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Research Article

Effects of N-Ethyl-N-Nitrosourea on semen quality traits of Nigerian local chicken

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Abstract: To improve the Nigerian local chicken (NLC), N-Ethyl-N-Nitrosourea (ENU) can be used to introduce mutations into the genome to characterise the genetic architecture of complex traits and develop novel trait models and lineages. The extent ENU is tolerated as an effective mutagen in chickens and the knowledge of its biology of action is however required before its application. This is measured by the induction and duration of sterility in the absence of lethality in male mice. The objective of this study was to characterise the ENU effects on the onset and duration of induced sterility through direct studies of sperm (count and motility) from mutagenized cocks. In a randomized design experiment, 22 mutagenized and 20 non-mutagenized (control) NLC cocks were intraperitoneally injected with 300mg ENU/kg body weight fractionated as 3 doses of 100mg, and sham respectively and administered in 3 successive weeks. Semen volume (ml), sperm concentration ($x10^6$/ml) and sperm motility were evaluated prior to ENU and Sham injected weekly up to week 13. Data were subjected to Mann-Whitney Wilcoxon at $\alpha_{0.05}$. The ENU significantly ($P < 0.05$) reduced sperm concentration and motility but had no significant effect on semen volume at week 2. The duration of sterility could not be determined in this study as treated cocks did not show complete fertility recovery and ENU administered can be used for mutagenesis in chickens at the examined dose and regime.

Keywords: N-ethyl-n-nitrosourea**;** Mutagenicity**;** Sterility**;** Fertility**;** Chicken

1. INTRODUCTION

N-Ethyl-N-Nitrosourea (ENU) may be useful for inducing mutations into the genome for the purpose of characterising the genetic architecture of complex traits, developing new models of health, production and disease and developing elite economically important trait lineages during improvement schemes in the Nigerian local chicken (NLC). ENU has not yet been applied to such studies in chickens, and its use will require stage-wise empirical studies to determine whether it is tolerated at the dose levels established in other species in which it has been used previously (Russell *et al.,* 1979; Hitotsumachi *et al.,* 1985; Massironi *et al.,* 2006) or other levels which are specific to the biology of chickens. Equally, a fundamental understanding of the mechanisms by which it acts in chickens will be required to confirm the established mechanisms and reveal species related idiosyncrasies of action.

An earlier dose optimization study (Adesina et al., 2017) indicated that 300 mg/kg ENU administered as fractioned dose of 100 mg/kg body weight over 3 successive weeks was the optimal dose of ENU for mutagenesis. This is as measured by induction and duration of sterility (confirmed through effects of sperm from mutagenized cocks on egg fertility) in the absence or near-absence of lethality in NLC, thereby mirroring the optima established in mice previously (Hitotsumachi *et al.,* 1985). The present study was conducted to further characterise the ENU effects on fertility (including the onset and duration of ENU induced sterility) through studies of sperm

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(count and motility) characteristics of mutagenized cocks. In conventional ENU mutagenesis protocol, to assess recovery of fertility in male, the males are set up in cages with females for a period of 8 – 10 weeks after the last injection of ENU (CSH, 2008). The consequential additional cost of feeding and maintaining the mutagenized males and their female counterparts could be minimised through direct evaluation of sperm characteristics of the mutagenized males. This would establish fertility recovery prior to the breeding of such animals to recover mutations. The utility of this approach was demonstrated by Yin *et al.* (2015) in mice where they showed that changes in sperm count reflected the profile of ENU induced histological damage and recovery in testes and also ENU effects on spermatogenesis and fertility. Knowledge derived from the present study has the potential to reveal whether sperm studies can complement or indeed replace established egg fertility studies in the determination of the effects of ENU on sterility.

2. MATERIALS AND METHODS

2.1 Experimental Site

The experiment was carried out at the poultry unit, in animal section of the Teaching and Research Farm, Kwara State University, Malete, Kwara State. The university is located in the north central zone of Nigeria and lies on geographical coordinates 8°42'0''N, 4°28'0''E.

