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Original article

Impact of Vitamin C and Hydroxyurea on the Reproductive System Health in Male Rats

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ABSTRACT

Hydroxyurea (HU) is a medication used to treat various diseases and is also being studied for its effects on spermatogenesis. Vitamin C (VC), an antioxidant, has been shown to protect sperm cells from oxidative stress, potentially improving fertility and sperm quality. In a study involving twenty-four adult male albino rats divided into four groups, Group 1 served as the control, Group 2 received 5 mg of vitamin C, Group 3 received 100 mg of hydroxyurea per body weight, and Group 4 received a combination of vitamin C and hydroxyurea to assess essential functions of the reproductive system, including hormone levels, antioxidant markers, oxidative stress indicators, histopathology, and identification of DNA damage. The study found that hydroxyurea significantly reduced testicular weight, SOD, CAT, and GSH while increasing FSH and MDA levels and causing abnormalities in sperm morphology. Hydroxyurea also caused apparent alterations in the histological structure of the testes and comet parameters. Rats treated with vitamin C showed a significant increase in absolute and relative epididymis weight compared to the control group. Moreover, vitamin C intervention reversed the adverse effects observed in rats fed hydroxyurea, indicating that low doses of vitamin C can protect against testicular damage.

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INTRODUCTION

Hydroxyurea (HU) and vitamin C (VC) have attracted attention in recent research for their potential effects on testicular function [1]. Hydroxyurea is widely used to treat hematologic diseases and certain malignancies by inhibiting DNA synthesis, which affects cells with high rates of proliferation [2, 3]. Reported adverse reactions to hydroxyurea include changes in blood cell counts, suppression of bone marrow function [4], and negative effects on spermatogenesis, testicular atrophy [5], reversible decline in sperm count, motility [6], and morphology. This medication also leads to alterations in chromatin structure in preleptotene spermatocytes [7]. Additionally, HU induces apoptosis in spermatogonia and early spermatocytes without any discernible negative impact on stem spermatogonia [8]. This suggests that spermatogenesis may recover after discontinuing HU treatment. Animal experiments show that HU dosages are significantly higher than the typical therapeutic range for individuals with sickle cell disease (SCD) [9].

VC, an essential nutrient renowned for its involvement in collagen formation, antioxidant protection, and immunological function, also crosses the domain of male reproductive health [10]. According to existing research, VC has been found to play a role in protecting sperm cells from oxidative stress, a condition that can have detrimental effects on fertility and the quality of sperm [11]. There is evidence suggesting a positive correlation between adequate VC consumption and enhanced sperm parameters [12]. However, it is crucial to maintain a balance, as excessive intake of VC, especially

through the use of supplements, has the potential to disrupt the delicate balance of testicular activity. Like any other nutrient, it is important to consume it in moderation in order to maximize the benefits and avoid any difficulties. Although sufficient consumption of VC appears to have positive effects on sperm quality and fertility, an excessive amount of this nutrient, especially when obtained through supplementation, may disrupt the delicate hormonal and cellular balance necessary for good testicular function [13]. The appropriateness of VC intake should consider individual differences, health status, and dietary patterns.

Achieving a comprehensive understanding of the impact of HU and VC on testicular health necessitates the incorporation of several perspectives. Although HU is not primarily intended for the testes, healthcare professionals should be aware of any potential secondary effects on male reproductive health, especially when the drug is used for a long period [14]. Striking a balance between the benefits of HU in the management of blood disorders and its possible impact on fertility requires a collaborative approach involving healthcare professionals and individuals seeking treatment. [15]. In light of the ever-changing landscape of medical knowledge, the impact of HU and VC on testicular function continues to be an area of active research. This study aimed to investigate the impact of VC on reducing the damage caused by HU in the testicular in Swiss albino male rats.

