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Evaluation of the Histomorphometric Evidences of Hydroxyurea-induced Testicular Cytotoxicity in Sprague-Dawley Rat

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ABSTRACT
Hydroxyurea (HDU) is approved for reducing the frequency of painful crises and the need for blood transfusions in adults with sickle cell disease who experience recurrent moderate to severe pain. Treatment with HDU is however, associated with known side effects such as cytotoxicity and myelosuppression. In the present study we evaluated the effect of a clinically relevant dose of HDU used in the treatment in sickle cell disease on the seminiferous tubules of rats. Adult male Sprague -Dawley rats were orally treated with 25 mg HDU kg^{-1} body weight/day for 28 consecutive days. Control rats received the vehicle for HDU which was normal saline 2.5 mL kg^{-1} body weight. Groups of rats were sacrificed variously on the next day, the 56th and the 112 day after the last dosing with HDU or saline. The *testis* were recovered, weighed and subjected to histopathology. The gross anatomical parameters assessed included the testicular weights and volumes while stereological parameters estimated includes diameter and cross-sectional area of the seminiferous tubules; number of profiles per unit area and numerical density of seminiferous tubules. The results show that treatment with HDU exhibited significant atrophic degeneration in the seminiferous tubules compared with controls. There was an initial manifestation of progressive worsening of the testicular profiles with passage of time, as the animals sacrificed on day 56 demonstrated greater toxicity than those autopsied a day after day 28. However, the animals sacrificed on day 112 showed some improvement in their testicular profiles, suggesting some degree of self-reversal or recovery of the effect. We conclude that HDU has a deleterious effect on the rat testis even at the clinically relevant dose used in management of sickle cell disease.

Key words: Hydroxyurea, morphometry, seminiferous epithelium, cytotoxicity, histopathology

INTRODUCTION
Sickle cell disease (also known as sickle cell anemia or sickle cell disorder) is a common genetic condition due to a haemoglobin disorder inheritance of mutant haemoglobin genes from both parents. The frequency of the carrier state determines the prevalence of sickle cell anaemia at birth. Nigeria which is by far the most populous country in Africa, has 24% of its population being carriers of the mutant gene and the prevalence of the sickle cell anaemia is about 20 per 1000 births (WHO, 2006).

Sickle Cell Disease (SCD) is characterized by a painful vaso- occlusive crisis resulting from the blockage of capillaries by the interaction of sickle erythrocytes, leukocytes, platelets and plasma

proteins with vascular endothelium (Smiley *et al.*, 2008). Currently there is no cure for SCD. However, HDU, an antineoplastic agent, is commonly used in the treatment of SCD (King, 2003). HDU increases fetal hemoglobin which has a higher oxygen carrying capacity and does not undergo sickling under low oxygen tension, thus improving some aspects of quality of life in patients suffering from moderate to severe SCD (Charache *et al.*, 1995, 1996).

As an antineoplastic agent, the specific action of HDU is on ribonucleotides reductase whose action is to reduce ribonucleotides to deoxyribonucleotides. Hydroxyurea impedes the latter reaction and limits DNA biosynthesis. This makes HU an S-phase-specific cytotoxic and antineoplastic agent that interrupts the cell cycle at the G1 and S phases (Yarbro, 1992). One of the major mechanisms of action of HDU is the production of reactive oxygen species (Vazquez-Sanchez *et al.*, 2009).

It has been postulated that HDU-induced organ toxicity could be due its metabolites particularly Carbamoyl nitroso. Carbamoyl nitroso is easily oxidized to form nitroxyl and nitric oxide. Carbamoyl nitroso may be involved in electron transfer, reactive oxygen species formation and oxidative stress. Thus carbamoyl nitroso, nitroxyl, nitric oxide and metal complexes of the parent drug are designated the main actors in the physiological effects which they mediate by lipid peroxidation (Cokic *et al.*, 2003).

Hydroxyurea is non-selective and treatment with HDU is suspected to be associated with side effects including cytotoxicity (or toxicity to cells) and myelosuppression (or reduced production of red blood cells, white blood cells and platelets) and hydroxyurea can damage DNA (genotoxic) (Perreault *et al.*, 2008; Friedrisch *et al.*, 2008).

Evenson and Jost (1993), Fritz and Hess (1996), Jorge *et al.* (2005) and Vazquez-Sanchez *et al.* (2009) have all carried out studies on the effects of HDU on the rat testis. However, none of these investigations extensively utilized the stereological tool to elucidate convincing histomophormetric evidences of HDU-mediated derangement of the testis. Previous reports also contain only early one-point findings. Furthermore, most of the previous studies made use of doses that are more that triple the clinical doses recommended for management of SCD patients.

