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Vitamin C Supplementation Protects Against Hydroxyurea - Induced Testicular Damage in Male Wistar Rats

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Abstract **Original Article**

Introduction: Hydroxyurea (HU) is used to treat cancers, sickle cell disease (SCD), and thalassemias but it induces reproductive toxicity through oxidative stress. Vitamin C, an antioxidant, has been shown to mitigate reproductive toxicity. This study examines Vitamin C's effects on HU-induced male reproductive toxicity.

Method: Thirty rats weighing 180-250g were divided into six groups (A, B, C, D, E, F) with 5 rats each. Group A was the control, Group B received 100mg/kg body weight of Vitamin C (low dose), Group C received 200mg/kg (high dose), and Groups D, E, and F received 300mg/kg body weight of HU. Groups E and F also received low or high dose Vitamin C, respectively. After six weeks, blood samples were analyzed for FSH, LH, and testosterone levels, and testis tissue was stained with Hematoxylin and Eosin.

Results: Results showed that neither HU nor Vitamin C co-treatment significantly affected FSH and LH levels. HU alone reduced testosterone significantly compared to the control, but this reduction was reversed with both low and high-dose Vitamin C co-treatment. HU caused degenerative changes in the testis, which were reversed only in the high-dose Vitamin C group.

Conclusion: The findings suggest that Vitamin C supplementation could help protect against HU-induced testicular damage and preserve fertility in patients undergoing HU treatment.

Keywords: Hydroxyurea, Male reproductive system, Oxidative stress, Reproductive hormones, Reproductive toxicity and Vitamin C.

INTRODUCTION

Hydroxyurea (HDU) is an antineoplastic drug commonly used to treat various cancers (such as melanoma, prostate cancer, ovarian cancer, transitional carcinoma of the urogenital tract, cervical cancer, and squamous cell carcinoma of the head and neck), as well as sickle cell disease and thalassemia. Its therapeutic effect is based on its ability to inhibit DNA synthesis by blocking the

ribonucleotide reductase enzyme, leading to a reduction in deoxynucleotide triphosphates (especially dATP) levels (1,2). In experimental animals and humans, HDU has also been linked to spermatogenic arrest, reduced sperm production, and male infertility] 3-10]

The cytotoxic effects of HDU are thought to be caused by the formation of carbamoyl nitroso, a metabolite that may contribute to the generation of reactive oxygen species (ROS), thereby inducing oxidative stress [11]. Excessive ROS production can harm spermatozoa, which is associated with male subfertility. The high concentration of unsaturated fatty acids in spermatozoa's plasma membrane makes them more susceptible to oxidative damage [12].

Vitamin C is a water-soluble antioxidant that protects cells from water-soluble free radicals. It also plays a role in reducing and regenerating oxidized forms of other vitamins [13]. As one of the most accessible and cost-effective non-enzymatic antioxidants, it is commonly used to reduce oxidative damage [14]. Vitamin C effectively neutralizes reactive oxygen species (ROS) and reactive nitrogen species (RNS) in the body [15]. This readily available, inexpensive, and relatively safe antioxidant is highly beneficial in reducing the harmful effects of various toxic substances [16]. Vitamin C has been shown to alleviate testicular toxicity induced by various substances, including lead, abamectin, crude oil, chlorpyrifos, and others, as demonstrated by earlier studies [17-21]. The study was designed to evaluate the effect of Vitamin C on HU-induced reproductive toxicity. This study was designed to evaluate the effect Vitamin C on Hydroxyurea-induced male reproductive dysfunction in rats.

MATERIALS AND METHODS

Experimental Animals:

Thirty adult male Wistar rats weighing between 180g-250g were used. The Wistar rats were kept in cages in the LASUCOM Animal House and maintained at room temperature with approximately 12 hours dark and 12 hours light cycle. The rats were provided with standard rat chow and water *ad labitum* during this study. This study was conducted following approval from Lagos State University College of Medicine Animal Research Ethics Committee with **Ref. No: AREC/2023/029.**

Drugs

Hydroxyurea and Vitamin C.

Hydroxyurea manufactured by Bond chemical Ind. Ltd, Oyo State, Nigeria and Vitamin C produced by Kunimed Pharmachem Ltd were commercially purchased for the study.

Experimental Design

The rats were divided into six groups (A, B, C, D, E and F) with 5 rats in each group. Group A was the control while groups B, C, D, E and F were the test groups. All drugs were administered via oral gavage. Group A was control. Group B received 100mg/Kg body weight of vitamin C (Low dose Vitamin C) once daily. Group C received 200mg/Kg body weight of vitamin C (High dose Vitamin C) once daily.

