In Vitro Effects of Cholecalciferol on Characteristics of Stored Semen of Broiler Breeder Roosters at Two Temperatures and Three Times

Homeyra Abdollahi Forg

Abstract

The aim of this study was to investigate the effects of adding different levels of cholecalciferol to stored semen of arboracres breed roosters. Fifteen breed roosters were bought from the Fars poultry complex and were habituated for ejaculation by abdominal massage for two weeks. Semen samples were collected from several roosters and stored for 12 h and then various doses of cholecalciferol were added to the diluted semen sample. Negative control treatment (no cholecalciferol and ethanol), positive control treatment (no cholecalciferol with ethanol) and 10, 20, 40 and 80 ng / mL solubilized ethanol diluted in semen dilution of the experimental treatments. The samples were diluted with Sexton at a ratio of 1 to 6. Each treatment had three replications. The samples were evaluated after 6, 12 and 24 h storage at 4 to 5 and 19 to 24 ° C. For each treatment, semen characteristics including motility, percentage of live sperm, percentage of abnormal sperm, percentage of membrane integrity and TBARS concentration were evaluated. The effect of cholecalciferol on percentage of motility sperm and live sperm was significant (P <0.05); The addition of 20 ng of cholecalciferol to the semen diluent improved sperm quality and the best storage temperature was 19-24 ° C for 6 hours. Overall, cholecalciferol had remarkable effects on some of semen charactristics in in vitro condition. But confirmation of these findings and their applicability to improved sperm storage and artificial insemination, in particular fertility improvement, requires further investigations.

Keywords: Sperm, Motility, Vitamin, Artificial Insemination

Introduction

The domestic poultry breeding industry relies on high reproductive efficiency to reproduce the best genetic strains worldwide, so high reproductive potential is one of the important foundations of the domestic poultry breeding industry (Etches, 2006). Recent advances in semen processing have led to the development of reliable methods of semen storage for 24 hours without decreasing fertility that can be used in the artificial insemination industry (Etches, 2006). For several years, the possibility of increasing reproductive productivity in different species and strains of birds has been investigated (Donoghue & Wishart, 2000; Blanco et al., 2000; Blesbois et al., 2005). Avian sperm show diverse physiological differences and metabolic requierments with mammals that diluting with the appropriate chemical composition, pH, and osmotic pressure

can provide conditions for maintaining greater production potential during in vitro accumulation (Lake, 1981). Semen diluents are essential due to increase in the number of inseminated birds per unit semen volume and enable sperm viability in long-term and short-term storage conditions (Mohan et al., 2015). Semen diluents are usually made based on the biochemical composition of semen (Lake, 1995) and isotonic because the osmotic pressure created by the solution may be inappropriate for the sperm cell (Senger, 2003). For the storage of sperm and semen, the diluent type and storage temperature are crucial (Getachew, 2016). Artificial insemination has been considered a valuable method in the poultry industry that has benefits as compared to natural mating including better male performance, lower costs (Benoff et al., 1981), and disease control (Blanco & Höfle, 2004). Success in artificial insemination depends on many structures such as the diluent chemical composition and the semen accumulation method (Lukaszewicz, 1988). One of the causes of different insemination methods is the change in sperm metabolism; in the way that sperm accumulate, sperm are exposed to oxygen, which can affect the lipid peroxidation in the seminal plasma membrane and free radical production (Wishart, 1984). In vitro semen accumulation also influences sperm motility, viability, and morphology.

Therefore, sperm evaluation after its accumulation is an important construct for evaluating artificial insemination (Lukaszewicz, 1988; Douard et al., 2004; Blesbois et al., 2005). Vitamin D3, or cholecalciferol, is a steroid hormone that affects sperm through the genomic and non-genomic pathways and increases sperm motility (Haussler et al., 2011). The vitamin D receptor is a steroid receptor found in most tissues and organs. This vitamin helps to strengthen bones and cell growth by controlling calcium and phosphorus balance by increasing the absorption of phosphorus and calcium from the intestines and reducing their excretion from the kidneys. It is one of the essential vitamins for the body and soluble in fat (Blesbois et al., 2005) and also acts as a multipurpose messenger molecule in the male reproductive system (Bouillon et al., 2008).The most important source of this vitamin is sunlight (Holick & Chen, 2008). Research in male mice has shown that vitamin D deficiency affects spermatogenesis (Sood et al., 1995). Also in the male body, vitamin D concentration was positively correlated with serum concentrations of androgen hormones (Wehr et al., 2010).

