Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.2, 2015

Effects of Oral Administration of Clomiphene Citrate and Frequency of Ejaculation on Ejaculate Characteristics of Chickens

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Abstract
The effects of clomiphene citrate (CC) and ejaculation frequency on semen characteristics of chickens were evaluated using 36 Black Nera cocks aged 24-28 weeks, and weighting 1.96kg to 2.39kg in a 4 x 3 factorial experiment, with clomiphene citrate dose at four levels (0mg, 5mg, 10mg and 15mg/kg body weight) and ejaculation frequency at three levels (once a week, twice weekly and thrice weekly) as factors. Ejaculate volume and colour, sperm motility, sperm concentration and total sperm per ejaculate generally increased progressively with increase in CC administration, with the 15mg CC group being significantly ($P<0.05$) higher than the control and 5mg CC groups. CC dose had no significant ($P>0.05$) effect on semen pH and morphologically abnormal sperm. Percent sperm motility increased significantly ($P<0.05$) while sperm concentration declined progressively for cocks ejaculated two and three times per week respectively, the difference between one and three ejaculations per week being significant ($P<0.05$). Semen volume, colour and pH as well as proportions of live and abnormal sperm were unaffected ($P>0.05$) by frequency of ejaculation. Ejaculation frequency, at each level of CC application, had no significant ($P>0.05$) effect on semen volume, semen colour score, sperm motility and total sperm per ejaculate. However, 15mg CC in combination with three ejaculations per week resulted in the highest numerical values of these characteristics, and in the lowest proportion of morphologically abnormal sperm. It was concluded that oral administration of 15mg CC kg^{-1} body weight alone or in combination with frequent ejaculations significantly improved semen output, sperm motility and % live sperm, and reduced the proportion of morphologically abnormal sperm in the cocks.
Keywords: chickens, clomiphene citrate, cocks, ejaculate, ejaculation frequency, semen

1. Introduction

Successful application of artificial insemination (AI) to commercial hatchery operations depends on the quantity and quality of sperm obtainable from breeder males (Zhang 2006). Higher semen output in terms of semen volume and sperm concentration determines the number of females that can be inseminated with it. While increased semen output can be achieved from breeder males through more frequent ejaculations (Zhang 2006; Fan *et al*. 2004; Klimowicz *et al*. 2005), evidence exists that some semen quality parameters decrease progressively with increase in the frequency of ejaculation (Santayana 1985; Nwachukwu *et al*. 2006; Onokwufor & Ezekwe 2006).

Clomiphene citrate (CC) is an orally-active non-steroidal fertility drug commonly used to induce ovogenesis in anovulatory women, and to stimulate spermatogenesis in oligozoospermic men. It exerts its effects by competitively binding to hypothalamic oestrogen receptors thereby blocking the negative feedback of oestrogen on gonadotropin production. Consequently, the hypothalamus produces its gonadotropin-releasing hormones (GnRH) which, in turn, cause the pituitary to produce follicle stimulating hormone (FSH) and luteinizing hormone (LH) (Taylor & Levine 2010). Leydig cells in the testes, in response to LH, produce testosterone which, together with FSH, induces spermatogenesis (Patankar *et al*. 2007; Zaman *et al*. 2009).

The efficacy of CC in increasing sperm output and enhancing fertility in males has sometimes been called to question (Sokol *et al*. 1998; Brown & Chakraborty 1988) due to the observation that not all males respond positively to its use even under the same conditions, and its supposed adverse effects on sperm function (Parinaud *et al*. 1993) and morphology (Shanis *et al*. 1991). Nevertheless, its efficacy in stimulating sperm production in freshwater fish (Kumar 1985), rabbits (Herbert *et al*. 2001), boars (Umeseobi 2006) and rams (Iheukwumere *et al*. 2008) has been demonstrated. However, not much has been reported about its use, either alone or in combination with frequent ejaculation, in enhancing sperm production, and how these factors will impact on semen characteristics in chickens. In this study, the effects of oral administration of clomiphene citrate and different ejaculation frequencies on semen characteristics of chickens in a tropical environment were investigated.