Group D received Hydroxyurea 300mg/kg body weight once daily. Group E received both Hydroxyurea 300mg/kg body weight and Low dose Vitamin C (100mg/Kg body weight). Group F received both Hydroxyurea 300mg/kg body weight and high dose Vitamin C (200mg/Kg body weight). The study carried for for six weeks.

Sacrifice of Male Rat, Blood Sample Collection and Removal of Reproductive Organs:

The rats were injected intraperitoneally with 100mg mg/kg body weight of ketamine and were observed for signs of anesthesia such as drowsiness and loss of consciousness after which they were sacrificed when reflexes were lost.

Blood samples of each rats was collected by cardiac puncture using 5mls syringe. The samples were placed in plain bottles and allowed to clot, followed by centrifuging at 2500rpm for 20 minutes using a

desktop centrifuge (Surgifriend centrifuge, Model SMBO-2, England). This process separates the sera from the blood cells. The sera were aliquoted into an eppendorf tube and stored at 20^0C. The frozen blood samples were used for hormonal assay. The testes were preserved in 10% buffered formalin.

Hormonal Assay:

Follicle Stimulating Hormone (FSH), Luteinizing Hormone (LH) and Testosterone Assay were carried out using commercially availabable Accubind ELISA microwells kit (monoband Inc. Lake Forest CA 92630, USA) according to manufacturer' instruction.

Heamatoxylin and Eosin (H&E) Tissue Staining

This was carried out as described by [22,23]. In this procedure, the collected organs previously stored in 10% formalin were cut in slabs of about 0.5cm thick transversely and fixed in 10% formalin for a day after which it was transferred to 70% alcohol (ethanol) to cause the transversely cut tissues to become dehydrated. The tissues were passed through 90% of alcohol and chloroform for different durations before they were eventually transferred into two changes of molten paraffin wax for 20minutes each in an oven of 570C. Serial sections were cut using rotary microtome at 5microns. Slides were prepared from these tissues. The slides were then de-waxed and passed through absolute alcohol (2 changes); 70% alcohol and then to water for five minutes. The slides were then stained with haematoxylin and eosin. Photomicrographs of stained slides were captured using a camera attached to a microscope.

Statistical Analysis

Data were expressed as Mean $\pm$ SEM (standard error of mean) where applicable and statistical analysis were carried out using one way analysis of variance (ANOVA) followed by multiple comparison using turkey's post-hoc test. Paired data analyzed using t-test were applicable. GraphPad Prism version 8.0.2 (GraphPad Software, Inc., La Jolla, CA, USA) statistical software was used for the analysis and $p<0.05$ was considered statistically significant.

RESULTS

The effect of Ascorbic Acid on reproductive hormones in rats treated with hydroxyurea

There was no statistical significant difference in FSH of the group treated with hydroxyurea alone (group D) when compared with the control or the groups co-treated with Vitamin C (F). High dose Vitamin C administered alone in group C caused statistical significant elevation in FSH when compared with group A ($p=0.005$), group B ($p=0.0004$), group D ($p=0.0003$), group E ($p=0.004$).

There was no statistical significant difference in LH of the group treated with hydroxurea alone (group D) when compared with the control or the group co-treated with vitamin C (F). High dose Vitamin C administered alone in group C caused statistical significant elevation in LH when compared with group A ($p=0.01$), group B ($p=0.003$), group D ($p<0.0001$), group E ($p=0.0001$), group F ($p<0.0001$).

High dose Vitamin C administered alone in group C caused statistical significant elevation in testosterone when compared with group A ($p=0.009$), group B ($p=0.002$), group D ($p<0.0001$), group E ($p<0.0001$), group F ($p<0.004$). There was statistical significant reduction in testosterone of the group treated with hydroxurea alone when compared with control .There was statistical significant reduction in testosterone of the group treated with hydroxurea alone (group D) when compared with the control, and the group co-treated with either low dose vitamin C (group E) ($p<0.0001$) or high dose vitamin C (F) ($p<0.0001$) and hydroxyurea.

