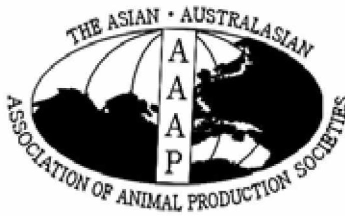

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Effect of *rc* Mutation on Semen Characteristics, Spermatogenic Tissues and Testosterone Profile in Blind Rhode Island Red Cockerels

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ABSTRACT : Seven *rc* mutant and seven normal male birds (Rhode Island Red suie, RIR) were used in this study to determine the effects of *rc* mutation on semen characteristics, testosterone profile and spermatogenic tissues. All birds were randomly selected at week 12 of age and housed in individual cages and were fed and watered *ad libitum*. The birds were exposed to a 14L:10D light cycle during experiment. Semen were collected at weeks 22 to 23 from each bird twice a week and evaluated for semen volume (SV), sperm concentration (SC), total sperm count (TSC), percent of sperm motility (%SM), dead sperm (%DS), and sperm metabolic activity (SMA). To determine the testosterone concentration (TC) in plasma, blood was collected at weeks 12, 16 and 18. Testicular tissue were collected, processed and evaluated for seminiferous tubule diameter (STD), round spermatid number (RSN), percent elongated sperm (%ES) and seminiferous tubules length (STL). Body weight (BW), comb weight (CW) and testes weight (TW) were weighted at the end of experiment (week 23). The SV, TSC and %SM were significantly higher in normal birds but the %DS was higher in blind birds ($p<0.05$). The SC did not differ significantly between the two groups but its value was higher in normal birds. The sperm metabolic activity in the first h of collection did not differ significantly between the two groups but after 24 h, the level of SMA in normal group was significantly higher ($p<0.05$). The level of TC did not differ significantly between the two genotype groups but normal birds had higher TC in all collections except the last one. The STD, RSN, %ES and STL in normal birds were higher when compared to blind birds but the differences were insignificant except for ES percent. The BW, CW and TW between the two groups did not differ significantly but the weights were higher in normal group compared to blind birds. Statistical analysis of semen characteristics, testosterone profile and histological factors were indicated detrimental effects of *rc* mutation in prepubertal RIR blind male birds due to lack of light. (**Key Words** : *rc* Mutation, Semen Characteristics, Spermatogenic Tissues, Testosterone Hormone, Blind Cockerel)

INTRODUCTION

A few studies have been done on genetic dysfunction of reproduction in chickens. The phenomenon of "rc" gene represents an autosomal recessive mutation which associated with degeneration of retinal photoreceptors and caused blindness in Rhode Island Red (RIR) chickens, first reported by Cheng et al. (1978). Similarly, incubation behaviour in chickens is not controlled by a major gene (or genes) on Z chromosome and there must, therefore, be major autosomal genes contributing to the expression of the behaviour (Romanov, 2001). Chicks homozygous for this mutation (rc^-/rc^-) hatched with a retina that appeared to be analogous, on a microscopic level, to normal (Rc^+/Rc^-) chick retina but the mutants were blind and did not have an electroretinographic (ERG) response (Cheng and Pang, 1989). As the chicks grew older, significant degeneration of the retinal photoreceptors started during the second week after hatching, and by two months after hatching, only very few photoreceptors remained in the central retina (Ulshafer and Allen, 1985a, b). Homozygous (Rc^+/Rc^-) and heterozygous (Rc^+/rc^-) chickens both have apparently normal vision (Cheng et al., 1980). Thus the rc^-/rc^- chickens have no ERG response and their rods and cones degenerate as they age. It is also reported that the rc^-/rc^- mutation in chicken not only affect the rods and cones of the retina, but also have significant differences in their heart, kidney, liver, thymus, thyroid and gonads as well (Rabkin and Cheng 1992; Cerruti-Sola et al., 1997). The objectives of this study were to determine the effects of rc^-/rc^- mutation on semen and gonadal characteristics and testosterone profile due to delayed gonadal maturation in comparison to Rc^-/Rc^+ males of the same age.

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MATERIALS AND METHODS

Birds

A breeding colony of fourteen male chickens of Rhode Island Red (RIR) strain with seven rc^{-}/rc^{-} mutant (blind) and seven Rc^{-}/Rc^{+} (sighted) as a normal group were used and maintained at Avian Genetics Laboratory of the University of British Columbia in Canada. All birds were housed in an individual cages and had access to free water and feed. The birds were exposed to a 14L:10D light cycle and trained for a 2 weeks period prior to the trail date.

