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Effect of Testosterone, Dihydrotestosterone, Estradiol and Cortisol on the Quality of Fresh and Cryopreserved Stallion Sperm

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ABSTRACT

The effect of steroid hormones on the quality of fresh and cryopreserve sperm has not been fully understood. This study aimed to evaluate the effect of testosterone, dihydrotestosterone, estradiol, and cortisol on the quality of fresh and cryopreserved stallion sperm. The study was conducted on 40 *Equus caballus* stallions, including Arab (n=20), Oryol trotting (n=4), Standardbred (n=4), and Soviet Heavy Draft (n=12) breeds. The average age of the experimental animals was 9.9 ± 0.7 years. We determined standard quality indicators in fresh and cryopreserved sperm and the concentration of steroid hormones in the blood plasma of stallions. Results of the study suggested a negative correlation between the level of testosterone with total (r=-0.41; p<0.01) and progressive (r=-0.44; p<0.01) sperm motility in cryopreserved sperm as well as in fresh sperm (r=-0.38; p<0.05 and r=-0.39; p<0.05 correspondingly). While the level of estradiol showed a positive correlation with survival rate in cryopreserved (r=0.35; p<0.05) and in fresh (r=0.33; p<0.05) sperm. Further, the level of cortisol in the blood plasma of stallions did not show any statistically significant correlations with the qualitative characteristics of sperm. A positive relationship was found between the concentration of dihydrotestosterone with the volume of ejaculate (r=0.37; p<0.05) and the total number of sperm in the ejaculate (r=0.43; p<0.01). Results of the study can be concluded that steroid hormones have different effects on the quality indicators of fresh and cryopreserved sperm of stallions and their concentration in the blood should be considered when selecting stallions for cryopreservation of sperm.

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1 Introduction

The expansion of assisted reproductive technologies in the horse breeding industry has facilitated the use of frozen sperm to preserve valuable genetic material. Cryopreserved sperm has several advantages, including long-term storage and long-distance transportation, rational use of semen, prevention of infection, and preservation of sperm from endangered species (Atroshchenko et al. 2017). However, freezing followed by thawing negatively affects the fertility of cryopreserved sperm compared to fresh ones (Atroshchenko et al. 2019b). The proportion of spermatozoa that survive after freezing-thawing is affected by their susceptibility to cold shock, cooling rate, diluent composition, and osmotic stress, and the functional state of spermatozoa after cryopreservation depend on the action of oxidative stress, membrane stability, membrane receptor integrity, and nuclear structure state (Watson 2000; Elkina et al. 2011). Various biologically active compounds can also influence sperm quality and cryostability, among which steroid hormones play a special role (Atroshchenko et al. 2019c; Martínez-Fresneda et al. 2019; Coloma et al. 2010).

Testosterone is an important regulator of cellular processes occurring in the stallion testes (Roser 2008), it is involved in maintaining the normal functioning of the blood-testis barrier (Meng et al. 2005), inducing meiosis (Holdcraft and Braun 2004; Larose et al. 2020) and regulating apoptosis of germ cells (Jeremy et al. 2021). The mechanism of testosterone participation in the process of spermatogenesis is known by binding to the androgen receptor, which is expressed in various types of testicular cells, such as Sertoli cells (Walker 2011), and this is realized both through classical intracellular mechanisms and through non-classical membrane mechanisms (Cooke et al. 2022). The expression of the androgen receptor varies at different stages of a horse's life and in different glands. An increased expression is observed in the vesicular gland of the fetus compared to the stallion and a lower expression in the bulbourethral gland in comparison to other glands (Ellerbrock et al. 2021).

Studies in recent decades have established the importance of estrogens as a paracrine-autocrine factor in the regulation of seminal gland function and spermatogenesis in stallions (Roser and Hughes 1992). The synthesis of estrogens from androgens is carried out with the participation of the aromatase enzyme (Robertson et al. 1999). Aromatase expression in stallion changes with age, as demonstrated by immunocytochemistry (Hess and Roser 2004), and it is significantly lower in the bulbourethral gland than in the other accessory sex glands at all horse`s life stages (Ellerbrock et al. 2021). Estrogen receptors in stallion testes are found in Leydig, Sertoli, and germ cells (Bilinska et al. 2006; Pearl et al. 2011) and both ER-α and ER-β are present in the accessory sex glands of the horse (Ellerbrock et al. 2021). The low level of estrogens in the blood of subfertile stallions is associated with a low concentration of spermatozoa in semen (Roser and Hughes 1992). The state of the endocrine function of the testis in stallions can be assessed by the concentration of testosterone and the total level of estrogens in the blood (Inoue et al. 1993). There is evidence of a positive correlation between the concentration of estradiol in the blood plasma of stallions and sperm motility (Hoffmann and Landeck 1999), and it is also known that estradiol increases the membrane transport of monosaccharides in spermatozoa (Ballesteros et al. 1983).