2. Materials and Methods

2.1 The Cocks and their Management

A total of 36 Black Nera cocks aged 24-28 weeks, and with body weights ranging from 1.96kg to 2.39kg were

used for this study at the Poultry Unit of the Teaching and Research Farm of the Faculty of Agriculture, Delta State University, Asaba Campus, Asaba, Nigeria. The study was carried out for 4 weeks from August to September, 2012. The cocks were managed on deep litter, and fed a commercial breeders diet containing approximately 2650 kcal/kg metabolizable energy, and 17% crude protein *ad libitum*. Cool, clean drinking water was also made available *ad libitum.*

2.2 Experimental Design and Drug Administration

The cocks were randomly allotted to 12 clomiphene citrate dose x ejaculation frequency combinations in a 4 x 3 factorial experiment, with clomiphene citrate dose at four levels (0mg, 5mg, 10mg and 15mg/kg body weight) and ejaculation frequency at three levels (once a week, twice weekly and thrice weekly) as factors.

The model of the experiment was:

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + e_{ijk}$$

where Y_{ijk} = the response variable (semen characteristic),

μ = the overall mean,

A_i = effect of the *i*th dose of clomiphene citrate (CC) (i = 1, 2, 3, 4),

B_j = effect of the *j*th frequency of semen collection (j = 1, 2, 3),

AB_{ij} = interaction effect of the *i*th CC dose and the *j*th frequency of semen collection, and

e_{ijk} = experimental error associated with the experimental determinations.

Each 50mg tablet of clomiphene citrate (CC) (Clomid®) was first dissolved in 3mL of sterile water pre-warmed to 20^0C, and made up to 5mL so that each mL of the mixture contained 10mg of active clomiphene citrate. Each cock in the 5mg, 10mg and 15mg CC kg^{-1} body weight groups received a volume (in mL) of the mixture equivalent to 0.5, 1.0 and 1.5 respectively, multiplied by its body weight, (in kg), administered as an oral douche. Oral administration of CC was done once daily (between 09.00 and 09.30 hours Nigerian time) for seven consecutive days.

2.3 Semen Collection and Evaluation

Semen was collected from the cocks by the dorso-lumbar massage technique of (Lake *et al*. 1985) for four weeks in line with the design of the experiment, after an initial two-week pre-experiment training period aimed at getting them acquainted to the massage technique of semen collection, and the collector. Ejaculates were physically and microscopically evaluated for semen colour or consistency, semen volume, semen pH, sperm motility, sperm concentration, total sperm per ejaculate, percent live sperm and percent of morphologically abnormal sperm as previously described by (Bratte *et al*. 2011). A total of 288 semen samples were evaluated.

2.4 Data Analysis

Data obtained were subjected to a two-factor factorial analysis of variance using the General Linear Model of IBM's SPSS Statistics (version 20) computer software (2009 – 2011). Significantly different means were separated using the post-hoc tests available in the same statistical package.

3. Results and Discussion

The effects of varying oral doses of clomiphene citrate (CC) on ejaculate characteristics of the Black Nera cocks appear in Table 1. The results indicate that clomiphene citrate produced significant variations in all ejaculate characteristics evaluated, except semen pH and morphologically abnormal sperm. Semen volume, sperm concentration and total sperm per ejaculate were similar ($P>0.05$) among the control, 5mg and 10mg CC groups, but were significantly ($P<0.01$) higher in the 15mg CC group. Semen colour score averaged between 3 (white) and 4 (cream), and generally increased with increase in CC dose, the control and 5mg CC groups being similar ($P>0.05$) (3.06 ± 0.13 and 3.20 ± 0.13 respectively) but significantly ($P<0.05$) lower in value than the 10mg and 15mg CC groups (3.66 ± 0.09 and 3.71 ± 0.08 respectively). The increase in semen/sperm output in terms of semen volume, sperm concentration, semen colour (or consistency) score and total sperm per ejaculate obtained at higher levels of CC administration show clearly that spermatogenic effects of CC already demonstrated in rams (Noseir 1998), rabbits bucks (Herbert *et al*. 2002), boars (Umeseobi 2006) and men (Zaman *et al*. 2009) are also demonstrable in cocks. Sperm motility was significantly ($P<0.05$) higher in cocks treated with CC (> 73%) than in the control group (65.63 ± 2.71%) while the proportion of live sperm of the control group was not significantly ($P>0.05$) different from those of the 10mg or 15mg groups, but was significantly ($P<0.05$) higher than that obtained from cocks which received 5mg CC. Semen colour observed in the control and CC-treated cocks were white-cream, and consistent with findings reported for seven strains of chickens reared in a tropical environment by Peters *et al*. (2008). Heller *et al.* (1969) had long observed that sperm concentration in men treated with CC was dose-dependent, and that low dosages of clomiphene (50 mg/day) caused an increase, and high doses (> 200mg/day) caused precipitous decreases. This earlier finding, which is consistent with the findings of Umeseobi (2006) that sperm concentration was higher ($P<0.05$) in rabbits treated with 6.25mg CC