Therefore, the aim of the present study was to probe the short, mid and long terms toxic characteristics of a clinically relevant dose of HDU on the *testis* of rats using simple but efficient stereological and morphometric tools.

MATERIALS AND METHODS

Chemicals: Hydroxyurea (®Hydrea, Bristol-Myers Squibb USA) was obtained from Juli Pharmacy, Ikeja, Lagos State, Nigeria in the month of March, 2009.

Animals and interventions: Experimental procedures involving the animals and their care were conducted in conformity with International, National and institutional guidelines for the care of laboratory animals in Biomedical Research and Use of Laboratory Animals in Biomedical Research as promulgated by the Canadian Council of Animal Care (1985). Further the animal experimental models used conformed to the guiding principles for research involving animals as recommended by the Declaration of Helsinki and the Guiding Principles in the Care and Use of Animals (World Medical Association. American Physiological Society, 2002).

The rats were procured from a breeding stock maintained in the Animal House of Lagos State University College of Medcine (LASUCOM). The animals were housed in well-ventilated wire-wooden cages in the Animal Facility of the Department of Anatomy, LASUCOM, Ikeja, Lagos. An approval was sought and obtained from the Departmental ad-hoc Ethical Committee. The rats were

maintained under standard natural photoperiodic condition of twelve hours of light alternating with twelve hours of darkness (i.e., L: D; 12:12) with room temperature of between 25 to 26°C and humidity of 65±5%. They were allowed unrestricted access to water and rat chow (Feedwell Livestock Feeds Ltd., Ikorodu, Lagos, Nigeria). They were allowed to acclimatize for 28 days before the commencement of the experiments. The weights of the animals were estimated at procurement, during acclimatization, at commencement of the experiments and twice within a week throughout the duration of the experiment, using an electronic analytical and precision balance (BA210S, d = 0.0001 g) (Satorius GA, Goettingen, Germany).

Thirty-two male adult (10 to 12 weeks old) Sprague Dawley rats weighing 170-175 g were used for this research work. The rats were randomly divided into four groups of eight rats each such that the average weight difference between and within groups did not exceed ±20% of the average weight of the sample population. Group A rats were given normal saline 2.5 mL kg^{-1} body weigh/day/orally for 28 days and then were sacrificed a day after. Group B animals were orally treated with 25 mg HDU kg^{-1} body weight/day for 28 days. This dosage being clinically relevant in the treatment of SCD (Masood *et al.*, 2007). These rats were sacrificed the day after the last HDU administration. Group C animals had orally 25 mg HDU kg^{-1} body weight/day for 28 days but were sacrificed on the 56th day (i.e., 8 weeks after the last HDU treatment), the duration of spermatogenesis in rat being 51.6-56 days (Heller and Clermont, 1964; Jegou *et al.*, 2002). Group D rats had similar treatment as those in groups B and C except that they were sacrificed on day 112 (i.e., the end of the 16th week) after the last HDU treatment.

Animal sacrifice and sample collection: The rats were at the time of sacrifice first weighed and then anaesthetized by placing them in a closed jar containing cotton wool sucked with chloroform anesthesia. The abdominal cavity was opened up through a midline abdominal incision to expose the reproductive organs. Then the *testis* were excised and trimmed of all fat. The *testis* weights of each animal were evaluated. The *testis* were weighed with an electronic analytical and precision balance (BA 210S, d = 0.0001- Sartoriusen GA, Goettingen, Germany). The testis volumes were measured by water displacement method. The two testis of each rat were measured and the average value obtained for each of the two parameters was regarded as one observation.

The testis was fixed in 10% formol-saline and histological slides prepared. However, prior to embedding, it was ensured that the sections were orientated perpendicular to their long axes and designated as “vertical sections”.

Determination of morphometric parameters: For each testis, five vertical sections from the polar and the equatorial regions were sampled (Qin and Lung, 2002) and an unbiased numerical estimation of the following morphometric parameters was determined using a systematic random scheme (Gundersen and Jenson, 1987).

The evaluation of the diameter was done with calibrated eyepiece and stage grids mounted on a light research microscope. Estimation of volume density of testicular components and number of seminiferous tubules were done on a computer monitor onto whom a graph sheet was superimposed and on which slides were projected from a research light microscope (Saalu *et al.*, 2008, 2010).

