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Intramuscular progesterone injection decreases sperm functionality and fertility in broiler breeder roosters

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Abstract Chicken seminal plasma contains a high content of sex steroids including progesterone (P4). According to a published study, in vitro supplementation of P4 to the semen resulted in decreased fertility in the White Leghorn chicken; however, it is not known if increased P4 production in vivo is associated with decreased sperm fertility. Therefore, the aim of the present experiment was to study the effect of P4 injection on semen characteristics and fertility in broiler breeder chickens. Eighteen roosters and 55 laying hens of the Cobb 500 strain at 47 weeks of age were used in this experiment. Initially, semen was collected twice a week for 3 weeks and semen characteristics including the semen volume, sperm concentration, sperm motility, percentages of live and abnormal sperm, and sperm membrane integrity (using the hypo-osmotic swelling test; HOST) were determined. Then semen from each rooster inseminated to 3 hens and we evaluated sperm penetration, egg fertility and fertility duration. Subsequently, the roosters were injected with P4 (150 mg of the long-acting medroxy- progesteroneacetate) in the pectoral muscle and the above-mentioned measurements repeated. Progesterone injection decreased the sperm motility by 10%, membrane functionality (integrity) by 40%, egg fertility rate by 24% and number of days to first non-fertile egg by 3 days. However, sperm penetration of the perivitelline membrane increased about 60% ($P<0.05$). More experiments are warranted for a clearer understanding of the role of progesterone in male chickens.

Keywords: fertility, progesterone, rooster, sperm

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Introduction

Hormones have essential role in sperm production, performance and secondary traits in roosters (Etches, 1996). Sexton (1974) showed that chicken sperm metabolism is influenced by various steroid hormones including testosterone (T), estradiol (E_2) and progesterone (P_4). Testosterone is of special interest because it exists in measurable and variable quantities in the seminal plasma (Anderson and Navara, 2011). Circulating androgen concentrations have been correlated with fertility in poultry species (Biswas *et al*., 2007). Cecil and Bakst (1988) demonstrated that there was no correlation between blood or seminal plasma testosterone concentration and sperm quality in turkeys; however in Leghorn cockerels, seminal testosterone concentration was correlated with the ejaculate volume and sperm concentration (Zeman *et al*., 1983). Progesterone and estradiol exist in measurable quantities in the male chicken and influence the reproductive system (Weil *et al*., 1999). Progesterone is part of the steroid biosynthetic pathways and small concentrations circulate in blood (Nieschlag *et al*., 2003). Progesterone and its receptors have important role in male physiology and behavior (Wagner, 2006) and P_4 receptors are detected in chicken embryonal testes (González-Morán *et al.,* 2008).

In White Leghorns, progesterone was the most predominant steroid quantified in seminal plasma, with a concentration ranging between 1.85 to 4.89 ng/mL. Testosterone and dihydrotestosterone (DHT) were present at lower concentrations and ranged between 0.01 to3.71 ng/mL and 0.43 to 1.13 ng/mL, respectively (Anderson and Navara, 2011). Progesterone concentration in blood plasma was positively correlated with semen P4 concentration. Neither blood plasma T nor DHT were correlated with those of seminal plasma. In addition, P_4 concentrations were significantly higher in seminal plasma than in blood plasma (Anderson and Navara, 2011), which contrasts with the findings inmammals, such as humans, in which seminal concentrations of T, DHT, androstenedione, E_2, P_4, and cortisol were significantly lower than their corresponding blood levels (Pohanka *et*

al., 2002).

Anderson and Navara (2011) reported that i*n vitro* progesterone treatment significantly decreased the ability of spermatozoa to penetrate the perivitelline layer (PVL). However, it is not known if *in vivo* increases in concentration of seminal plasma P_4 would impair sperm functionality. Therefore, the aim of the present experiment was to study the effect of intramuscular P_4 injection on semen characteristics and sperm fertilizing ability, judged by PVL penetration, in broiler breeder roosters and the duration of fertility after artificial insemination.