Semen characteristics

Semen volume (SV) : All 14 males were trained for a 2 week period prior to the trail date to allow them to adapt to their new surroundings and handling for semen collection. Collections were taken from all birds at 22 weeks of age by massaging method (Burroms and Quinn, 1937). The ejaculate from each bird was collected in a cryovac vial and the SV was recorded to the nearest 0.01 ml by the use of a tuberculin syringe.

Percent sperm motility (%SM) : The collected semen transferred to a test tube and diluted to a solution of one part semen to three parts BPSE (Beltsville Poultry Semen Extender) buffer solution (Sexton, 1977). The solution was mixed gently by inverting the test tubes 2 or 3 times then the small drop of diluted semen was placed on the warmed (40°C) microscope slide with a sterilized wire loop. A cover slip was placed on the droplet to prevent from drying out. Several fields of the prepared slide were examined under a light microscope (400×G). Motility assessments ranged from O (no movement) to 4 (very active movement) as outlined by Wilox (1956). Slight modification was made when ranking falls between two designated by adding a plus (e. 2^{+}). Sperm motility was assessed at both 0 and 24 h after collection. The diluted semen was stored at 4°C.

Percent live sperm (%LS) and percent abnormal sperm (%AS) : An aliquot of the 1:3 diluted semen samples were then diluted further to a final 1:79 dilution with BPSE. A drop of this diluted solution was mixed with a drop of eosin -nigrosin (E-N) stain on a dry microscope slide and a smear was made (Mortimer, 1994). Under 1,000×magnification (oil immersion), 200 sperm were counted and the number of dead and abnormal sperm (dead sperm = eosin permeable and stained pink; live sperm = eosin impermeable and white color) were recorded and expressed as %LS and %AS (Mortimer, 1994; Wolfe, 1977). The abnormalities were included: looped, double, coiled and broken tails; oval-shaped, spatula-shaped, elongated, filamentous and double heads and membrane destruction. The semen analysis was repeated with semen samples stored for 24 h.

Sperm concentration (SC) and total sperm per ejaculate (TS) : An aliquot of the 1:3 diluted semen was further diluted with BPSE to a final 1:99 dilution. Semen concentration (10^9 sperm per ml) was determined counting the number of spermatozoa in a Neubauer hemocytometer. The concentration was determined using the equation: SC (10^9 per ml) = number of sperm counted × multiplication factor × the dilution factor; TS (10^9 sperm) = semen concentration × semen volume (Hafez, 1976; Mortimer, 1994).

Sperm metabolic activity (SMA) : An aliquot of the 1:3 diluted semen samples were analyzed for their metabolic activity (percent Formazan production) at 1 and 24 h after collection, according to the method outlined by Chaudhuri and Wishart (1988). After each samples' absorbance was determined in Shimadzu spectrophotometer, the n mol of Formazan produced during the reaction were calculated using the previously established regression equation for the wavelength of 520.

$$A520 = -0.0105+0.0132X$$

The SMA (n mol of Formazan/min/10^9 sperm/ml) of a particular sample = X/20/sperm concentration/25.6

Histological characteristics

At the end of study, the birds were weighed and killed by pentobarbital anesthesia and immediately subjected to postmortem examination. The comb and testes from each bird were removed and weighed accordingly. For histological study, right testes immediately placed in %10 buffered formalin and divided approximately to 10×10×5 mm pieces and left them in the solution for 3 days. Following fixation, the specimens were subsequently dehydrated through graded alcohols and xylene then infiltrated and embedded in paraffin wax according to Arshami and Ruttle (1988). Tissues were sectioned at 4 microns and stained with hemotoxyline and eosin (HE) for histological examination. For quantitative evaluation in each bird, 20 randomly chosen, circular seminiferous tubules diameter (STD) were measured in microns by the use of a calibrated micrometer eyepiece. The number of round spermatid (RS) and elongated sperm (ES) in one gram of testis were counted and the total length of seminiferous tubule (LST) was measured in each tubule group according to Brillard and McDaniels (1986) and Blazak and Fechheimer (1981).

Blood sampling

Blood samples were collected by vacutainer tubes on 12, 16 and 18 weeks of age then the serum was separated by centrifugation and freezed at -20°C for testosterone concentration later.