In the male reproductive system, the vitamin D receptor and its fuel-producing enzymes (Blomberg Jensen et al., 2010) are expressed in Sertoli cells, sex cells, Leydig cells, spermatozoa, and reproductive tract epithelial cells that are expressed in species such as humans, mice and rooster there are slight differences (Blomberg Jensen et al., 2012). Goodarzi et al. (2019) showed that adding 96 mg/ml of calcitriol to the diluent improves sperm quality and calcitriol also has significant effects on partridge sperm parameters in vitro (Goodarzi et al., 2019). Little is known about the dilution properties and the effect of temperature and storage time on rooster semen accumulated and diluted. Therefore, in the present study, by adding different concentrations of cholecalciferol to the diluent Sexton and Semen and its effect on the semen accumulated characteristics of the arborecores strain at two temperatures (4 to 5 ° C and 19 to 24 ° C) and three times 6, 12 and 24 hours.

Materials and Methods

This study was conducted at the Research Station of Animal Science department, School of Agriculture, Shiraz University (Shiraz, Iran). Fifteen poultry breeding rooster were prepared from Fars poultry complex at the age of 32 weeks. At the beginning, the roosters were habituated for

ejaculation for two to three weeks (Burrows & Quinn, 1937). After each ejaculation, all samples were diluted with Sexton's diluent (Sexton & Fewlass, 1978) at a ratio of one to six based on previous concentration determination. Each specimen was then assigned to one of two storage temperatures of 4 or 25 ° C and evaluated at 6, 12 and 24 h intervals. After 12 h of sampling, ethanol-soluble cholecalciferol (Bello et al., 2013) was added to the experimental treatments and semen characteristics 6, 12, and 24 h after refrigerated storage (4 to 5 ° C) and room temperature. (19 to 24 ° C) were evaluated. The experiment was repeated three times. Semen samples were centrifuged to obtain basal vitamin D concentration and the cholecalciferol concentration was measured by ELISA (Engvall & Perlmann, 1971). As the seminal volume and timing considerations were limiting factors, the evaluations were conducted at 3 individual days. The percentages of motile sperm, live sperm (eosin-nigrosin staining), abnormal sperm, incidence of hypoosmotic swelling (**HOS)**, and thiobarbituric acid reactive species (**TBARS**) concentrations were determined (Akhlaghi et al., 2014a). A number of 12 replicate samples were evaluated within each calcitriol concentration-storage time-storage temperature combination treatment. Sperm forward motility was determined by placing 10 μL of diluted sample on a prewarmed (37◦C) slide with a cover slip using a Zeiss (Jena, Germany) light microscope (×400 magnification). Sperm viability and abnormality were evaluated using diluted samples stained with an eosin-nigrosin mixture. Unstained spermatozoa were recorded as live. Detached heads, bent tails, coiled tails, and double tails were recorded as abnormal spermatozoa (Akhlaghi et al., 2014b). Sperm membrane integrity was determined by the HOS test. A portion of diluted semen (10 μL) and 40 μL of hypoosmotic solution (100-mOsm sodium citrate solution) were incubated at 37◦C for 15 min, a droplet was placed under the cover slip, and at least 200 sperm were evaluated using a light microscope (×1,000 magnification). Sperm cells with swollen and curled flagella were considered normal (Akhlaghi et al., 2014b). Lipid peroxidation was quantified as TBARS concentration (nM/mL) in the seminal plasma, using thiobarbituric acid (**TBA**) and trichloro-acetic acid (**TCA**) substances. Briefly, a sample of seminal plasma (500 μL) was mixed with 1 mL TBA-TCA solution [7.5 g TCA+ 0.187 g TBA+ 1250 mL 25% HCl in 50 mL distilled water (v/v)], and placed for 15 min in a water bath (100◦C). After cooling and centrifugation for 20 min at 604 × g, the supernatant was separated and the absorbance was read at 535 nm by UV spectrophotometry (Unico 1200, China; Saemi et al., 2012).