MATERIALS AND METHODS

Designing experiments and administering drugs

Twenty-four male albino rats, weighing between 170 and 220 g, were obtained from the Animal Breeding House of the National Research Centre (NRC) in Dokki, Giza, Egypt. The rats were housed in polypropylene cages, with six rats per cage, and were given free access to food and water under controlled conditions, which included a 12-hour light cycle, a temperature of 23°C, and a minimum relative humidity of 48%. The rats were acclimated for a week before the experiment began. The rats were divided into four groups based on the dosage of the substance, with six rats in each group. The rats were administered the substances via oral gavage on a daily basis. Group 1, the control group, was not administered any medicine; Group 2 was administered 5 mg of VC therapy; Group 3 was administered HU capsules containing 100 mg, and Group 4 was administered a combination of VC + HU therapy at the same dosages as groups 2 and 3. Before administration, HU 100 mg and 5 mg of VC were dissolved in purified water and administered orally to the rats for 30 days. Following the treatment period, the rats were fasted overnight and then anesthetized with sodium pentobarbital. Blood samples and biochemical tests were taken to evaluate testicular function.

Assessing sperm parameters

The motility of sperm was analyzed by cutting the caudal epididymis and examining at least 200 sperm from each animal in five microscopic fields per sample. Similarly, the left caudal epididymis was dissected, cultured, and fixed with formaldehyde. The total number of sperm counts was quantified and expressed as counts per mL as the method described by [16, 17, 18]. Counting was done under a light microscope at 40X magnification and expressed as a million/ml of suspension [19, 20]. Sperm morphology and viability were assessed using eosin-nigrosine staining, with a total of 100 spermatozoa observed in each sample to detect any abnormalities, as estimated by [20-23].

Biochemical analysis of serum

Samples were prepared according to the guidelines of each test, and were processed as follow:

Hormones in serum

The testosterone levels were measured following the method outlined by Södergard and colleagues [24], which was also confirmed by Vermeulen and others [25]. The samples were evaluated using a single assay, which included serum hormone assays and immunofluorometric assays (Delfia, Wallac Oy, Finland) for FSH and LH levels, as previously described by Page and team [26]. The intra-assay and inter-assay coefficients for LH and FSH were 5.6% and 13.9%, respectively. All samples were batched and measured in a single assay.

Oxidative stress biomarkers enzymes in testicular tissues

A spectrophotometer was utilised to analyse the activity of an enzyme called SOD (superoxide dismutase) in a solution containing Tris, EDTA-Na2, and pyrogallol. The enzyme activity was measured at a wavelength of 420 nm, as estimated by Marklund and Marklund [27]. CAT (catalase) activity was calculated using the technique described by Sinha [28]. The fluorescence of the glutathione (GSH) assay was determined using a fluorescence spectrophotometer, following the method outlined by Hissin and Hilf [29]. The tissue MDA (malondialdehyde) level was estimated by measuring the

fluorescence intensity of the n-butanol layer and the standard solution. The measurement of content was performed using the Jasco spectrofluorometer method of Yagi [30] and the method estimated by Buege and Aust [31].

Histopathological studies

Histological analysis, following the method of Russell [32], includes the use of rat testicular tissues that have been fixed in Bouin's solution, processed, embedded in paraffin, and cut into micron-thick sections. Blue nuclei were stained, and purple, while cytoplasm is stained using hematoxylin and eosin staining for microscopic analysis, which includes detection of abnormalities and pathological examination.

Comet assay

To measure the induced DNA damage, we used single-cell gel electrophoresis (SCGE) following the method detailed by Tice [33]. In the comet assay for testicular tissues, small portions of the tissue were turned into a solution of individual cells. These cells were then embedded in an agarose gel on a microscope slide, lysed to remove cellular membranes, subjected to electrophoresis to separate fragmented DNA from intact DNA, and finally stained with fluorescent dyes to visualise DNA fragments. The resulting comet formations were then observed using a fluorescent microscope to quantify the DNA damage.