Table 1: The Effect of Ascorbic Acid on Reproductive Hormones in Rats Treated With Hydroxyurea

	Control	Vitamin C 200mg/kg	Vitamin C 400mg/kg	Hydroxyurea (HDU) 300mg/Kg	Vitamin C 200mg/kg + HDU	Vitamin C 400mg/kg + HDU
Follicle stimulating hormone (FSH)(nmol/l)	0.6 ± 0.05^{a}	0.49 ± 0.02^{a}	1.06 ± 0.16^{b}	$0.48\pm0.03^{a}c$	0.59 ± 0.07^{a}	0.46 ± 0.02^{a}
Luteinizing Hormone(LH) nmol/l)	0.99 ± 0.09^{a}	0.94 ± 0.03^{a}	1.32 ± 0.07^{b}	0.69 ± 0.08^{c}	0.82±0.03ac	0.73 ± 0.04^{ad}
Testosterone (nmol/l)	4.64 ± 0.21^{a}	4.39 ± 0.42^{a}	$6.05\pm0.0.27^{b}$	1.05 ± 0.12^{c}	3.94 ± 0.22^{a}	4.49 ± 0.22^{a}

The results are expressed as Mean +SEM. The data with different superscripts (a, b, c and d) are statistically significant ($p<0.05$) when compared across groups.

Effect of Vitamin C on H&E-Stained Sections of the Testis in Hydroxyurea-Treated Rats (Figure 1)

Group A (Control)

The seminiferous tubules appear normal, showing spermatozoa at different stages of development. Sertoli cells and Leydig cells are present.

Group B (Low-Dose Vitamin C)

The testicular architecture remains normal, with intact seminiferous tubules containing spermatozoa at various developmental stages. Sertoli cells and Leydig cells are also observed.

Group C (High-Dose Vitamin C)

Histological examination reveals normal seminiferous tubules with active spermatogenesis. Sertoli cells and Leydig cells are present, similar to the control group.

Group D (Hydroxyurea Only)

Severe degenerative changes are evident in the testes, characterized by complete destruction of the seminiferous tubules and failure of spermatogenesis. Most tubules exhibit vacuolation.

Group E (Hydroxyurea + Low-Dose Vitamin C)

Marked testicular degeneration is observed, with extensive seminiferous tubule destruction and impaired spermatogenesis. Vacuolation is prominent in most tubules, similar to the Hydroxyurea-only group.

Group F (Hydroxyurea + High-Dose Vitamin C)

The seminiferous tubules appear largely intact, showing spermatozoa at various developmental stages. Sertoli cells and Leydig cells are present, suggesting a protective effect of high-dose Vitamin C against Hydroxyurea-induced testicular damage.