than their counterparts treated with 12.50mg of the drug, is at variance with results obtained in this study which indicate that 15mg CC produced a significantly ($P<0.05$) higher mean sperm concentration ($3.06 \pm 0.22 \times 10^9$/mL) than the control and the lower dosages of CC ($< 2.50 \times 10^9$/mL). Differences in responses of different species and strains of animals to the drug, locational differences and differences in nutrition and management may have contributed to this disparity. Clomiphene citrate also reduced the proportion of morphologically abnormal sperm (Table 1), although the differences were not significant ($P>0.05$).

Table 1. Effect of Clomiphene Citrate on Ejaculate Characteristics of Black Nera Cocks (Mean ± SE)

Ejaculate Characteristics	Clomiphene citrate dose (mg/kg body weight)			
	0	5	10	15
Semen Volume (mL)	0.20 ± 0.01^b	0.16 ± 0.01^b	0.18 ± 0.01^b	0.34 ± 0.02^a
Semen Colour Score (1 – 4)*	3.06 ± 0.13^b	3.20 ± 0.13^b	3.66 ± 0.09^a	3.71 ± 0.08^a
Sperm Motility (%)	65.63 ± 2.71^b	73.54 ± 1.59^a	75.66 ± 1.48^a	76.08 ± 0.90^a
Sperm Concentration (x 10^9/mL)	2.19 ± 0.20^b	2.38 ± 0.19^b	2.43 ± 0.19^b	3.06 ± 0.22^a
Total Sperm/Ejaculate (x 10^9)	0.46 ± 0.06^b	0.41 ± 0.04^b	0.50 ± 0.06^b	1.00 ± 0.11^a
Semen pH	7.63 ± 0.07	7.69 ± 0.08	7.67 ± 0.07	7.73 ± 0.06
Live Sperm (%)	78.19 ± 0.28^a	76.98 ± 0.71^b	78.22 ± 0.21^a	78.49 ± 0.16^a
Abnormal Sperm (%)	8.34 ± 0.10	8.18 ± 0.08	8.28 ± 0.11	7.98 ± 0.08

*Watery = 1, Opalescent = 2, Milky = 3, and Creamy = 4; SE = Standard Error
a, b: Within each row, means with different superscripts differ significantly ($P < 0.05$).

Means (± SE) of ejaculate characteristics at one, two and three ejaculations per week are presented in Table 2. Semen volume, colour score and pH as well as proportions of live and abnormal sperm were unaffected ($P>0.05$) by frequency of ejaculation. Semen samples from two and three ejaculations per week had significantly ($P<0.05$) higher proportions of motile sperm (73.54 ± 1.59% and 75.66 ± 1.48% respectively) than those from cocks ejaculated once weekly (65.63 ± 2.71%). This is in conformity with the findings of Fan *et al*. (2004) with Taiwan country chickens that weekly motile sperm production increased with increase in ejaculation frequency when the chickens were subjected to 1, 2, 3 or 6 ejaculations per week, but contrary to those of Onokwufor & Ezekwe (2007) who found no effect of frequency of semen collection on motility and proportion of abnormal sperm. Sperm concentration declined progressively from $2.77 \pm 0.15 \times 10^9 mL^{-1}$ for cocks ejaculated once a week to 2.35 ± 0.16 and $2.07 \pm 0.23 \times 10^9 mL^{-1}$ for cocks ejaculated two and three times per week respectively, the difference between one and three ejaculations per week being significant ($P<0.05$). Similar progressive decline was reported by Onokwufor & Ezekwe (2007) for different phenotypes of Nigerian local cocks, and Zahraddeen *et al*. (2005) for two breeds of turkeys.