Statistical analysis

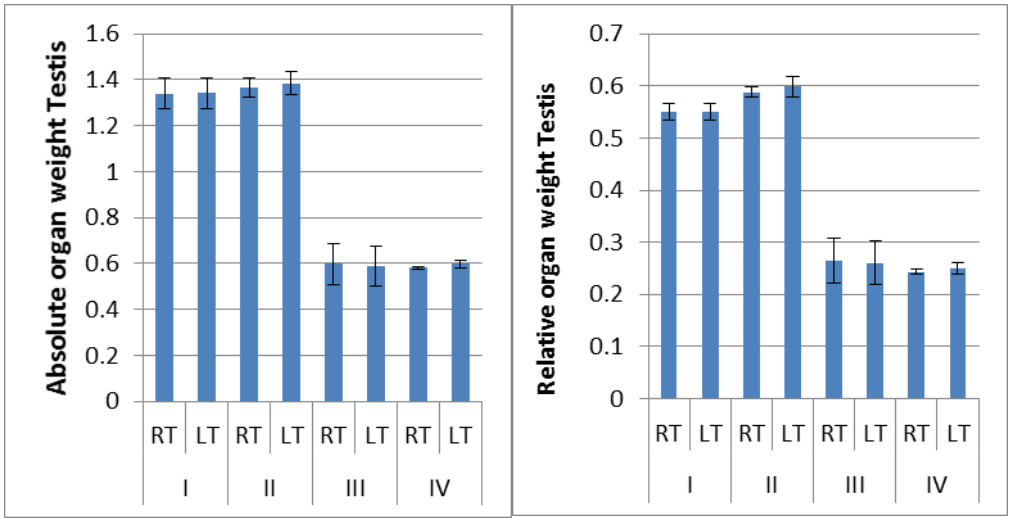
The data was analysed using the SPSS program (version 20) with a one-way ANOVA followed by Duncan's multiple range test (DMRT). The data was expressed in the format of mean ± standard error (SE). Statistically significant P values were considered as < 0.01 and 0.05.

RESULTS

The results of the current study indicate that exposure to HU and oral administration of VC and their combinations for 30 days did not produce any mortality in Wistar rats.

Genital organs weight

The results in Figure 1 show a significant reduction in testicular weight and relative weight (right & left) in groups III (HU with 100 mg) and group IV (VC with 5 mg + HU with 100 mg) after 30 days of the experiment compared with the control group. In group II, there was also a significant increase ($p<0.05$) in absolute and relative left epididymis. Furthermore, group IV showed a significant increase in absolute and relative right epididymis at ($p<0.05$) and ($p<0.01$), respectively, compared to the control group, as detailed in Table 1.



(**a**) (**b**)

Figure 1. The impact of oral administration of VC, HU and a combination of HU+VC at the same doses on the absolute and relative testes weights of the rats.

Table 1. Effect of HU and VC on Absolute and relative epididiymis weight of the rats.

Treatments	Absolute		Relative	
	LE	RE	LE	RE
I	.1150±.00764	.1033±.00667	.048333±.0030732	04233±.002883
II	.1400±.01125*	.1200±.00966	.061167±.0058276*	.05217±.004600
III	.1017±.00833	.0983±.00833	.045000±.0034157	.04317±.004053
IV	.1050±.00224	.1333±.00333*	.043850±0014259	.05833±001667**

*Values are means ± SE. Significantly different from control (P< 0.05). *Significantly different from control (P< 0.01). **SE: Standard Error.*

Sperm quality analysis

The analysis of different sperm parameters revealed variations in sperm motility, concentration, percentage of live sperm, and abnormality between the control and treated groups. The findings indicated that the administration of a 100 mg/body weight dose of HU led to a significant decrease in both sperm motility ($p < 0.05$) and percentage of live sperm ($p < 0.01$), along with a notable increase in abnormal sperm ($p < 0.01$) compared to the control group, as illustrated in Figure 2.