Dihydrotestosterone is a product of testosterone reduction under the action of 5α-reductase. Like testosterone, dihydrotestosterone also binds to androgen receptors and triggers protein synthesis in the cell, but dissociates more slowly from the receptor (Wilson 2001). The effect of dihydrotestosterone on spermatogenesis is currently being discussed. Since the concentration of dihydrotestosterone in the testes of rats is 5 % of the concentration of testosterone (Turner et al. 1984), while in humans it is only 2 % (Jarow and Zirkin 2005), some researchers believe that dihydrotestosterone does not play a significant role in the process of spermatogenesis (Kang et al. 2014). How dihydrotestosterone affects the reproductive function of stallions remains unexplored.

Glucocorticoids are steroid hormones that are produced in response to stress. Some researchers believe that glucocorticoids can inhibit reproductive function (Wiest et al. 1988; Claus et al. 2005; Rengarajan and Balasubramanian 2007). Thus the concentration of cortisol in blood plasma is higher in Japanese black beef bulls during puberty with abnormal fresh sperm (Weerakoon et al. 2020). However, a positive correlation between cortisol and testosterone levels had been reported in the blood of stallions (Villani et al. 2006). Further, in vitro effects of cortisol on bull sperm have been characterized by increased sperm motility (Cheng et al. 1980). In this regard, the question of the effect of cortisol on the quality indicators of stallions' sperm remains open and given that at the moment there is no literature data on the effect of cortisol on the quality indicators of cryopreserved sperm. Therefore, this study aimed to evaluate the effect of testosterone, dihydrotestosterone, estradiol, and cortisol on the quality of fresh and cryopreserved stallion sperm.

2 Materials and Methods

2.1 Animals and Semen Collection

The study was performed on the livestock of the All-Russian Research Institute for Horse Breeding (ARRIH, Ryazan Region, Russia) and the Tersk Stud Farm N169 (Stavropol Region, Russia), Perevozsky and Pochinkovsky studs (Nizhny Novgorod region, Russia). All manipulations with animals were carried out according to the Law of the Russian Federation on Veterinary Medicine No. 4979-1 (14 May 1993) and the “European

Convention for the protection of vertebrates used for experimental and other scientific purposes" ETS No. 123 (18 March 1986). The protocol of the present investigation was approved by the Local Ethics Committee of the All-Russian Research Institute for Horse Breeding (ARRIH), Ryazan Oblast, Russia.

Studies were conducted on 40 breeding stallions, of which 20 stallions of the Arabian breed, 12 of the Soviet Heavy-Draft, 4 of the Standardbred, and 4 of the Oryol trotting breed. The average age of the experimental animals was 9.9 ± 0.7 years.

Stallions were kept in individual stalls. The stallions received hay, oats, and granulated compound feed with added minerals under established standards and were exercised for at least 1 h daily.

All stallions were used in natural mating. Sperm from stallions was obtained during the breeding season (February-May) with an interval of 48 hours for an artificial vagina (ARRIH model, Ryazan, Russia) for a mare in heat. After a long period of sexual rest (10 days or more), three ejaculates were taken from stallions at 48-hour intervals, the first two ejaculates were not used in studies, and the third ejaculate was taken for research since the sperm characteristics in the first two ejaculates after prolonged sexual rest may differ from the sperm indicators inherent in this stallion.

2.2 Sperm Examination

Immediately after receiving the sperm, the gel was removed, and the sperm was filtered using a sterile gauze filter. This was followed by the estimation of the volume of the ejaculate, concentration of spermatozoa in 1 ml sperm, total and progressive motility, total sperm number (TNS), and the number of sperm with progressive motility (TNS PM) in the ejaculate, as well as the survival of sperm under the regime of hypothermic storage of diluted semen at +4 °C (Atroshchenko et al. 2020**)**. The ejaculate volume (in ml) after filtration was determined using a measuring cylinder while the sperm concentration was measured using the SDM1 photometer (Minitube GmbH, Tiefenbach, Germany). The Argus CASA computer analysis system (ArgusSoft LTD, Saint Petersburg, Russia) was used to assess the progressive (PM) and total (TM) sperm motility.