Table 2. Effect of clomiphene citrate on ejaculate characteristics of Black Nera cocks (Mean ± SE)

Ejaculate Characteristics	Number of ejaculations per week		
	1	2	3
Semen Volume (mL)	0.23 ± 0.01	0.21 ± 0.02	0.24 ± 0.03
Semen Colour Score (1 – 4)*	3.39 ± 0.08	3.45 ± 0.10	3.40 ± 0.14
Sperm Motility (%)	65.63 ± 2.71^b	73.54 ± 1.59^a	75.66 ± 1.48^a
Sperm Concentration (x 10^9/mL)	2.77 ± 0.15^a	2.35 ± 0.16^{ab}	2.07 ± 0.23^b
Total Sperm/Ejaculate (x 10^9)	0.63 ± 0.05	0.52 ± 0.07	0.57 ± 0.12
Semen pH	7.63 ± 0.07	7.69 ± 0.08	7.67 ± 0.07
Live Sperm (%)	78.19 ± 0.28	76.98 ± 0.71	78.22 ± 0.21
Abnormal Sperm (%)	8.34 ± 0.10	8.18 ± 0.08	8.28 ± 0.11

*Watery = 1, Opalescent = 2, Milky = 3, and Creamy = 4; SE = Standard Error
a, b: Within each row, means with different superscripts differ significantly ($P < 0.05$).

Table 3. Interaction effects of ejaculation frequency and clomiphene citrate on ejaculate characteristics of Black Nera cocks (Mean±SE)

		Ejaculate Characteristics							
Clomiphene citrate (mg/kg BW)	Ejaculations per Week	Semen Volume (mL)	Semen Colour*	Sperm Motility (%)	Sperm Conc. ($x10^9$/mL)	Total Sperm/ Ejaculate ($x10^9$)	Semen pH	Live Sperm (%)	Abnormal Sperm (%)
0	1	0.22 ± 0.02	3.08 ± 0.18	66.80 ± 3.20	2.51 ± 0.29	0.59 ± 0.10	7.56 ± 0.10	80.04 ± 0.39	6.52 ± 0.52^{a}
	2	0.17 ± 0.02	2.94 ± 0.24	65.63 ± 5.47	2.00 ± 0.30	0.35 ± 0.05	7.75 ± 0.11	80.13 ± 0.55	5.53 ± 0.55^{ab}
	3	0.19 ± 0.05	3.29 ± 0.32	61.43 ± 8.57	1.46 ± 0.39	0.24 ± 0.05	7.57 ± 0.20	80.86 ± 0.26	4.50 ± 0.50^{b}
5	1	0.17 ± 0.01	3.48 ± 0.15	74.00 ± 2.58	2.68 ± 0.25^{a}	0.48 ± 0.05	7.69 ± 0.13	78.24 ± 1.33^{b}	5.54 ± 0.41
	2	0.16 ± 0.02	3.17 ± 0.24	74.67 ± 2.15	2.23 ± 0.33^{ab}	0.34 ± 0.05	7.67 ± 0.13	80.01 ± 0.27^{a}	5.17 ± 0.44
	3	0.16 ± 0.03	2.38 ± 0.34	70.00 ± 3.27	1.73 ± 0.46^{b}	0.33 ± 0.12	7.75 ± 0.16	79.25 ± 0.53^{ab}	4.88 ± 0.57
10	1	0.18 ± 0.02	3.44 ± 0.16	74.80 ± 2.52	2.71 ± 0.32	0.54 ± 0.09	7.64 ± 0.10	80.24 ± 0.23	6.38 ± 0.56^{a}
	2	0.16 ± 0.02	3.86 ± 0.10	76.43 ± 1.69	2.03 ± 0.18	0.37 ± 0.08	7.79 ± 0.11	80.50 ± 0.27	5.00 ± 0.57^{b}
	3	0.23 ± 0.04	3.87 ± 0.21	77.14 ± 1.84	2.24 ± 0.32	0.57 ± 0.18	7.57 ± 0.20	79.57 ± 0.97	4.50 ± 0.50^{b}
15	1	0.35 ± 0.03	3.54 ± 0.14	76.54 ± 1.10	3.16 ± 0.33	0.98 ± 0.16	7.73 ± 0.09	80.50 ± 0.20	4.94 ± 0.31
	2	0.32 ± 0.04	3.82 ± 0.12	74.71 ± 3.55	3.05 ± 0.38	0.95 ± 0.18	7.76 ± 0.11	80.88 ± 0.20	4.41 ± 0.28
	3	0.39 ± 0.08	3.97 ± 0.09	77.50 ± 1.63	2.79 ± 1.54	1.08 ± 0.36	7.63 ± 0.18	79.63 ± 0.60	4.00 ± 0.00