Materials and methods

Eighteen broiler breeder roosters and 55 laying hens of the Cobb 500 strain at 47 weeks of age (obtained from Fars Poultry Breeder Complex) were housed in individual cages in an environmentally-controlled room (16L:8D photoperiod). Commercially prepared diets for broiler breeder roosters and hens were fed (150-160 g per day per rooster and 130-140 g per day per hen, respectively) and water was freely available during the experiment. Seminal samples were collected via abdominal massage after an adaptation period, and sperm characteristics and fertility were determined for each rooster before and after progesterone treatment.

Semen evaluation before progesterone treatment

Before P4 treatment, semen was collected twice a week for 3 weeks and semen characteristics were determined. Seminal volume was measured in graduated collecting tubes. Sperm forward motility was assessed by placing a portion of ejaculate diluted with 2.9% sodium citrate solution (1:100) on a slide, using a Zeiss (Jena, Germany)compound light microscope (400 × magnification), equipped with a warm stage (37°C). Sperm viability and abnormality were determined, using a portion of ejaculate stained with eosin- nigrosin solution, by observing100 spermatozoa per slide. Spermatozoa with detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, and protoplasmic droplets were considered as abnormal (Pursel *et al.,* 1972). Sperm concentration was determined using a Neubauer hemocytometer.

The hypo-osmotic swelling test (HOST) was used to evaluate sperm plasma membrane integrity. Briefly, a microtube, containing a portion of semen (10µL) and 70 µL NaCl solution (100 mOsm), was placed in a water bath (39°C) for 10 min. A small sample was then transferred on a microscope slide and the HOST-reacted spermatozoa were counted using light microscopy (1,000 × magnification); the percentage of spermatozoa with a swollen "bubble" around the curled flagellum was determined by counting 100 cells per slide (Fonseca *et al.,* 2005).

Artificial insemination

Semen from each rooster was diluted (1:1) with the Sexton's diluents (Etches, 1996) and inseminated into three hens on two consecutive days. Eggs were collected for two days after the second insemination for evaluation of sperm penetration assay of the perivitelline layer. Eggs collected from days 3 to 7 after the second insemination were used for fertility determination. Artificial insemination was repeated on the 8th day, and the eggs were collected again on days 9 and 10 for determination of sperm penetration, and from days 9 to 15 for fertility evaluation, and until the laid egg was non-fertile egg.

Perivitelline layer (PVL) sperm penetration assay

Determination of PVL sperm penetration assay was carried after modification of the procedure described by Bramwell *et al.* (1995). The egg was opened and the albumen was separated from the yolk which was then placed in a glass container with the germinal disc positioned on the top. The excess albumen was removed from the PVL by tissue paper. The germinal disc (blastodermic area) was removed with scissors and immediately rinsed in 1%NaCl solution to remove excess yolk from the membrane. The PVL was then placed on a microscope slide followed by the addition of some egg albumin, fixed by adding3-4 drops of 10% formalin for 1 hour, and rinsed with 1% NaCl solution. The fixed PVL was ten replaced with periodic acid-Schiff and allowed to dry at room temperature. The inner PVL holes were counted using a light microscope (OSK856861, Japan) at 400 ×. The blastodermic area was located on each slide and centered in the field of vision (area = 40 mm^2) and the number of holes within this area counted.

Semen and fertility evaluation after progesterone treatment

Each rooster was intramusculary injected with 150 mg medroxy-progesterone acetate (MPA), a long acting progesterone (Depo-provera, Provedic injectable suspension, 150 mg/ml, Caspian Medical Suppliers, Iran) in the pectoral muscle. One week after injection, semen collection and evaluation, and fertility determination were carried out as described for the stage before P_4 treatment.