Statistical analysis: All data were expressed as Mean±SD of number of experiments (n = 8). The level of homogeneity among the groups was tested using two-way Analysis of Variance (ANOVA) as done by Snedecor and Cochran (1980). Where heterogeneity occurred, the groups were

separated using Duncan Multiple Range Test (DMRT). A value of $p<0.05$ was considered to indicate a significant difference between groups (Duncan, 1957).

RESULTS

Body weights, testis weights and volumes: Table 1 shows that there was a significant ($p<0.05$) decrease in the testis weight, testis weight/body weight ratio and testis volume in the HDU treated groups that were sacrificed the next day and on day 112 after withdrawal of HDU (i.e., groups B and D, respectively). Animals in group C which had HDU but were autopsied on day 56 after the last dosing exhibited a more significant ($p<0.001$) reduction in the testis weight, testis volumes and testis/body weight ratios (0.38±0.1 g, 0.37±0.2 g and 0.002, respectively) when compared to those of the control group (1.25±0.2 g, 1.25±0.6 g and 0.006).

Testis histology: The representative sections of the seminiferous tubules of control rats demonstrated fairly circular or oval profiles with normal seminiferous epithelium and abundant spermatozoa stocked in their lumen, with interstitium well outlined (Fig. 1). The histological profiles of the testis of animals that were HDU- treated and sacrificed on day 112-post treatment approximated those of the control rats except for their slightly more distorted cytoarchitecture (Fig. 4). Treated rats that were sacrificed on the next day and day 56 after last dosing (i.e., groups B and C, respectively) showed greater distortions in their testicular histology (Fig. 2 and 3). There were evidences of degenerative changes in their seminferous epithelium characterized by hypospermatozoa formation, interstitial oedema and vacuolization of the interstitium. As shown in Fig. 3, group C animals exhibited a remarkably worst testicular cytoarchitecture.

Testis morphometry: The morphometric evaluation of the rat testis as shown in Table 2 demonstrated that the tubular diameter, the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the

Table 1: The changes in gross anatomical parameters of sprague dawley rats

Treatment groups	Initial body weight (g)	Final body weight (g)	Body weight diff. (g)	Testis weight (g)	Testis volume (mL)	Testis wt./ body wt. ratio
A	170.7±4.0	178.6±4.3	8.1	1.25±0.2	1.25±0.6	0.006
B	173.2±1.8	169.3±4.5	5.1	0.80±0.1*	0.83±0.3*	0.004*
C	170.4±3.7	145.6±3.6	24.8**	0.38±0.1**	0.37±0.2**	0.002**
D	171.5±3.4	167.5±4.3	4.1	0.83±0.2*	0.85±0.3	0.005

*, **Represent significant decrease at $p<0.05$ and $p<0.001$, respectively when compared to control values. Values are Mean±SEM (n = 8)

Table 2: The changes in morphometric parameters of sprague dawley rats

Treatment groups	D (μm)	Ac ($\times10^3$μm^3)	N_A ($\times10^{-8}$ μm^{-2})	N_V ($\times10^{-10}$ μm^{-2})
A	201.31±8.14	37.50±6.12	34.16±7.15	14.15±4.91
B	134.35±14.20[a]	19.33±6.31[a]	22.46±3.22[a]	10.57±4.23[a]
C	61.41±10.33[b]	4.41±1.24[b]	9.43±1.22[b]	5.63±2.64[b]
D	139.35±8.12[a]	25.65±1.04[a]	24.41±1.32[a]	9.33±1.52[a]

[a,b]Represent significant decrease at $p<0.05$ and $p<0.01$, respectively when compared to control values; Values are Mean±SEM (n = 10)
D = Tubular diameter, Ac = Cross- sectional area of tubules, NA = No. of tubules propfile per unit area, Nv = Mean numerical density of seminiferous tubules

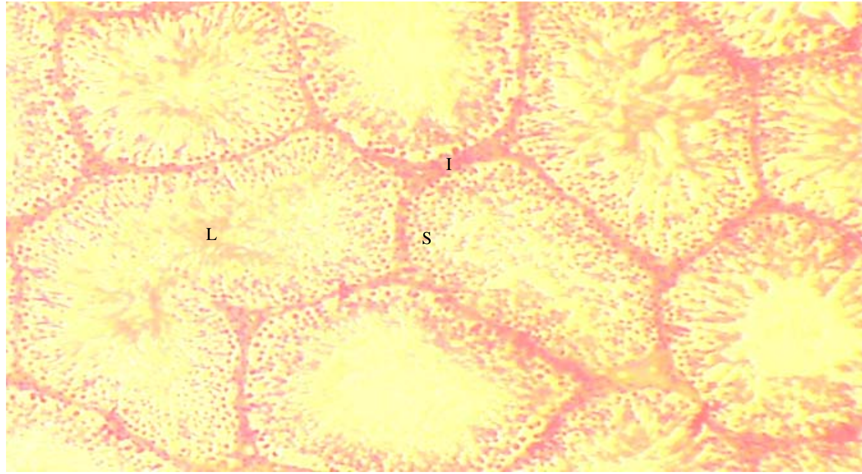

Fig. 1: Cross-section of testis of rat treated with normal saline (2.5 mg/kg/day/ orally for 28 days and then sacrificed the next day (CONTROLS). Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