* Watery = 1, Opalescent = 2, Milky = 3, and Creamy = 4; BW = Body weight; Conc. = concentration; SE = Standard Error
a, b: Means with different superscripts in the same column within the same clomiphene citrate dose differ significantly ($P < 0.05$).

The interaction effects of clomiphene citrate dose and ejaculation frequency on semen characteristics of the Black Nera cocks are presented in Table 3 and shown graphically in Figure 1a – 1h. Although ejaculation frequency, at each level of CC application, had no significant ($P>0.05$) effect on semen volume, semen colour score, sperm motility and total sperm per ejaculate, 15mg CC in combination with three ejaculations

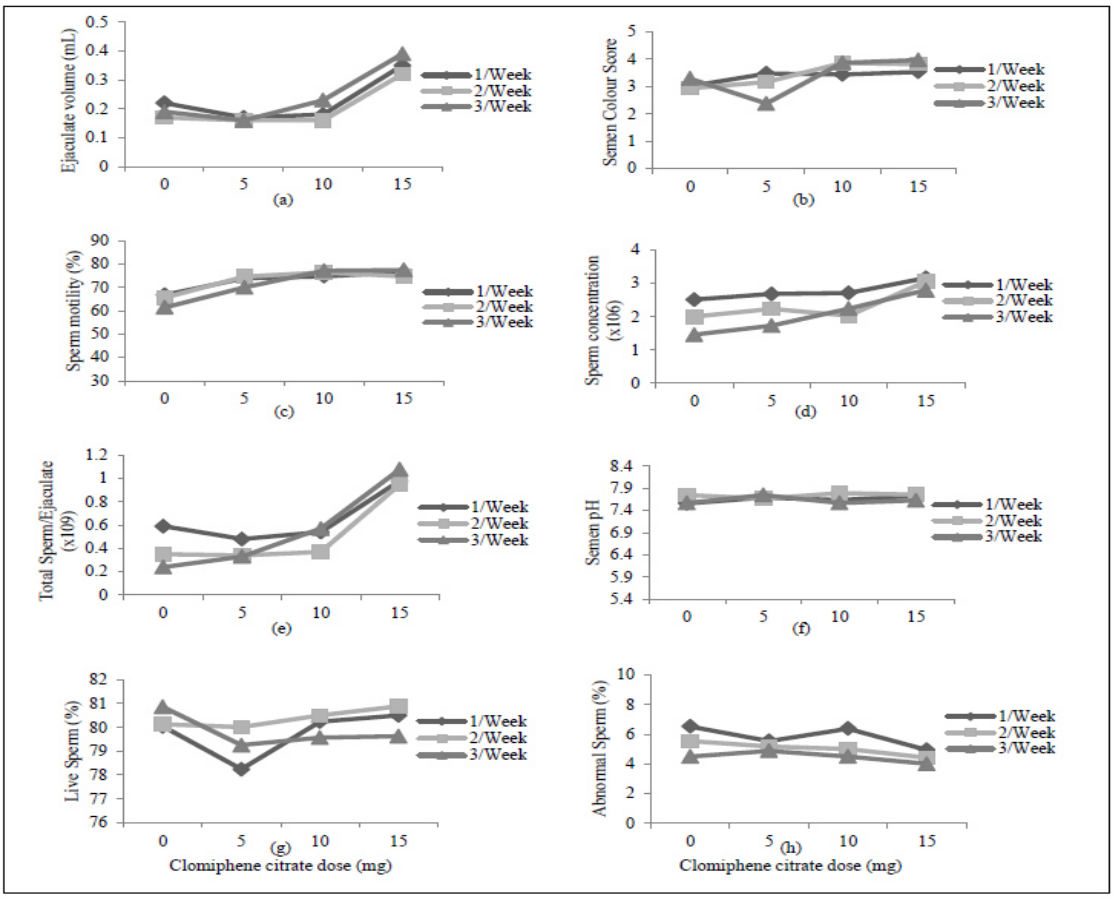

Figure 1: Interaction effects of clomiphene citrate dose and frequency of ejaculation on (a) semen volume, (b) semen colour score, (c) sperm motility, (d) semen pH, (e) sperm concentration, (f) total sperm per ejaculate, (g) live sperm and (h) abnormal sperm.

per week resulted in the highest values of these characteristics, and in the lowest proportion of morphologically abnormal sperm (Table 3). Differences in sperm concentration between ejaculation frequencies were significant in cocks which received 5mg CC, with cocks ejaculated once/week being significantly ($P<0.05$) superior to those ejaculated thrice weekly, but similar to those ejaculated twice weekly. The non-significant effect of ejaculation frequency on the quantitative characteristics (ejaculate volume, sperm concentration and total sperm per ejaculate) is an indication that the rate of spermatogenesis, even in the control birds which did not receive CC, was high enough to make up for the depletion of sperm which may have arisen from two or three ejaculations per week. This probably lends credence to the assertions of Fan *et al*. (2004) and Etches (1996) that a sexually active rooster produces approximately 3 billion sperm daily, and may require as much as 6 or more ejaculations weekly to deplete.

