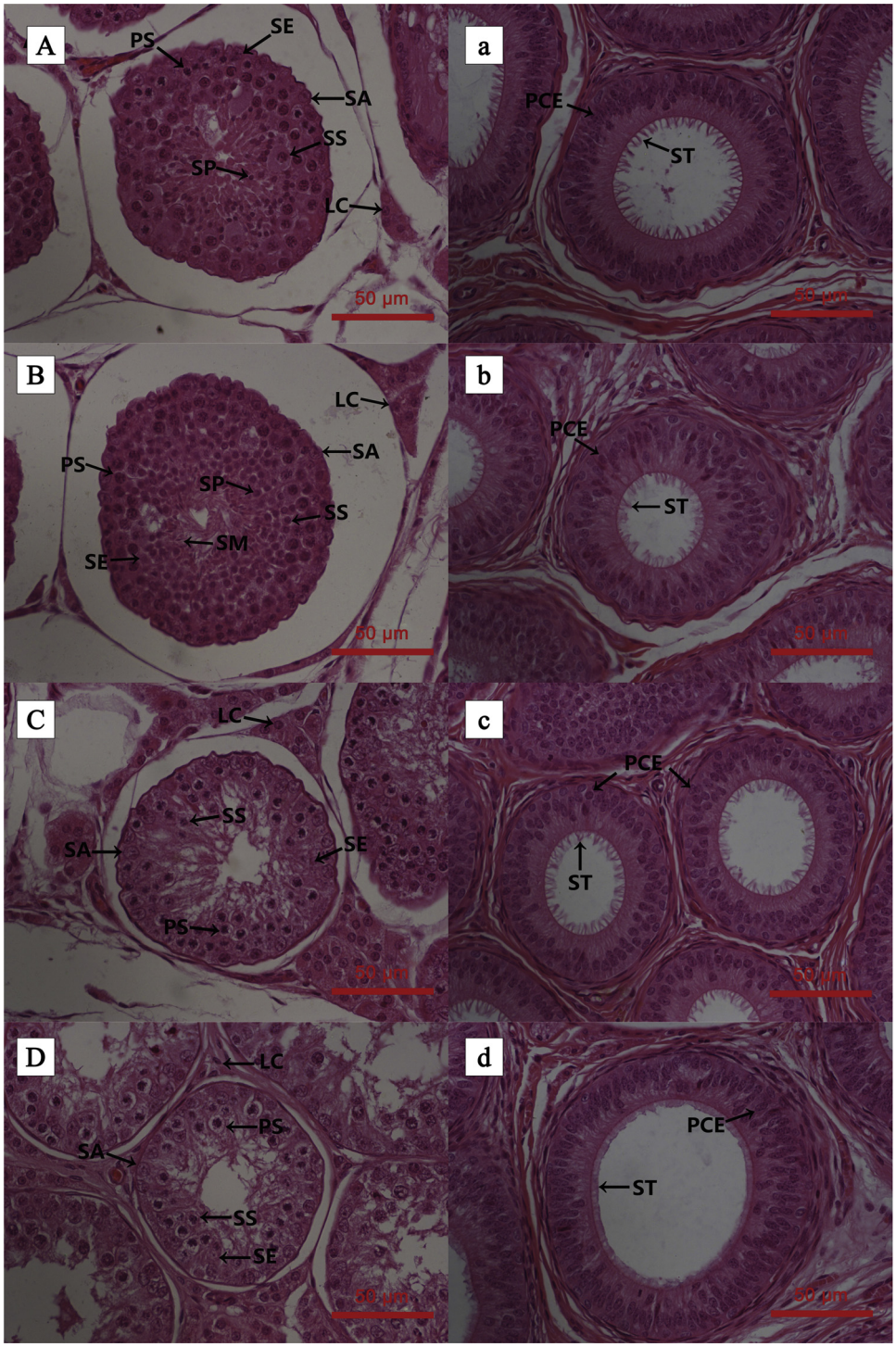

Fig. 1. Photomicrographs of testes and epididymides from fertile foxes stained with H&E. The testicular seminiferous tubules of (A) blue fox, (B) silver fox, (C) HSB, and (D) HBS are shown in the left lane, and epididymidis of (a) blue fox, (b) silver fox, (c) HSB, and (d) HBS are shown in the right lane. The presence of sperm and spermatocytes inside the testes are labeled with arrow lines. SE, Sertoli cell; LC, Leydig cell; SA, spermatogonium; PS, primary spermatocyte; SS, secondary spermatocyte; SP, spermatids; SM, sperm; ST, stereocilia; PCE, pseudostratified columnar epithelium; Scale bar, 50 μm (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Table 1
Morphological variables of testis and serum hormone concentrations of silver foxes, blue foxes and the hybrids during the pre-breeding period.