Table 1. The effects of Cholecalciferol in diluent, storage time, and storage temperature on the in vitro seminal characteristics of Broiler Breeder Roosters

	Cholecalciferol concentration in diluent1						*P*-value						
trial	VD_{0+}	VD_{0-}	VD_{10}	VD_{20}	VD_{40}	VD_{80}	cholecalciferol	Time	Temp	Cholecalciferol × Time	Cholecalciferol × Temp	Time × Temp	Cholecalciferol × Temp× Time
Motility (%)	51.9[b]	49.7[b]	49.7[b]	63.1[a]	57.0[ab]	50.2[b]	0.0109	<0.0001	<0.0001	0/0422	<0.0001	<0.0001	0.0005
Viability (%)	57.9[c]	66.7[bc]	76.1[ab]	75.1[ab]	79.5[a]	78.2[a]	0.0145	0.0378	<0.0001	NS	NS	0.0265	NS
Abnormality (%)	7.0	6.1	7.2	6.3	7.3	7.7	NS	NS	<0.0001	NS	NS	NS	NS
HOS[2] (%)	17.6	16.3	16.9	19.2	15.9	15.9	NS	NS	0.0002	NS	NS	NS	NS
TBARS[3] (nM/mL)	3.0	3.1	3.4	2.8	2.8	2.6	NS	<0.0001	0.0014	NS	NS	NS	NS

[a–c]Within rows, least squares means with different superscript(s) differ significantly ($P < 0.05$).

VD_{0+}: diluted semen with ethanol without adding cholecalciferol to the diluent, VD_{0-}: diluted semen without cholecalciferol, VD_{10}-VD_{80}: Diluted semen samples containing 10, 20, 40 and 80 ng /mL cholecalciferol in diluent, respectively.

[2]Incidence of hypoosmotic swelling.

[3]TBARS: Thiobarbituric acid reactive substances.

This study was conducted based on factorial experiment in completely randomized design. Cholecalciferol was measured at six levels, temperature at two levels and time at three levels. Data were analyzed with GLM procedure (SAS, 2013). effects of Cholecalciferol concentration, time, temperature, and their interactions. The following statistical model was used:

$$y_{i1k} = \mu + \alpha_i + \beta_j + \delta_k + \alpha\beta_{ij} + \alpha\delta_{ik} + \beta\delta_{ik} + \alpha\beta\delta_{ijk} + e_{jjk}$$

where Y_{ijk} = Observations of the forested species studied, μ = mean, α_i = fixed effect of treatment i, βj = fixed effect of time j, δ_k = fixed effect of temperature k, $\alpha\beta_{ij}$ = fixed interaction effect of treatment i with time j, $\alpha\delta_{ik}$ = fixed interaction effect of treatment i with temperature k, $\beta\delta_{ik}$ = fixed interaction effect of time j with temperature k, $\alpha\beta\delta_{ijk}$ = fixed interaction effect of treatment i with time j, and temperature k, e_{jjk} = random error. Differences among means were examined using the least significant difference ($P < 0.05$).

RESULTS

The aim of this study was to investigate the effects of adding different levels of cholecalciferol (Vitamin D3) to stored semen in breeding rooster in order to increase targeted sperm storage and to increase semen storage time. The results showed that the effect of adding cholecalciferol to semen thinner on reproductive semen was significant on the motility and percentage of live sperms, so that the sperm motility of the experimental groups was significantly higher than the control group. At 24 ° C at 6 h, the sample had 20 ng/ml of cholecalciferol (T2) and also at 24 h of treatment had 40 ng/ml of cholecalciferol (T3), which was consistent with the results of Goodarzi et al. (2019). They showed that addition of vitamin D to the diluent improves the motility of partridge sperm (Goodarzi et al., 2019). The vitamin D receptor is expressed in different parts of the

sperm (Corbett et al., 2006) and when vitamin D binds to the receptor on the sperm it activates phospholipase C and produces inositol triphosphate which in turn opens the calcium channels and increased intracellular concentration of calcium (Blomberg Jensen et al., 2012), which al., 2012) and vitamin D deficiency in mice, rats and pigs reduced sperm motility, increased sperm abnormalities and decreased fertility rates (Sood et al., 1995; Kwiecinski et al., 1989; Kinuta et al., 2000; Audet et al., 2004; Hamden et al., 2008). Research has shown that vitamin D administration improved sperm motility in fertile, low-fertile men (Blomberg Jensen et al., 2011; 2012) and adding vitamin D to sperm increased 5–10-fold basal calcium concentrations in the sperm. (Uhland et al., 1992). Also, mice lacking the vitamin D receptor in sperm have been reported to have decreased sperm count and motility and ultimately reduced fertility (Kinuta et al., 2000).