2.2 Safe Use of ENU

ENU is highly toxic and mutagenic, both properties are however inactivated by KOH treatment (CSH Protocols, 2008). The use of N-Ethyl-N-Nitrosourea as approved was consistent with published protocols (CSH Protocols, 2008) for containment and decontamination.

2.3 Personal Protective Equipment

Personal Protective equipment (including face mask (3M®, USA); hand gloves; safety goggles; rubber boots and; coveralls) was used at all times by operators in areas where ENU was handled, ENU mutagenized birds were kept, and ENU contaminated materials were handled, decontaminated and disposed.

2.4 Containment

The experiment was conducted in a highly restricted pen house to ensure that unauthorised persons were not exposed to ENU contamination. The pen house floor was lined with rubber sheets, overlaid with tissue paper bedding.

2.5 Decontamination and Disposal

All beddings, including accumulated faecal droppings were packed from animal pens daily, soaked in KOH solution (which inactivates ENU), and incinerated. All needles, syringe and containers used in the application of ENU were soaked in KOH solution for 24 hours and thereafter incinerated. Birds treated with ENU were euthanized and incinerated at the end of the experiment period.

2.6 Experimental Birds and Management

Forty–two Yoruba ecotype cocks of 25 weeks old, aged 25 weeks, were used for this study. They were wing tagged, leg banded, and housed in individual cages. They were fed a commercial grower diet containing 15% crude protein and 2850 kcal/kg ME (Top Feeds®, Nigeria) based on a restriction-feeding schedule for male breeders (Leeson & Summer, 2009) throughout the experimental period. All routine management, medication and vaccinations were strictly followed (Sola-Ojo *et al.,* 2011). The cocks were randomly distributed into two treatment groups, comprising 22 cocks in the 300 mg ENU group and 20 cocks in the Sham (control) group.

2.7 Preparation of ENU and Sham (Control) Stocks

ENU (Sigma – Aldrich UK) and Sham stocks were prepared as previously described (Adesina *et al.,* 2017).

2.8 Administration of ENU

Twenty – two cocks were weighed using 10kg capacity Top loading scale (Avery Weigh– Tronix UK) and administered with a weekly intraperitoneal (i.p) injection of 300 mg ENU given as a fractionated dose of 100 mg/kg weekly body weight over a period of 3 weeks (300D/3R). Also, 20 cocks assigned to the Sham (control) group were weighed and injected with 0mg ENU (Sham), administered as fractionated doses over 3 successive weeks (0D/3R). Intraperitoneal administration of treatment or Sham was performed as described by Adesina *et al.* (2017).

2.9 Data Collection

Semen volume, sperm motility and sperm cell concentration per unit volume (ml) were measured

prior to ENU administration, three days after ENU administration, and weeks after over a period of 11 weeks.

Semen was collected early in the morning as prescribed by Leeson and Summer (2009) by use of the method of Burrows and Quinns (1937), as described by Getachew (2016). **S**emen volume was measured using a tuberculin syringe (Insulin Syringe) as described previously by Churchill *et al.* (2014). Sperm motility was analysed within 1 hour of collection as described by Peters et al. (2008).

The sperm cell concentration per volume (ml) was measured by the procedure of Peters et al. (2008) and Churchill et al. (2014) using a Neubauer Haemocytometer. An aliquot of semen was mixed with buffered formalin (diluting fluid containing 5-gram sodium bicarbonate ($NaHCO_3$), 1 ml neutral formalin and 100ml distilled water) at a ratio of 1:25. A drop of diluted semen was loaded into the chamber of the haemocytometer by placing the tip of the pipette in the V-shaped groove of the haemocytometer. Capillary action drew the fluid into the chamber. The sample was allowed to settle for 2 – 3 minutes, covered with a glass cover slip and placed on a light microscope at a magnification of x 400. The sperm cell concentration per volume (ml) was then calculated using the formula:

$C = 50{,}000 \times N \times Df$

Where, C = Sperm cell concentration per volume (ml)

N = Number of sperm cells counted

Df = Dilution factor

2.10 Experimental Design and Statistical Analysis

The design of the experiment was a completely randomized design (CRD). The sperm motility score was analysed using the Mann – Whitney Wilcoxon test. The data on semen ejaculate volume and sperm cell concentration per volume (ml) were subjected to Kolmogorov – Smirnov test of normality and Levene's test of equality of variance (Field, 2009). The data were subsequently subjected to non - parametric analysis using SPSS version 22 (SPSS, 2013) (Mann – Whitney Wilcoxon test and Wilcoxon rank test) due to their significant departure from normality and significant heterogeneity of variance.