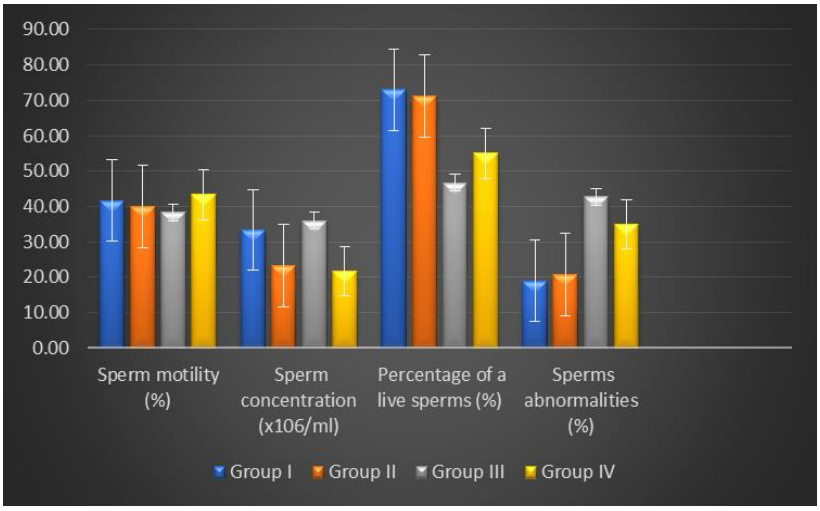


Figure 2. The impact of orally administering VC, HU and a combination of HU+VC at the same doses on the sperm quality of rats.

In Figure 3, there was a noticeable rise in sperm abnormalities (head and tail) at a significance level of $p<0.01$ in the group treated with HU alone compared to the control group. These sperm anomalies are shown in Figure 4 and include sperm with amorphous head, sperm with amorphous head and without tail, sperm with folded head, sperm without a head, sperm with cut head, and sperm with folded tail.

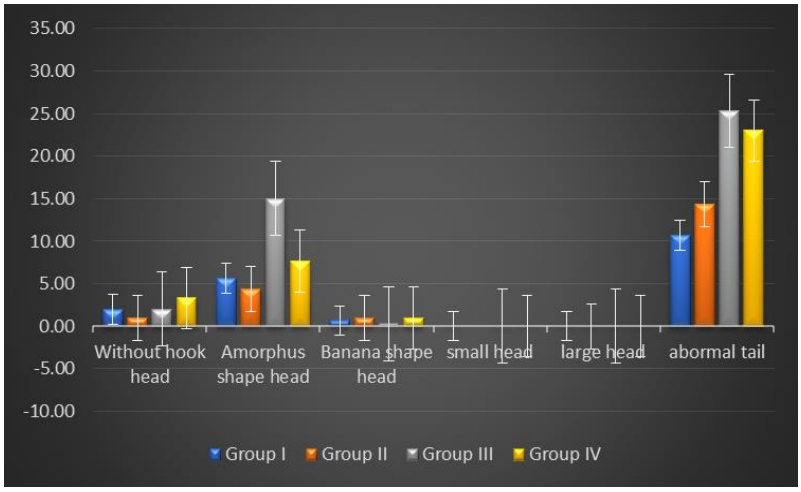


Figure 3: The impact of oral administration of VC, HU and a combination of HU and VC at the same doses on sperm abnormalities in rats.