Mean percent live sperm exceeded 78% in all CC x ejaculation frequency combinations, and showed no significant interaction except in the 5mg CC group in which cocks ejaculated twice weekly had a significantly ($P<0.05$) higher percent live sperm than those ejaculated once/week (Table 3; Figure 1 (g)).

The interaction between CC dose with ejaculation frequency on the proportion of morphologically abnormal sperm was also significant at 0mg and 10mg CC levels in which three ejaculations per week significantly ($P<0.05$) lowered percent abnormal sperm compared with one ejaculation per week (Table 3; Figure 1 (h)) most probably because increase in ejaculation frequency is associated with production of younger sperm compared to stored, older sperm associated with less frequent ejaculations.

The close proximity of the ejaculation frequency lines in the illustrations for semen volume (Figure 1(a)), semen colour score (Figure 1(b)), sperm motility (Figure 1(c)), sperm concentration (Figure 1(d)), and total sperm per ejaculate (Figure 1(e)), which also increased markedly at the 10mg and 15 mg CC doses show that increases in these semen traits were more a function of effects of CC dose rather than frequency of ejaculation at the higher levels of CC administration. Patankar *et al.* (2007) had reported that CC improved spermatogenesis, and manifested it by improving sperm count, sperm motility and to a certain extent morphology of the sperm.

No significant ($P>0.05$) variations in ejaculate pH were observed in all CC x ejaculation frequency combinations (Table 3; Figure 1(f)).

4. Conclusion

Clomiphene citrate, administered alone at 15mg per kg body weight for 7 consecutive days, or in combination with as much as three ejaculations per week, significantly increased sperm output, improved sperm motility and decreased proportion of morphologically abnormal sperm in the cocks.

Acknowledgement

The authors are grateful to Mr. Peter Odili and his team at the Physiology Laboratory of the Faculty of Agriculture, Delta State University, Asaba Campus, Asaba, Nigeria for their invaluable contributions to the success of this study.

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