Statistical analysis

Data for semen parameters (SV, %SM, %LS, %AS, SC, TS and SMA) were analyzed by Least Squares ANOVA

Table 1. Semen characteristic of normal (control) (No. = 7) and blind (*rc* mutant) (No. = 7) prepubertal male chickens[1]

Characteristic		Normal (Rc^+/Rc^+)	Blind (rc^-/rc^-)
Semen volume (ml)		0.80±0.04[a]	0.41±0.04[b]
Semen concentration (10^9 per ml)		1.52±0.10[a]	1.42±0.10[a]
Total sperm count per ejaculate (10^9 sperm)		1.39±0.16[a]	0.72±0.16[b]
Sperm motility (%)		2.22±0.11[a]	1.52±0.11[b]
Live sperm (%)		79.01[a]	71.10[b]
Abnormal sperm (%)		13.55[a]	16.90[b]
Sperm metabolic activity	1st hour	10.93±0.78[a]	9.58±0.80[a]
	24th hour	11.72±0.73[a]	8.85±0.73[b]

[a, b] Means (±SE) within a row with the same superscript are not significantly different (p>0.05).

[1] Three collections of semen were made on week 12, 16 and 18 of age.

Table 2. Histological characteristic of normal (control) and blind (*rc* mutant) testis prepubertal male chickens

Genotype	Round spermatid (No. per g)	Elongated sperm (%)	Seminigerous length (m)	Seminiferous diameter (μ)
Normal (No. = 7) (Rc^-/Rc^-)	1.95±0.73[a]	83±60[a]	196.8±32[a]	252.6±47.4[a]
Blind (No. = 7) (rc^-/rc-)	0.52±0.41[a]	43±80[b]	98.8±32[a]	209.9±53.3[a]

[a, b] Means (±SE) within a row with the same superscript are not significantly different (p>0.05).

with the help of statistics software JMP (SAS, 1996). Percentages were arc-sine transformed before analysis. Mean testosterone for different group at 12, 16 and 18 weeks was analyzed by student t-test. Data from histological study were analyzed by CRD test.

RESULTS AND DISCUSSION

Semen characteristics and sperm metabolic activity

The results of this study showed lower semen volumes, total sperm per ejaculate, percent sperm motility and percent live sperm with higher percent abnormal sperm in blind male chickens when compared to normal birds ($p<0.05$). Sperm concentration and SMA in the first hour of collection were slightly higher in normal birds but at 24 h after collection the SMA was significantly increased (Table 1).

Our finding indicated that, the quality of semen characteristics of blind birds due to *rc* mutation reduced significantly. Cerruti et al. (1997) were reported that the gonads of blind birds (male and female) showed delay maturity in comparison to normal birds. Other studies were reported that *rc* mutation decreases cGMP activity and releases intracellular calcium (Semple-Rowland et al., 1996). In fact, calcium is needed for testicular cell membrane excitability (Becker and Deamer, 1991). Therefore, the possibility that *rc* mutation may affect on delay sperm maturation and degeneration is certain. It has been suggested that Ca^{2+} dependent regulatory protein "calmodulin" is involved in acrosomal biogenesis (Poccia, 1994). The latest study supports our finding about higher percent abnormal sperm and lower percent live sperm in *rc* mutant birds.

Froman and McLean (1996) stated that sperm motility at body temperature in chicken is Ca^{2+} dependent. The effect of Ca^{2+} is so strong that its presence in high concentration can overcome the temperature-induced loss of motility (Ashizawa and Wishart, 1987). In our study, the lower sperm motility in blind chickens may resulted from both low cGMP concentration and calcium regulation in comparison to normal chickens ($p<0.05$). Our finding showed sperm concentration and metabolic activity in the first hour of collection did not reduced significantly in blind chickens ($p<0.07$). This could be due to overall reduction of sperm production in blind chickens versus normal chickens. But the average total sperm count per ejaculate and SMA after 24 h in normal birds induced significantly ($p<0.05$). Marini and Goodman (1969) found positive correlations between sperm concentration and motility; semen volume and sperm motility and percent live sperm; but negative correlations between semen volume and percent abnormal sperm ; sperm motility and percent abnormal sperm and oxygen utilization ($p<0.05$). Therefore, chickens with *rc* gene may have lower semen quality from that without mutation.