Statistical analysis

Data on sperm characteristics and fertility duration were analyzed using the Proc Mixed (SAS, 2010). Data on sperm penetration of the perivitelline layer and egg fertility rate were analyzed by the paired t-test and Proc Genmod, respectively. The level of significance was set at $P \leq 0.05$.

Results

Progesterone treatment had a detrimental effect on sperm motility, membrane integrity (percent HOST-reacted sperm), sperm penetration of PVL, fertility rate and duration of fertility in broiler breeder roosters (Table 1). Progesterone treatment did not affect seminal volume and sperm concentration, viability and morphology (Table 1).

A significant time by treatment interaction was recorded for sperm concentration (P = 0.0005; Table 2), sperm motility (P = 0.0107; Table 3) and percent HOST-reacted spermatozoa (P = 0.0052; Table 4).In the birds injected with progesterone, sperm concentration decreased ($P < 0.05$) at weeks two (690×10^6) and three (1058×10^6) compared with the first (1757×10^6) week (Table 2). The percentages of motile spermatozoa in the semen at weeks 1 and 2 post-injection were about 15 and 24 percentage points lower than the comparable weeks before progesterone injection (Table 3). The percentage of spermatozoa with intact plasma membrane (HOST-reacted) decreased by about 35 percentage points during weeks 1 to 3 post-injection compared with the values recorded for weeks 1 to 3 before injection (Table 4).

Discussion

In line with in vitro progesterone treatment of semen (Anderson and Navara, 2011), intramuscular P4 injection decreased sperm membrane integrity, motility and fertility in the present experiment.

There was about 40% increase in sperm concentration one week after P4 injection compared with the corresponding pre-injection week. This decreased by about 40% at two weeks post-injection that was numerically lower than the value obtained at week two pre-injection. The reason for such variation in sperm concentration is not known; however, considering the non-significant effect of treatment (Table 1) and the interaction effect of treatment and time on sperm concentration (Table 2), and the duration of spermatogenesis in roosters it is likely that duration of treatment might not have been long enough for P4 to affect spermatogenesis the lasts 13 to 15 days in roosters (Etches, 1996). However, there are reports in the literature indicating the deleterious effects of progesterone on male reproduction in other species. Mice lacking P4 receptors had larger testes with higher numbers of Leydig and Sertoli cells, and produced more sperm (Lue *et al*., 2013). Injection of medroxy-progesterone acetate (MPA) plus testosterone inhibited spermatogenesis in old men (Nieschlag *et al.,* 2003). Short-term injection, but not long-term treatment, of MPA and testosterone decreased sperm concentration in young men (Mclachlan *et al.*, 2002). Injection of MPA alone resulted in decreased number of motile and normal spermatozoa in men (Meyer *et al*., 1985). Megestrol acetate, a synthetic progestin, decreased spermatogenesis in zebrafish (Han *et al*., 2014). In rams, combination of P4 and 6-alpha-methyl-17alpha-hydroxyprogesterone acetate decreased the volume of semen and sperm production, while increasing the abnormal spermatozoa in the semen; however, the effects of hormonal treatment decreased with time (Ericsson and Dutt, 1965).

The percentage of motile spermatozoa in the semen of roosters decreased as a result of P4 treatment, as also shown *in vitro* by Anderson and Navara (2011). This could be due to the pro-oxidative properties of P4 as increased production of the reactive oxygen species decreases sperm viability and motility (Lamirande *et al.,* 1997). It has also been shown that P4 causes sperm hyperactivity, associated with sperm capacitation, in mam-

Table 1. Effect of intramuscular progesterone injection on reproductive parameters in broiler breeder roosters (LSmeans ± SE)