3. RESULTS AND DISCUSSION

The initial semen volumes prior to ENU administration in birds administered with 0 mg ENU (sham) and 300 mg ENU were not significantly (P = 0.41) different (Table 1). There was no significant (P > 0.05) effect of ENU on semen volume from week 0 to 3 and from weeks 6 to 11. There was significantly (P = 0.01) lower semen volume in the 300 mg ENU group relative to the Sham group at weeks 4 and 5 (Table 1).

The initial sperm cell concentration of NLC cocks in the sham group (0 mg ENU) did not differ significantly (P > 0.05) from that of the 300 mg ENU group (Figure 1). Also at week 1, there was no significant (P > 0.05) difference in the sperm cell concentration between the two groups. However, there was significant (P < 0.05) difference between the two groups at weeks 2 – 11. It was observed that there was a sharp drop in sperm cell concentration of the two groups at week 2 post-ENU administrations (0 mg = 620.25 x 10^6/ml and 300 mg = 372.44 x 10^6/ml). There was a slight increase in sperm cell concentration in both groups at week 3 post-ENU administration (0 mg = 925.31 x 10^6/ml and 300 mg = 446.88 x 10^6/ml), it however plummeted in birds administered with 300 mg ENU at week 4 (319.32 x 10^6/ml) from which point gradual increment was observed up till week 7 (412.95 x 10^6/ml). The sperm cell concentration again plummeted to 257.27 x106/ml at week 9 and subsequently a gradual increment was observed till week 11 (Figure 1). The results however showed that there was no significant (P > 0.05) change in sperm cell concentration in the 300 mg ENU group from week 4 to week 11 and the sperm cell concentration remained significantly lower than that of the control (Figure 1).

At weeks 1, 4 – 9 and 11, birds that received 0 mg ENU had significantly (p < 0.05) higher sperm motility score than birds that received 300 mg ENU (Figure 2). In contrast, there was no significant difference in motility scores at weeks 2 and 3. In the 300 mg ENU group, the nadir of semen motility occurred at week 4 (Figure 2). From week 4 onwards, there was a trend towards recovery of motility in the 300 mg ENU group to week 11 at which no significant (P > 0.05) difference was observed between sham and 300 mg treatment groups.

Table 1: Effect of ENU on Semen Volume in Nigerian Local Chicken.

	Median Semen Volume (ml)					
Week	0 mg (n = 20)	300 mg (n = 22)	U	Z	P	r
-1	0.40	0.40	187.50	- 0.84	0.41	0.13
0	0.40	0.40	202.50	- 0.47	0.65	0.07
1	0.35	0.40	152.00	- 1.77	0.08	0.27
2	0.40	0.40	198.00	- 0.57	0.59	0.09
3	0.40	0.40	186.00	- 0.88	0.41	0.14
4	0.30	0.25	126.50	- 2.44	0.01	0.38
5	0.40	0.30	121.50	- 2.55	0.01	0.39
6	0.40	0.30	172.00	- 1.24	0.22	0.19
7	0.40	0.30	154.00	-1.70	0.09	0.26
8	0.30	0.40	209.50	- 0.27	0.79	0.04
9	0.40	0.40	203.00	- 0.44	0.67	0.07
10	0.30	0.30	219.00	- 0.03	0.99	0.01
11	0.30	0.30	187.00	- 0.85	0.40	0.13

N.B: U = Mann- Whitney Wilcoxon test statistic, Z = Z – Score, P = P – value, r = Effect size, Significance at $P \leq 0.05$