Testicular tissue development

Histological examination showed a reduction of STD and LST of testis in blind birds when compared to normal birds. The percent of seminiferous tubules filled with ES in blind birds was significantly lower than normal birds ($p<0.05$). The number of RS per gram of testis was lower in blind birds in comparison to normal birds (Table 2).

The effects of *rc* mutation showed alteration of testicular tissues in blind birds. The histological differences in blind birds severely affected on STD, LST, ES and RS when compared to Rc^-/Rc^+ birds, which indicating genetic defects at the cellular level. Reduction number of ES and RS and the length of LST and STD indicating delay sexual maturation and development in rc^-/rc^- males. These finding

Table 3. Concentration of testosterone in plsma in normal (control) (No. = 7) and blind (rc mutant) (No. = 7) prepubertal male chickens (ng/ml)

Age (wk)	Normal (Rc^+/Rc^+)	Blind (rc^-/rc^-)
12	0.89±0.15[a]	0.58±0.10[a]
16	1.07±0.22[a]	0.47±0.15[a]
18	1.78±0.32[a]	1.19±0.47[a]

[a, b] Means (±SE) within a row with the same superscript are not significantly different ($p>0.05$).

Table 4. Body weight, comb weight and testis weight of normal (control) and blind (*rc* mutant) prepubertal male chickens

Genotype	Comb weight (g)	Testis weight (g)	Body weight (kg)
Normal (No. = 7) (Rc^+/Rc^+)	16.62±3.6[a]	33.03±5.64[a]	2.58±0.12[a]
Blind (No. = 7) (rc^-/rc^-)	12.96±4.43[a]	16.05±5.42[a]	2.22±0.07[a]

[a, b] Means (±SE) within a row with the same superscript are not significantly different ($p>0.05$).

agrees with Cerruti et al. (1977), who also found retarded maturation and poor differentiation of the germinal cells in young rc^-/rc^- male birds. Similarly, Rabkin and Cheng (1992) were reported abnormalities of sarcolemmal membrane from cardiac myocyte of blind birds. Pelech et al. (1983) were reported that high levels of phosphatidylcholine are required by newborn and young animals for growth and development. Therefore, *rc* mutant gene may inhibited normal growth of STD, LST, ES and RS by disturbing the physiological environment for development of spermatocytes. More detailed investigation of ulterastracture of spermatogenesis is needed to determine the exact side of *rc* mutant gene.

Testosterone profile, weight of body, comb and testis

There was no significant difference for plasma testosterone between the two groups but normal birds showed higher testosterone concentration in the first 3 weeks (Table 3). Weights of body, comb and testis between the two groups did not differ significantly but the normal group had higher weights (Table 4).

It has reported that testosterone profile in young male fowls sharply increases on week 10-12 then slows down to week 18th and after that decreases slowly (Ogawo et al., 1989). Our finding showed similar pattern for testosterone level through week 18th in normal birds but the blind birds due to genetic defect and delay maturity, had lower testosterone concentration up to week 18th. Similarly, Driot et al. (1979) were reported lower concentration of testosterone in plasma of hemi castrated cockerels when compared to intact birds. The level of testosterone concentration in hemi castrated birds was raised up to week 22[sec] where the intact birds were up to 20 weeks. In our study, the level of testosterone concentration in blind birds showed similar results but the low level of testosterone pertained to small number of birds thus the differences were insignificant.

Since the plasma concentration of testosterone in blind birds increases during growth period (Ogawo et al., 1998), therefore BW will increase accordingly. Results of this study revealed, increase of BW in normal birds during course of study. Basically similar pattern showed for CW, which has increased in normal birds due to induction of testosterone concentration and BW. Similarly, the TW in control birds was much higher than blind birds at week 20 but it did not differ significantly. Actually, the TW in young birds depends of the amount of light and stage of growth (Kumoran and Turner, 1949; Mather and Wilson, 1964). In our study, the blind birds were unabeled to receive direct light due to *rc* mutation so the TW was lower.

Evaluation of semen characteristics, histological examination of testicular tissue and testosterone profile in Rc^+/Rc^- and rc^-/rc^- birds revealed numerous morphological changes in SV, %SM, %LS, %AS, SC, TS, SMA, STD, RS, ES and LST and testosterone concentration. Results of this study are suggested that *rc* mutation in male birds may reduce fertility and be detrimental to reproductive performance. Further study is needed in both sexes of *rc* mutant birds to determine the exact role of light on reproductive function and performance in chickens.

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