	Before injection	After injection	P-value
Semen volume (mL)	0.5 ± 0.01	0.5 ± 0.02	0.29
Sperm concentration (10^6/mL)	1117 ± 80	1169 ± 98	0.58
Live sperm (%)	88.9 ± 1.1	88.0 ± 1.2	0.53
Abnormal sperm (%)	3.3 ± 0.3	2.9 ± 0.3	0.35
Motile sperm (%)	78.6 ± 2.5	67.7 ± 3.5	0.01
HOST-reacted sperm (%)	55.2 ± 1.5	13.6 ± 1.8	0.0001
Sperm penetration rate	34.1 ± 5.3	53.2 ± 12.3	0.05
Fertile eggs (%)	93.9	69.7	0.05
Fertility duration (days)	11.3 ± 0.5	8.1 ± 0.5	0.05

Table 2. Interaction effect of progesterone and time (week) on sperm concentration (10^6 /mL) in broiler breeder roosters (LSmeans ± SE)

Week	Before injection	After injection
1	$^{A}1192.7 \pm 123.5_{b}$	$^{A}1757.9 \pm 169.9_{a}$
2	$^{A}1106.7 \pm 129.3_{a}$	$^{B}691.7 \pm 147.9_{a}$
3	$^{A}1052.7 \pm 111.6_{a}$	$^{B}1058.8 \pm 133.3_{a}$

A,a: In each column (A, B) or row (a, b), means with common letter (s) are not different (P > 0.05).

Table 3. Interaction effect of progesterone and time (week) on percent motile spermatozoa in broiler breeder roosters (LSmeans ± SE)

Week	Before injection	After injection
1	$^{A}76.2 \pm 4.1_{a}$	$^{AB}60.9 \pm 6.6_{a}$
2	$^{A}82.8 \pm 4.8_{a}$	$^{B}58.8 \pm 6.1_{b}$
3	$^{A}76.8 \pm 4.2_{a}$	$^{A}83.6 \pm 5.5_{a}$

A,a: In each column (A, B) or row (a, b), means with common letter (s) are not different (P > 0.05).

Table 4. Interaction effect of progesterone and time (week) on percent HOST-reacted spermatozoa in broiler breeder roosters (LSmeans ± SE)

Week	Before injection	After injection
1	$^{AB}53.9 \pm 2.3_{a}$	$^{A}17.1 \pm 3.0_{b}$
2	$^{A}61.1 \pm 2.3_{a}$	$^{A}11.6 \pm 2.6_{b}$
3	$^{B}50.6 \pm 2.0_{a}$	$^{A}12.2 \pm 2.4_{b}$

A,a: In each column (A, B) or row (a, b), means with common letter (s) are not different (P > 0.05).

mals (Wu *et al*., 2006). On the other hand, the platelet-activating factor (PAF), a pro-inflammatory mediator, is present in the mammalian spermatozoa (Harper, 1989), and Bladi *et al*,. (1995) showed that short-term incubation of human spermatozoa with P4 and A23187 ionophore increased the sperm PAF production; with low concentrations of PAF significantly decreasing the sperm motility and acrosome reaction. It was also shown that L659, 989 (a PAF-receptor antagonist) inhibited P4-induced acrosome reaction (Krausz *et al.,* 1994).

Progesterone significantly decreased sperm membrane integrity (functionality). Sex steroid receptors play an important role in male poultry reproduction (González-Morán *et al.*, 2008). Progesterone receptors have been detected in human sperm (De Amicis *et al.,* 2011), with rapid effects of P4 in sperm being mediated through surface P4 receptor (Luconi *et al.,* 2004). In human sperm, nanomolar concentrations of P4 resulted in the activation of Ca^{2+} channels which are pH-dependent, have P4 receptors, and are activated by intracellular pH and extracellular P4 (Lishko *et al.,* 2011).