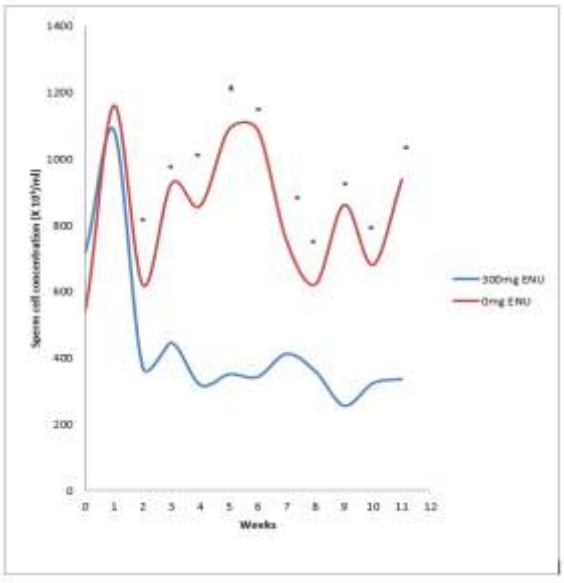

Figure 1: The Effect of ENU (administered intraperitoneally) on Sperm Cell Concentration in Nigerian Local Chicken

*Significant ($P \leq 0.05$) difference (Mann – Whitney Wilcoxon Test)

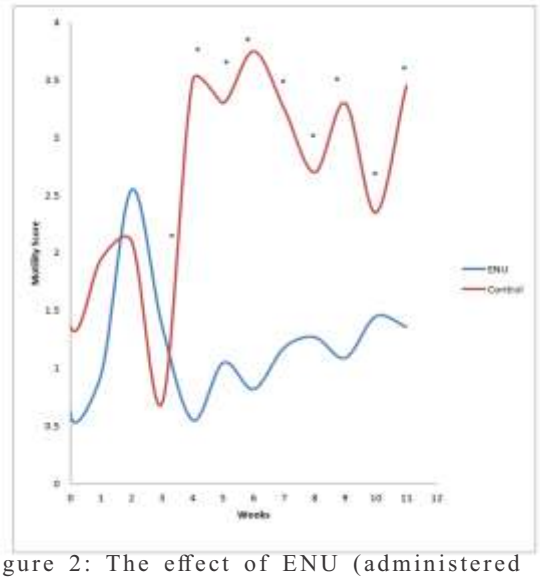

Figure 2: The effect of ENU (administered intraperitoneally) on Sperm Motility in Nigerian Local Chicken

*Significant ($P \leq 0.05$) difference (Mann – Whitney Wilcoxon Test)

N-Ethyl-N-Nitrosourea's Effects on Chicken Semen Quality.

The semen volume in Sham group NLC in this study was within the normal range (0.2 – 0.5ml) described previously for chicken (Churchill *et al.,* 2014 and Getachew, 2016), indicating that the data was reliable. The data showing that semen volume of birds in the 300mg ENU group was also within the normal range and did not differ from that of the sham group over the period of study indicating that in the NLC, at the dose administered, ENU does not act through effects on semen volume.

The initial increase in sperm cell concentration ($x10^6$/ml) in the first week irrespective of treatment group (Sham and 300 mg group) followed by a dramatic reduction could be adduced to the co-dislodgement of mature spermatozoa and maturing spermatids from the epithelium of the vas deferens, due to the toxic effect of the ENU (Hitotsumachi *et al.,* 1985) and ethanol (a component of the ENU buffer and the Sham) or both, on sperm cells; resulting in increased germ cell desquamation and inactive seminiferous tubules (La Vignera *et al.,* 2013). The severity of sperm count reduction (week 1 – 2) in birds administered with 300mg ENU compared to those that received 0 mg ENU could be attributed to the effect of ENU over and above the acute and reversible effect of ethanol. The specific effect of ENU is adduced to toxic effects causing spermatogonia death (Hitotsumachi *et al.,* 1985; Kennedy & O'Bryan, 2006) following first injection of ENU. The similar sperm cell concentration in the control and ENU groups in the first week of ENU administration was consistent with Yin *et al.* (2015), though differences between treatment and control (sham) groups were observed at week 2 in contrast to Yin *et al.* (2015), who reported that there was no significant difference in the sperm count of the control and experimental groups within the first two weeks after injection of ENU in mice. According to Yin *et al.* (2015), a reduction of sperm count in the experimental group continued till week 8, pointing to inter-species similarity in the response to ENU, though in the chicken, a nadir was observed in week 9 which may point to a slightly delayed recovery relative to the mouse. This delay in the chicken is brought into sharper contrast, given that spermatogenesis in Aves (12.77 days - Lin & Jones, 1992) takes almost half the time required in mice (Oakberg, 1956; Perrard *et al.,* 2016) indicating that despite the lapsation of a greater number of spermatogenesis cycles (circa 4 cycles) in chickens over an 8-week period relative to 2 cycles in mice over the same period, sperm concentration had still not recovered in the former.