2.2.1 Sperm survival test

After diluting the sperm with a lactose-chelate-citrate-yolk medium (LCCY) containing 3.5% glycerin to a sperm concentration of 45-50 million/ml in samples, the sperm was placed in a refrigerator at a temperature of +4 ° C. Sperm motility was assessed at 24-hour intervals. The survival rate of spermatozoa was defined as the time during which spermatozoa retain a PM of at least 5% under the hypothermic sperm storage regime.

2.3 Freezing and thawing of sperm

Sperm was frozen in liquid nitrogen vapors in aluminum tubes with a volume of 20 ml according to the standard methodology of the All-Russian Research Institute of Horse Breeding (Atroshchenko et al. 2019a; Naumenkov and Roman'kova 1971). Cryopreserved sperm were thawed in a water bath at a temperature of +40°C for 90 seconds. After thawing, the total and progressive motility and survival of sperm were determined using the above methods.

2.4 Blood Plasma Samples

A blood sample from the jugular vein was taken twice during the sperm collection period during the breeding season from each stallion. Vacuum tubes for taking venous blood "Vacuette"(5 ml, 13×100 mm) series "Premium" with a clot activator and gel (Greiner Bio-One GmbH, Austria) were used for this purpose. For this, blood samples were taken before morning feeding and centrifuged at 400 g for 20 min and plasma was stored at -20 °C until analysis was performed.

2.5 Determination of Hormones

Determination of testosterone, estradiol, and cortisol was performed using a corresponding commercial CLIA kit (DRG Instruments, Marburg, Germany) on a chemiluminescent analyzer Immulite 2000 (Siemens Healthcare Diagnostics Inc., USA), while dihydrotestosterone was measured using a commercial ELISA kit (DRG Instruments, Marburg, Germany) on a Multiskan ELISA analyzer ("Thermo Labsystems OU", Vantaa, Finland).

2.6 Statistical Analysis

Statistical analysis was performed using the program Statistica 10 and "Microsoft Office Excel 2016" (StatSoft Inc., USA). The nonparametric Mann – Whitney U-test and the Spearman coefficient (Rs) were used to evaluating the rank correlation in the study groups. The results were presented in the format Median (Quartiles). Differences were considered statistically significant at $p < 0.05$.

3 Results and Discussion

The results of measuring the quality of stallions' sperm before and after cryopreservation are shown in Table 1. The table shows a statistically significant decrease in the total and progressive motility after cryopreservation of sperm, as well as a decrease in sperm survival at +4 °C ($p < 0.01$). Cryopreservation with subsequent thawing of sperm has a negative impact on the integrity of sperm membranes: there are damages associated with temperature shock, cell defects associated with the extracellular and intracellular formation of ice crystals, oxidative and osmotic

stress (Khan et al. 2021). This also leads to an increase in the number of ultrastructural organelles damage affecting sperm motility and survival (Atroshchenko et al. 2019b; Aurich et al. 2020; Greiser et al. 2020).

Table 1 Qualitative characteristics of stallion sperm before and after cryopreservation

Indicator	Sperm	
	Fresh	Thawed
The volume of ejaculate, ml	36.65	-
Sperm concentration, million / ml	235.70	-
TNS, billion	8.27	-
TNS PM, billion	4.64	-
Total motility, %	69.50*	40.66
Progressive motility, %	60.80*	28.32
The survival rate at T +4 ° C, hour	127.70*	72.00

n=40; Median, * p< 0.01.

The results of measuring the concentrations of hormones in the blood plasma of stallions are shown in Table 2. It is known from the literature that the concentration of testosterone in healthy stallions normally varies from 0.04 to 1.02 ng/ml (Haffner et al. 2010; Seale 2010; Hind et al. 2021; Basiru et al. 2022), while the median estradiol concentration in healthy stallions normally varies from 13.40 - 66 pg/ml (Haffner et al. 2010; Basiru et al. 2022). The normal cortisol concentration in healthy stallions varies from 19.88 - 70 ng/ml (Tamanini et al. 1983; Villani et al. 2006; Haffner et al. 2010). Results of the measuring hormone concentrations in the current study are approximately in the specified range. Therefore, the studied stallions were within the physiologically acceptable limits for the studied indicators.

The analysis of the rank correlation between the concentration of steroid hormones in the blood plasma of the studied stallions (n = 40) and the quality of fresh and thawed sperm was carried out separately for each studied hormone (Table 3). A negative correlation was found between testosterone concentration with total motility, as well as with progressive motility in fresh and thawed sperm. Further, in the case of estradiol, a positive correlation was reported between the hormone concentration with sperm survival rate in fresh and thawed sperm. The study of dihydrotestosterone correlations also showed a direct correlation between the volume of ejaculate and the total number of sperm in the ejaculate while in the case of cortisol, statistically significant results were not obtained.