In the present study, P4 injection to roosters significantly increased sperm penetration of the perivitelline membrane. In an *in vitro* study, Anderson and Navara (2011) showed that hens inseminated with the semen samples to which 0.4 nanogram P4 had been added produced eggs in which the perivitelline membrane was penetrated with fewer number of spermatozoa. Such inconsistencies may be due to the differential effects of P4 under the *in vivo* and *in vitro* conditions of these experiments. This could also be due to differences in the concentrations of P4 that affected the spermatozoa in these experiments. The basal level of P4 in semen samples to which P4 was added was not reported by Anderson and Navara (2011), nor can it be calculated from their data; despite correspondence with these authors, they did not provide us with the information we requested. We encountered an unprecedented problem in measuring the plasma level of P4 in the experimental roosters, which does not allow for a comprehensive comparison of our findings with those of Anderson and Navara (2011).

Decreased sperm fertility (by 24%) and duration of production of fertile eggs (by 3 days) were consistent with decreases in sperm motility (by 10%) and the percentage of HOST-reacted spermatozoa (40%) due to P4 treatment of the roosters.

The roosters used in the present experiment were 47 weeks old broiler breeder roosters when the trial started, which may respond differently to P4 treatment than the younger ones as shown in men (Mclachlan *et al.*, 2002); therefore, further experimentation on different age birds is also warranted. More experiments, using larger number of roosters, are need to more clearly determine the effect of progesterone on reproductive parameters in roosters.

Conclusions

Sperm motility, membrane functionality, fertility and duration of fertility decreased as a result of intramuscular medroxy-progesterone acetate injection to broiler breeder roosters in this preliminary study. More detailed experiments are needed to determine the relationship of seminal and blood plasma levels of progesterone with seminal characteristics and fertility in roosters. This may find application in screening the roosters for fertility.

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تزریق درون ماهیچه‌ای پروژسترون جنبایی، یکپارچگی غشا و باروری اسپرم خروس را کاهش می‌دهد

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چکیده پلا سمای منی ماکیان دارای هورمون‌های ا ستروییدی از جمله پروژ سترون ا ست. طبق یافته‌های منت شر شده، افزودن پروژ سترون به منی موجب کاهش باروری در ماکیان لگهورن سفید شد، هر چند رو شن نیست که افزایش تولید درون تنی پروژسترون نیز با کاهش باروری همراه است یا نه. بنابراین، هدف این آزمایش بررسی اثر تزریق پروژسترون بر ویژگی‌های منی و باروری در خروس های مولد گوشتی بود. این آزمایش، با ۱۸ قطعه خروس و ۵۵ قطعه مرغ سویه‌ی کاب ۵۰۰ و در سن۴۷ هفته انجام شد. نخست، نمونه‌های منی ۲ بار در هفته برای ۳ هفته جمع‌آوری شدند و ویژگی‌های منی شامل حجم، غلظت، جنبایی، در صد ا سپرم‌های زنده، نابهنجار و یکپارچگی غشای ا سپرم (آزمون یکپارچگی غشای ا سپرم HOST) ارزیابی شد، سپس نمونه منی هر خروس به ۳ مرغ م شخص تلقیح شد و ویژگی‌های نفوذ ا سپرم به غشای پری ویتلین، باروری و تداوم باروری ارزیابی شد، ۱۵۰ میلی‌گرم پروژسترون طولانی اثر با نام مدروکسی پروژسترون استات در ماهیچه‌ی سینه‌ی خروس‌ها تزریق شد و اندازه‌گیری‌های گفته شده تکرار شدند. اثر تزریق پروژسترون بر حجم منی، غلظت اسپرم، درصد اسپرم‌های نابهنجار و زنده معنی‌دار نبود. تزریق پروژسترون جنبایی اسپرم‌ها را ۱۰ درصد، یکپارچگی غشای اسپرم را۴۰ درصد، نرخ باروری تخم‌مرغ‌ها را ۲۴ درصد و شمار روز تا نخستین تخم‌مرغ نابارور را ۳ روز کاهش داد. در هر روی، نرخ نفوذ ا سپرم به غ شای پری ویتلین ۶۰ در صد افزایش یافت ($P < 0.05$). شناخت بهتر نقش پروژسترون منی در تولید مثل خروس نیازمند پژوهش‌های بیشتری است