The sperm cell concentration of 809.21 x 10^6/ml reported for birds in the control group was lower than the range of 3, 000 – 7, 000 x 10^6/ml reported for chicken by Peters *et al.* (2008), Churchill *et al.,* (2014) and Getachew (2016) but higher than the value of 750 x 10^6/ml cells /ml observed by Machal and Krivanek (2002) in birds at age 28 - 35 weeks, which were similar in age to cocks used in the current study.

The acute effect of ENU and sham administration on semen concentration between weeks 1 and 2 was mirrored by an acute reduction in sperm motility in the same time window, showing that in the chicken, effects of ENU transcend potentiation of acute reduction in the rate of sperm maturation, and extend to effects on functional properties of mature spermatozoa. The reduction in sperm motility in the sham group may be adduced to the effect of ethanol (alcohol) in the Sham. Alcohol has previously been shown to reduce sperm motility and other sperm parameters (Dare *et al.,* 2002), and the effects may be partially reversible upon discontinuation of alcohol consumption (La Vignera *et al.,* 2013).

The use of 95% ethanol in the current study to dissolve ENU prior to i.p administration in chicken was consistent with standard ENU usage protocol (CSH, 2008). However, the inclusion of 95% ethanol in the control group (sham) had not been previously reported. Hence, the current data fortuitously provide fresh insights, showing that the initial and acute reduction in sperm parameters following ENU administration (week 1 – 2) here and likely in published studies (Yin et al., 2015) is in large part due to the matrix or medium in which ENU is dissolved, and specifically ethanol as a component of that matrix. Further, the data shows that recovery from the effects of alcohol toxicity on sperm parameters is evident between weeks 2 and 3, following administration. Also, the main effects of ENU in potentiating an initial reduction in sperm quality parameters is modest relative to the effects of alcohol. Significantly, ENU is central to the persistence of reduced sperm quality characteristics associated with its mutagenic effect beyond weeks 2 – 3 to week 11.

The result revealed that a rebound of sperm motility commenced in week 5, following a nadir in week 4. The increase in sperm motility score of birds administered with 300 mg ENU from week 5 through successive weeks to week 11, conforms with the observations of Yin *et al.* (2015) in mice, indicating a similarity in the temporal programme of

fundamental biology governing recovery from ENU toxicity between Rodentia and Aves. Though in the latter, recovery is incomplete in the period. The basis of delayed full recovery in the chicken bears examination in subsequent studies.

4. CONCLUSION AND RECOMMENDATION

This study provides novel evidence in chickens that, the effect of ENU on relative-sterility and recovery, which are associated with its mutagenic effects, are detectable through the determination of sperm parameters. Furthermore, it sheds light on the temporal profile of ENU action in the chicken. Specifically, it shows that ethanol, a matrix component of the ENU administration buffer, is chiefly responsible for the initial adverse effect of ENU treatment protocols on sperm parameters between the first and second week of ENU administration. The key ENU effect on sperm parameters is detectable from weeks 2-3, following exposure and persists to week 11, while a nadir is observed at week 4. In the chicken, a failure of complete recovery of sterility at week 11, following ENU exposure points to fundamental differences in response relative to mice and the underlying mechanisms for this observed difference should be examined. Sperm parameters should be used as surrogate indicators of the mutagenic effect of ENU in chickens because they reveal effects on sterility (which are associated with mutagenicity in the established literature in mice).

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Ethical Statement:

All experimental protocols were conducted in compliance with institutional guidelines on use of animals in scientific experiments and within terms approved under University of Ilorin Ethical Review Committee licence UERC/ASN/2015/117.