Cholecalciferol has genomic and non-genomic effects. Non-genomic effects of cholecalciferol in the short-term and long-term non-genomic effects of these findings may be a result of either or one of these effects (Haussler et al., 2011; Blomberg

led to an increase in motility of capacitated of human spermatozoa (Aquila et al., 2008; Mendeluk & Rosales, 2016). In vitro vitamin D improved sperm bindind to oocyte and initiated acrosomal reaction (Blomberg Jensen et

Jensen & Dissing, 2012). Semen plasma vitamin D may be involved in sperm motility regulation and 1 and 25-dihydroxycholecalciferol by increasing the synthesis of cAMP (Costa et al., 2011) and protein kinase A (Jueraitetibaike et al., 2019) and subsequently phosphorylation. Protein becomes axonal and flexes and flexes (Esposito et al., 2004). Between calcium, the production of oxygen radicals resulting from irreversible aerobic activity and ATP production is also closely related (Brookes et al., 2004). Calcium stimulates ATP production by affecting mitochondria (Brookes et al., 2004). When intracellular calcium concentrations are high, sperm motility is inhibited because mitochondrial respiratory motility is much more sensitive than intracellular calcium relative to glycolysis (Breitbart & Nass-Arden, 1995).

Table 2. The interaction effect of calcitriol concentration in diluent, storage time, and storage temperature on the sperm motility (%) Broiler Breeder Roosters sperm

	Storage temperature (˚C)					
	4 to 5			19 to 24		
Storage time (h)	6	12	24	6	12	24
Cholecalciferol concentration (μg/mL)1						
T_0^+	$^{A}88.3$	$^{AB}91.6$	$^{A}93.3$	$^{C}13.3^{b}$	$^{BC}31.6^{a}$	6.6^{b}
T_0^-	$^{AB}85.0$	$^{A}95.0$	$^{AB}91.6$	$^{C}11.6^{b}$	$^{C}13.3$	13.3
T_1	$^{AB}81.6$	$^{AB}81.6$	$^{AB}80.0$	$^{C}35.0^{a}$	$^{AB}45.0^{a}$	10.0^{b}
T_2	$^{ABC}76.6$	$^{AB}88.3$	$^{AB}90.0$	$^{A}67.5^{a}$	$^{AB}50.0^{a}$	6.6^{b}
T_3	$^{C}62.5$	$^{AB}80.0$	$^{AB}86.6^{a}$	$^{B}37.0^{a}$	$^{A}65.0^{a}$	13.3^{c}
T_4	$^{BC}66.6^{b}$	$^{B}73.7^{a}$	$^{B}73.3$	$^{B}37.5^{a}$	$^{AB}42.5^{a}$	7.5^{b}
SEM	6.98	6.98	6.98	6.98	6.98	6.98