Table 2 Concentration of testosterone, dihydrotestosterone, estradiol and cortisol in stallion blood plasma.

Hormone	Concentration
Testosterone (ng/ml)	0.50
Dihydrotestosterone (pg/ml)	197
Estradiol (pg/ml)	42.22
Cortisol (ng/ml)	37.34

Median, (n=40).

A positive correlation was reported between the concentration of dihydrotestosterone and testosterone in the blood plasma of stallions (r = 0.37; p = 0.018), and these results are in agreement with the findings of Hoffmann and Landeck (1999) those who reported a similar relationship in the spermoplasm of stallions. This may be because testosterone is a precursor for the synthesis of dihydrotestosterone (Wilson 2001). A positive correlation was also obtained between the concentration of estradiol and dihydrotestosterone in blood plasma (r = 0.40; p = 0.011). The findings of the current study are contradictory to the findings of Hoffmann and Landeck (1999) who did not find any correlation in stallion seminal plasma however they have not investigated these correlations in the blood plasma. Whether dihydrotestosterone affects the synthesis of estradiol or vice versa, or whether some

Table 3 Spearman's correlation coefficient (R_S) between sperm quality indicators and the blood plasma concentration of steroid hormones in stallions

Hormone	Indicators of sperm quality	R_S	p value
Testosterone	Total motility (TS)	-0.41	0.009
	Progressive motility (TS)	-0.44	0.004
	Total motility (F)	-0.38	0.016
	Progressive motility (F)	-0.39	0.012
Dihydrotestosterone	Ejaculate volume	0.37	0.018
	TNS	0.43	0.006
Estradiol	The survival rate at T +4 ° C (TS)	0.35	0.028
	The survival rate at T +4 ° C (F)	0.33	0.040

Abbreviations: TS- thawed sperm; F-fresh sperm, TNS- total number of spermatozoa

third factor simultaneously acts on the synthesis of these hormones in stallions remains unknown.

Further, statistically nonsignificant correlations were reported between the age of the studied animals and the concentration of hormones in the blood plasma, but a negative correlation between the survival rate at T +4 ° C of fresh sperm and the age of stallions ($r = -0.32$; $p = 0.042$).

Seale (2010), found a positive correlation between the concentration of testosterone in the blood plasma of stallions and progressive motility in fresh sperm, while the results of the current study suggested a negative correlation between the total and progressive motility of fresh stallion sperm and the concentration of testosterone. Similarly, negative correlations were obtained between the concentration of testosterone in blood and sperm motility in cryopreserved sperm and these results contradict with the findings of previous research. Coloma et al. (2010) revealed that high levels of testosterone in the *Iberian ibex* blood plasma were associated with a decrease in progressive sperm motility and a decrease in acrosome and membrane integrity, and this relationship is characteristic only for the frozen-thawed sperm. Bóveda et al. (2021) also found a reduction in the concentration of testosterone in the blood plasma of *Iberian ibex* and it increased with the sperm cryostability duration increases and vice versa. Moreover, these changes most likely occur at the final stage of sperm maturation in cauda epididymis and secretion of the sperm and may be associated with remodeling of the protein and lipid components of the sperm membrane. Epididiosomes (Nixon et al. 2019) and extracellular vesicles (Leahy et al. 2020) may play a key role in the changes described above. In a series of recent experiments, it was demonstrated that *in vitro* incubation of small ruminants spermatozoa in a testosterone medium leads to a decrease in the acrosome integrity in thawed sperm. The authors of the study also suggested that testosterone negatively affects the resistance of sperm to cryopreservation and its destabilizing effect directly depends on the concentration *in vivo* (Martínez-Fresneda et al. 2019).

The molecular mechanisms of testosterone's influence on the quality characteristics of sperm have long been discussed. Shivaji and Jagannadham (1992) attempted to evaluate the direct effect of testosterone on fluidity, aggregation, fusion, and leakage of human and hamster spermatozoa membranes and reported a very little effect of the testosterone on the selected indicators. Purohit et al. (2000) have observed that supraphysiological doses of testosterone administered to mice can cause oligospermia and alter the liquid-crystalline configuration of sperm membranes (Purohit et al. 2000).