Items	SF(n = 20)	BF(n = 20)	HBS(n = 15)	HSB(n = 15)	P-value
Testicular volume (cm^3)	4.32 ± 0.27	3.95 ± 0.25	3.44 ± 0.41	3.91 ± 0.41	0.369
Relative weight of bilateral testis (%)	0.15 ± 0.01	0.13 ± 0.01	0.19 ± 0.02	0.17 ± 0.02	0.073
Diameter of seminiferous tubule (μm)	202.86 ± 1.69 [a]	187.82 ± 2.10 [b]	149.62 ± 1.58 [c]	176.26 ± 1.90 [d]	< 0.001
FSH (ng/mL)	14.77 ± 1.29 [a]	15.67 ± 1.29 [a]	20.93 ± 1.29 [b]	19.83 ± 1.28 [b]	0.032
LH (ng/mL)	0.93 ± 0.07 [a]	0.87 ± 0.07 [a]	1.16 ± 0.07 [b]	1.17 ± 0.07 [b]	0.024
Testosterone (pg/mL)	3330.37 ± 121.89 [a]	3451.27 ± 121.89 [a]	1973.47 ± 121.89 [b]	868.12 ± 86.19 [c]	< 0.001
Estradiol (pg/mL)	42.94 ± 1.95 [a]	41.42 ± 1.95 [ab]	34.60 ± 2.51 [b]	22.74 ± 1.95 [c]	< 0.001
PRL (ng/mL)	49.38 ± 1.91 [a]	51.52 ± 1.71 [a]	60.97 ± 2.21[b]	61.27 ± 2.21 [b]	0.002

Values are presented as mean values ± SEM; Comparisons were done among rows (different foxes); Differences ($P < 0.05$) are indicated by different superscript letters.

hybrid compared with the purebred foxes and, therefore, this is not the determinant of impaired spermatogenesis. There were smaller diameters of the seminiferous tubules in the hybrids as compared with the purebred foxes, however, and this was associated with the disrupted spermatogenic processes in the hybrids.

Furthermore, histological observations of testes provided evidence for the many spermatogonia, primary and secondary spermatocytes in pure-species males, while there were few spermatogonia, primary spermatocytes and no secondary spermatocytes in the HSBs and HBSs, which is consistent with findings in previous studies of fox hybrids during the breeding season (Yang et al., 2016).

Whereas failures at the early stages of sperm development could be more likely to result in complete reproductive sterility, failures in the latter stages of spermatogenesis may result in at least some sperm production by hybrid males. Furthermore, failures in the later stages of sperm development could result in sperm that are viable, but of low quality (Wunsch and Pfennig, 2013).

Results of previous studies indicated that meiosis in HSBs foxes was arrested at prophase I (Andersen Berg, 1984; Nyberg, 1980), which was similar to the findings in the present study. Fox hybrid reproductive sterility may, therefore, result from failures at the early stages of spermatogenesis, and as a result, the hybrids are completely sterile.

Similar results were obtained in studies of hybrid males of other species. Due to the smaller testis size, less seminiferous tubule volume density, and fewer germ cells, the total length of seminiferous tubules in mules is smaller than in donkeys (Neves et al., 2002). Although the mean number of seminiferous tubules was greater in stallions and hinnys than Jack donkeys, the mean proportion of seminiferous tubules was less in hinnys which resulted from the smaller diameter of the seminiferous tubules. The mean number of spermatogonia and spermatocytes per unit area was less in the hinny testis and there were no spermatids or mature spermatozoa cells detected. These results indicated that defects in seminiferous tubule development and structure occur in the testis of hinnies (Han et al., 2016). In cattle-yak cross animals, the lumen contained no sperm and most seminiferous tubules were devoid of germ cells (Wishart et al., 1988). Throughout from the basement membrane to the lumen of seminiferous tubule, abundant spermatogenic cells were present in yak testis including round or lengthened spermatids in the lumen. In contrast, only a monolayer of spermatogenic cells were detected that were attached to the basement membrane in cattle-yak cross testis, meanwhile, there were very fewer spermatocytes and almost no round spermatids or sperm cells present in the lumen of the seminiferous tubule. Spermatogonia, therefore, were the main type of germ cells present in the testes of cattle-yak cross animals, whereas all types of germ cells were present in purebred yak testis (Cai et al., 2017).