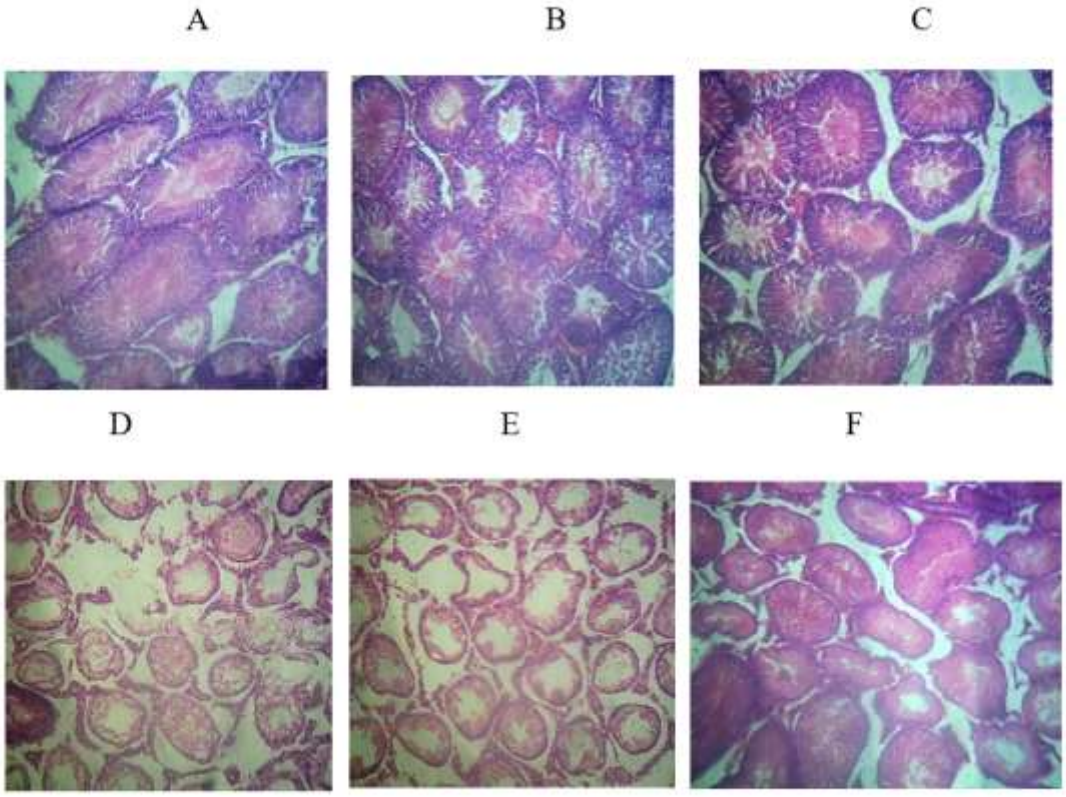

Fig 1: Photomicrograph of the H/E stained section of the testes of group A to F. Mag x100. Group A to C shows normal seminiferous tubules of the testes with present successive stages of spermatogenesis. Group D and E showed reduced seminiferous epithelium, reduced stages of spermatogenesis and seminiferous lumen also showed absent spermatozoa. Group F shows normal seminiferous tubules of the testes with present successive stages of spermatogenesis.

DISCUSSION

HU did not significantly affect the production of both FSH and LH when compared with controls rats in this study. Our findings however disagree with earlier reports of Virguos *et al.*[10] and Abdulhadi *et al.* [9]. Virguos *et al.* [10] reported significant elevation of both FSH and LH while Abdulhadi *et al.* [9] a significant suppression in the production of both hormones when compared with controls. These findings imply HU may not have significant effect on the gonadotropin production. Both low and high dose vitamin C did not significantly improve the production of FSH and LH in the HU-treated rats in this study, although high dose Vitamin C caused significant elevation in both hormones when compared with HU treated groups in this study.

HU caused a significant reduction in testosterone in the exposed rats and this finding aligns with previous study [9]. Vitamin C however reversed the toxic effect as evidenced by statistical significant elevation in testosterone in the groups co-treated with HU and either low dose or high dose Vitamin C when compared with the group treated with HU alone. The ability of vitamin C to improve testosterone in this study align with several reproductive toxicity where vitamin C have been demonstrated to improve testosterone production [17-21].

HU caused degenerative changes in the test is affecting all stages of spermatogenesis. Above effect of HU aligns with earlier reports of its effect on testis [9,10).Hydroxyurea induces this damage by generating generates reactive oxygen species (ROS) as a byproduct of its metabolism, leading to oxidative stress. This oxidative stress damages cellular macromolecules, including lipids, proteins, and DNA, ultimately resulting in cellular dysfunction and apoptosis and this manifests as impaired spermatogenesis (24-26) as seen in this study. This effect was ameliorated by Vitamin C at a dose of 400mg/kg body weight but not at a dose of 200mg/kg body weight. Possible mechanism by which Vitamin C ameliorated this toxic effect of HU is because of its crucial role in neutralizing ROS generated by HU and protecting cells from oxidative damage. Vitamin C improved testicular histopathology and this suggests that vitamin C not only protects against oxidative damage but also supports the recovery of spermatogenic cells and the maintenance of testicular function. The restoration of testicular architecture and spermatogenesis in the vitamin C-treated groups underscores its potential as a therapeutic agent in mitigating drug-induced testicular toxicity. It appears the ameliorative effect of Vitamin C on HU induced testicular toxicity is dose dependent. This ameliorative effect of vitamin C on testicular degenerative changes align with previous studies on the ability of this antioxidant on several testicular toxicity Vitamin C has been shown to alleviate testicular toxicity induced by various substances, including lead, abamectin, crude oil, chlorpyrifos, and others, as demonstrated by earlier researchers [17-21]. The testis plays important roles in the male reproductive system and this include testosterone production and spermatogensesis. The reduced testosterone in HU-treated rats further confirms the impairment of testicular function caused by the degenerative changes in the rats while elevated Testosterone in the groups treated with both Vitamin C and HU is a confirmation of restorative action of Vitamin C on testicular function.

In summary, the results of this study have significant clinical implications, particularly for patients undergoing hydroxyurea therapy. Hydroxyurea is widely used in the treatment of sickle cell anemia, chronic myeloid leukemia, and other conditions. However, its testicular toxicity poses a challenge, especially for male patients of reproductive age. The findings suggest that vitamin C supplementation could be a viable strategy to protect against hydroxyurea-induced testicular damage and preserve fertility in these patients.

Conflicts of Interest

The Authors declare that they do not have any competing interests regarding this article

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Nil

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