Similarly, Calzada et al. (1988) found that the incubation of human sperm in testosterone changes the configuration of the plasma membrane and induces the depolarization of the membrane. Testosterone has also been shown to increase the molecular transport of carbohydrates across the sperm membrane in the *in vitro* study of Ballesteros et al. (1983). According to Cai et al. (1994), dihydrotestosterone is synthesized under the action of 5α-reductase. Analysis of the men's sperm with homozygous mutation of 5α-reductase showed that dihydrotestosterone regulates the volume and viscosity of sperm, but does not affect the total number of viable spermatozoa, however, the authors of the study do not exclude that a small concentration of dihydrotestosterone is still necessary for normal spermatogenesis. In addition, there was a decrease in the total number of viable spermatozoa, sperm concentration, and volume in healthy men with reduced levels of dihydrotestosterone due to the use of finasteride, a 5A-reductase-2 inhibitor, and these indicators were restored to normal levels when the drug was discontinued (Amory et al. 2007). This is showing positive correlations between the concentration of dihydrotestosterone with the volume of sperm ejaculates and the total number of sperm in ejaculates.

In the analysis of the correlation between blood plasma estradiol and stallion sperm quality indicators, we obtained a positive correlation between the concentration of the hormone with the survival rate of sperm in fresh and thawed sperm. Previous studies have found a positive effect of estradiol on sperm motility. Mesbah et al. (2022) found that adding 3 µM estradiol concentrations to the goat semen extender increased survival rate, sperm integrity, and progressive motility through cryopreservation, and this process seems to be calcium-dependent. Hoffmann and Landeck (1999) reported a positive correlation between the concentration of estradiol in the blood plasma of stallions and sperm motility. In addition, the role of aromatase/estrogens in the acquisition of sperm motility in men was also established (Lambard et al. 2004; Carreau et al. 2009). Reduced sperm count and sperm motility have been described in men with genetic aromatase deficiency (Rochira et al. 2005) and in knockout mice (O'Donnell et al. 2001). Previously, it was shown that estradiol enhances the molecular transport of glucose and fructose across the sperm membrane (Ballesteros et al. 1983) and since these monosaccharides are the main sources of energy for sperm (Varner et al. 2016), they can be used to provide energy for movement. This mechanism likely underlies the increase in sperm motility and, possibly, survival rate under the influence of estradiol.

The effect of cortisol on reproductive function in many studies is considered through its effect on testosterone synthesis. It was found that in some species, glucocorticoids inhibit reproductive function by inhibiting the expression of proteins involved in testosterone biosynthesis (Claus et al. 2005; Rengarajan and Balasubramanian 2007). In the case of stallions, it has also been suggested that cortisol affects the testes by inhibiting the production of testosterone (Wiest et al. 1988). In other studies, there was a positive correlation between cortisol and testosterone levels in the blood of stallions (Villani et al. 2006). The findings of

Bishop et al. (1999) and Borg et al. (1991) are contradictory to the findings of previous researchers and suggested that cortisol does not affect the synthesis of testosterone in bulls and boars. Also, Seale (2010), found no statistically significant correlations between cortisol levels and the concentration of testosterone in the blood plasma of stallions, as well as indicators of the quality of fresh sperm. Deichsel et al. (2015) showed that an increase in cortisol concentration in stallions in response to physical activity and the introduction of adrenocorticotropic hormone does not affect the quality of sperm, which indicates good protection of stallions' testes from the effects of glucocorticoids (Deichsel et al. 2015). In mature stallions, this is probably achieved by oxidizing active cortisol to inactive cortisone using the enzyme 11β-hydroxysteroid dehydrogenase (Draper and Stewart 2005). This may explain the results of this study, in which a statistically significant correlation was not reported between the concentration of cortisol and the quality of fresh and cryopreserved sperm.

Conclusions

Results of the current study suggested that the level of testosterone in the blood plasma of stallions negatively correlates with total and progressive sperm motility in fresh and cryopreserved sperm. We also obtained a positive correlation between estradiol concentration with the survival rate in fresh and cryopreserved sperm. It is known that these hormones can affect spermatozoa at the molecular level; the mechanism of their effect on sperm cryostability requires further study. Results of this study also suggested that dihydrotestosterone affects the fertility of stallions, since a positive relationship was found between the concentration of the hormone with the volume of ejaculate and the total number of spermatozoa. We did not get statistically significant results for cortisol which may be due to the good protection of the stallion testes from the action of this glucocorticoid. Results of the study can be concluded that the studied steroid hormones have different effects on the quality of stallion sperm and their concentration in the blood should be taken into account when selecting animals for cryopreservation of sperm.

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Conflicts of interest and financial disclosures

The authors declare no conflict of interest.

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