The testicular function is influenced by the hormonal milieu generated by the hypotalamus-pituitary axis. Testosterone actions in the testis in relation to the regulation of spermatogenesis have been describe in review manuscripts (Mäkelä et al., 2018; Thirumalai and Page, 2018). It has been suggested that following withdrawal of intra-testicular testosterone in rats, round spermatids do not develop further by transitioning between Stages 7 and 8 of spermiogenesis and, therefore, cannot complete the elongation process (O'Donnell et al., 1996; Sofikitis et al., 1999). This effect may be mediated by the loss of the adhesion of the spermatids with the sustentacular Sertoli cells (Zirkin, 1998). It has been reported that there is a release during Stage 8 of spermiogenesis of round spermatids within the epididymal lumen of rats with a lesser intra-testicular testosterone milieu (Sofikitis et al., 1999). Decreases in intra-testicular testosterone concentrations can lead to an increased apoptosis of germ cells (Kim et al., 2001). Evidence from many species supports the view that estrogen has an important role in the regulation of testicular development and function (Kula et al., 2001; Pentikäinen et al., 2000). Not only is testis size and sperm production affected by endogenous estrogen secretion in boars, but inhibition of estrogen synthesis can increase the values for these variables by more than 20% (At-Taras et al., 2006). This delay in testicular development of boars with lesser estrogen concentrations is consistent with results in studies of the bank vole that indicate administration of estradiol accelerates testicular maturation and onset of spermatogenesis (Gancarczyk et al., 2004). In this present study, mean serum testosterone and estradiol concentrations in silver foxes and blue foxes were greater than that in HBSs and HSBs, which suggested that lesser concentrations of testosterone and estradiol can be associated with apoptosis of germ cells and inhibition of the completion of spermatogenesis.

There are FSH receptors located on Sertoli cells, whereas LH receptors are present on Leydig cells, which synthesize and release testosterone. Sertoli cells produce factors necessary for the progression of spermatogonia development into spermatozoa. The hormones, FSH and LH, affect the germ cell fate, and if there is suppression of concentrations of these hormones there is an increase in germ cell apoptosis (Sofikitis et al., 2008). There are inconsistent thoughts regarding the role of FSH in the regulation of spermiogenesis. There are results of studies that indicate there is FSH stimulation of early spermatogenic stages including spermatogonial

proliferation and meiosis. The FSH concentrations and sperm counts correlated inversely in the primary and secondary infertility groups (Gowri et al., 2010), and these findings are consistent with other reports in the literature (Matzkin et al., 1990; Abbaticchio et al., 2009). Exogenous FSH administration in combination with or without human chorionic gonadotrophin in infertile men led to an improvement in sperm counts (Baccetti et al., 1997). With the increase of testosterone, there is a reduction in LH release due to the negative feedback of testosterone at the hypothalamus inhibiting the release of GnRH. The serum concentrations of FSH and LH are inversely associated with sperm concentration, motility, morphology (Meeker et al., 2006), and mean testicular size (Takihara et al., 1987). Another hormone having a potential role in spermiogenesis is prolactin. Testicular regression coincides with the annual increase in prolactin secretion (Smith et al., 1985). In the present study, the mean serum FSH, LH, and prolactin concentrations in purebred silver foxes and blue foxes were less than that in HBSs and HSBs, which suggested that FSH, LH, and prolactin concentrations are inversely associated with spermatogenesis.

5. Conclusions

In conclusion, results of the present study indicate that fox hybrid reproductive sterility may result from failures at the early stages of sperm development, and the hybrids are completely sterile.

Conflicts of interest

None.

Acknowledgments

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