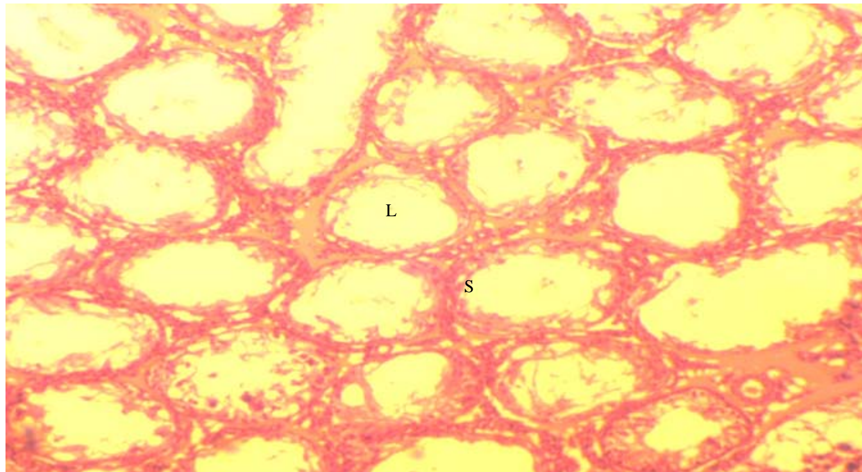

Fig. 2: Cross-section of testis of rat treated with hydroxyurea 25 mg kg^{-1} body weight/day orally for 28 days and were sacrificed the next day. Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

treated animals that were sacrificed the next day (134.35±14.20 µm, 19.33±6.31 × 10^3 μm^3, 22.46±3.22 ×10^{-8} μm^{-2} 10.57±4.23 ×10^{-10} μm^{-2}) and on day 112 after HDU withdrawal (139.35±8.12 µm, 25.65±1.04 × 10^3 μm^3 24.41±1.32 ×10^{-8} μm^{-2} 9.33±1.52 ×10^{-10} μm^{-2}) were significantly ($p<0.05$) reduced as compared to the control animals (201.31±8.14 µm, 37.5±6.12 × 10^3 μm^3 34.16±7.15 ×10^{-8} μm^{-2} 14.15±4.91 ×10^{-10} μm^{-2}).

However, there was an even greater statistically significant ($p<0.01$) reduction in the tubular diameter, the cross-sectional area of the tubules, the number of tubular profiles per unit area and the mean numerical density of seminiferous tubules of the animals that were treated with HDU but

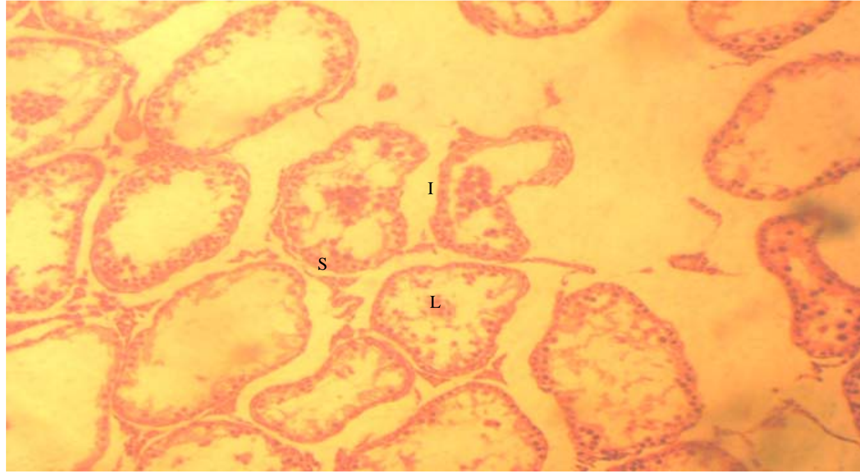

Fig. 3: Cross-section of testis of rat treated with Hydroxyurea 25 mg/kg body weight/day orally for 28 days and were sacrificed on day 56 after the last dosing. Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