The effect of cholecalciferol on the percentage of viable spermatozoa was significant ($P < 0.05$). This result is also in line with the results of Goodarzi et al. (2019) who showed that the effect of vitamin D supplementation on semen dilution improved the percentage of live sperm (Goodarzi et al., 2019). Control (T_0^+) treatment with 40 and 80 ng /mL dilutions had a significant difference in the percentage of live spermatozoa. Sperm viability was higher in 80 ng/ml Cholecalciferol at 12 ° C and 12 h and 40 ng/ml Cholecalciferol at 6h. Researchers stated that vitamin D_3 had an adverse effect on human sperm viability and function (Blomberg Jensen et al., 2010) and vitamin D deficiency on spermatogenesis in male mice (Sood et al., 1995) and caused abnormalities in human spermatozoa (Blomberg Jensen 2012). Low fertility rates in animals (such as mice, rats, and pigs) appear to be due to the negative effect of vitamin D deficiency on sperm motility and sometimes sperm morphological abnormalities and reduced fertility rates (Kwecinski et al., 1989; Sood et al., 1995; Kinuta et al., 2000; Audet et al., 2004; Hamden et al., 2008). Vitamin D supplementation improved the quality of human sperm and was able to increase testosterone concentration (Bosdou et al., 2019). Increased semen temperatures have been reported to increase sperm metabolism and decrease sperm viability, and it may be argued that cholecalciferol levels may delay sperm aging by antioxidant and metabolic rate effects. Overall, it can be concluded that the addition of vitamin D to partridge semen diluent (Goodarzi et al., 2019) was significant in arboracres producing sows and improved the viability and viability of spermatozoa. No significant differences were observed in the percentages of abnormal sperm, HOST and TBARS between treatments. However, Gooderzi et al. (2019) found an in vitro study of partridge semen on abnormal sperm percentage and TBARS (Goodarzi et al., 2019). The sperm plasma membrane has a large amount of long chain fatty acids with multiple double bonds (PUFAs) that make the cell susceptible to peroxidative damage, thereby destroying the integrity of the sperm membrane. Therefore, the use of antioxidant supplements improved sperm membrane integrity (Aurich et al., 1997). Vitamin D was expected to improve sperm membrane integrity due to its antioxidant properties, but the addition of vitamin D in the present study had no effect on HOST. Our results were similar to those of Goodarzi et al. (2019) in which their study did not show a significant effect of vitamin D supplementation on HOST semen of partridge (Goodarzi et al., 2019). Perhaps the concentration of vitamin D used in these two studies may not have been appropriate to improve HOST. It seems that sperm accumulation has a negative effect on the percentage of abnormal sperm and TBARS concentration in birds, and these results need further investigation. In this study, semen TBARS concentration decreased at 12 h and increased at 24 h. Overall, treatments with cholecalciferol had lower TBARS concentrations. (1997) showed that administration of vitamin D to vitamin D deficient rats reduced the level of lipid peroxidation in rat serum and liver, which confirms our findings (Kuzmenko et al., 1997). Goodarzi et al. (2019) reported that the effect of vitamin D supplementation on semen of partridge prevented lipid peroxidation and decreased TBARS concentration. They reported that both 96 and 192 µg of calcitriol significantly reduced the concentration of seminal TBARS (Goodarzi et al., 2019). In the present study, different concentrations of vitamin D had no effect on the reduction of semen TBARS concentration, which is likely to affect the concentration or species. The researchers reported that sperm exposure to extracellular reactive oxygen species (ROS) increased cAMP within the cell and this effect was inhibited by superoxidase dismutase (SOD)

(Zhang & Zheng, 1996). Sperm by the NADPH oxidase system at the plasma membrane surface of the sperm and NADPH-dependent redox-oxidation system in the mitochondria can produce ROS; in fact, mitochondria are the major source of ROS production in sperm (Saleh & Hcld, 2002; Fanaei et al., 2011). Calcium also reduces ROS production under natural conditions of mitochondrial complexes and only when calcium increases ROS production when these complexes are inhibited (Brookes et al., 2004). Since cholecalciferol is likely to alter cellular activity by increasing intracellular calcium concentration, it is also likely that cholecalciferol may have antioxidant properties by mediating calcium ion.

DISCUSSION

In this study, addition of cholecalciferol in vitro improved the kinetic parameters and the percentage of live semen accumulated in spermatozoa at two temperatures and three times. the samples appear to have a motility increase at 4 ° C at all times and 24 ° C at 12 h, but at 24 ° C and 24 ° C, the kinetic decreases, and the level of 20 ng of cholecalciferol in diluent showed the highest percentage of motilty. Therefore, if the goal is to maintain semen for 6 hours and simultaneously add chelcalciferol to the diluent, the findings suggest a level of 20 ng/mL chelcalciferol in the diluent. Also, at high concentrations of 40 and 80 ng of cholecalciferol was associated with an increase in viable spermatozoa, and despite the antioxidant properties of cholecalciferol, semen TBARS concentration was not affected by its addition. Due to the lack of in vitro research, especially in accumulated conditions, and the effects of vitamin D on in vitro semen conditions, further experiments are needed to improve inoculation conditions, improve fertility and confirm these findings, as well as their applicability.