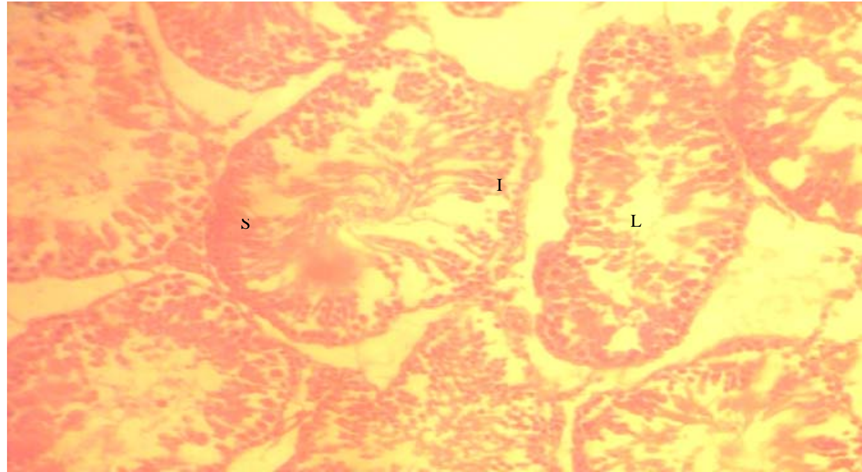

Fig. 4: Cross-section of testis of rat treated with Hydroxyurea 25 mg/kg body weight/day orally for 28 days and were sacrificed on day 112 after the last dosing. Showing the seminiferous tubules; L: Lumen; S: Seminiferous epithelium; I: Interstitium; Stains: haematoxylin and eosin; Mag: x400

autopsied on the 56th day after the last dosing (61.41±10.33 μm, $4.41\pm1.24 \times 10^{8}\mu m^{3}$ $9.43\pm1.22 \times10^{-8}$ μm^{-2}, $5.63\pm2.64 \times10^{-10}\mu m^{-2}$).

DISCUSSION

Several investigators (Evenson and Jost, 1993; Wiger *et al.*, 1995; Li *et al.*, 2008) have reported that administration of HDU can decrease the testicular weight of rats. Present study also showed that the absolute testicular weights, testicular weight/body weight ratio and testicular volumes of

rats that were administered HDU were significantly ($p<0.05$ or $p<0.001$) lower than those of the control group. This could be attributed to severe parenchyma atrophy in the seminiferous tubules following HDU challenge.

Results from this study showed that there was a varying degree of testis histological and morphometric derangement in all groups of animals that had HDU administration. The testis of these animals exhibited a general destruction coupled with derangement of cells of the seminiferous epithelium. Qualitative assessment revealed a significant reduction in their diameter, cross-sectional area, number of profiles and numerical density when compared to the control. These were also the findings by several researchers (Evenson and Jost, 1993; Fritz and Hess, 1996; Jorge *et al.*, 2005; Vazquez-Sanchez *et al.,* 2009) even though their investigative animal models utilized doses of HDU those were not clinically relevant for the treatment of SCD. Present findings in this respect are however, at variance with those of Singh and Taylor (1981) who had earlier reported no noticeable effects on the testicular structure or on general physiology of animals following HDU chemotherapy. Similar findings were reported from our laboratory using other antineoplastic agents (Saalu *et al.*, 2007, 2009).

The mechanism of HDU toxicity to organs including the testis is still not well understood. However, it has been postulated that it could be due its metabolites particularly Carbamoyl nitroso. Carbamoyl nitroso is easily oxidized to form nitroxyl and nitric oxide. Carbamoyl nitroso may be involved in electron transfer, reactive oxygen species formation and oxidative stress.

Worthy of note is also the findings that the manifest testicular histo-morphometric damage progressively worsened with passage of time for some period after cessation of HDU treatment. Oxidative stress and lipid peroxidation (self-propagating) mechanism of HDU cytotoxicity could provide a plausible explanation this outcome. The dramatic reduction in the testicular injury observed for animals that were autopsied on day 112 after withdrawal of HDU could mean that by that time there was already a degree of reversal of the HDU-induced testicular cytotoxicity.

CONCLUSION

We conclude that administration of even clinically relevant doses of HDU induces morphological and morphometric derangement of the testis of Sprague-Dawley rats. Furthermore, there is a progressive worsening of testicular derangement with time even after the stoppage of HDU therapy, but that a certain amount of self-reversal of the HDU-induced testicular toxicity is a possibility after a prolonged period of time.

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