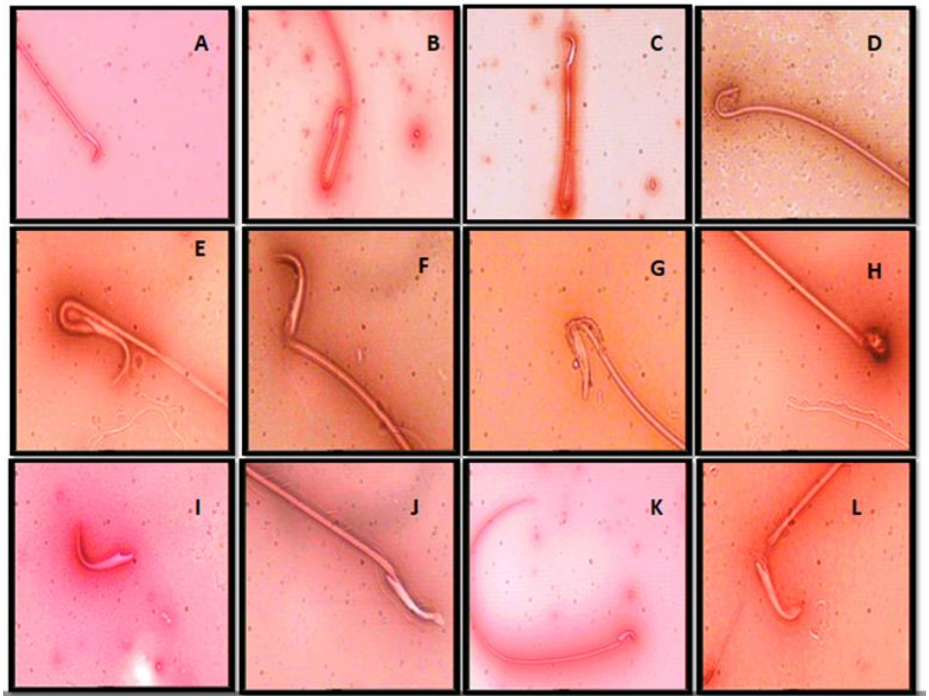


Figure 4: Light microscopy of normal and abnormal rat spermatozoa. All spermatozoa were classified using standard morphologic assessment. Normal spermatozoan (control) (A); common tail defects (B-D) [Sperm with folded tail (B), Sperm with bent tail (D)]; misshapen head or common head defects (E-I) sperm with an elongated head (E+F), sperm with irregularly shaped head (G), Globozoospermia or round-headed sperm with absent acrosome (H), Sperm with absent tail (I)]; acrosome defect (J); Sperm with small head and tail (K); mid-piece defects (L).

Biochemical results

The impact of VC and HU on FSH, LH, and testosterone levels in the serum is summarized in Table 2. Administration of VC in rats led to significant increases in FSH and testosterone levels compared to the control group ($p < 0.05$). There were no significant changes in LH levels observed in any of the treated groups compared with the control group.

Table 2. Effect of HU and VC on FSH, LH and Testosterone levels in sera of male rats.

Treatments	FSH	LH	Testosterone
I	2.100±.108	1.542±.193	5.750±.250
II	3.180±.407*	1.900±.103	6.650±.221*
III	4.080±.230**	1.880±.500	5.500±.168
IV	3.350±.170*	1.252±.126	6.450±.193*

*Values are means ± SE. Significantly different from control (P< 0.05). *Significantly different from control (P< 0.01). **SE: Standard Error.*

The results of the testicular antioxidant status and lipid peroxidation are shown in Table 3. The exposure of rats to HU for 30 days led to a significant decrease in testes SOD and GSH activities ($p < 0.01$) and in CAT activity ($p < 0.05$). Additionally, there was a significant increase in MDA activity ($p < 0.01$). However, combining VC with HU significantly improved these parameters.

Table 3. Effect of HU and VC on Oxidative Stress biomarkers in Testis tissues of male rats.

Treatments	SOD	CAT	GSH	MDA
I	922.50±24.988	79.50±4.699	4.345±.211	99.250±6.209
II	887.25±15.255	92.25±4.479**	3.587±.207	94.250±4.028
III	647.75±24.709**	73.75±2.954*	2.545±.192**	165.500±14.413**
IV	931.25±12.598	91.25±7.431**	4.030±.389	117.750±2.015*

*Values are means ± SE. Significantly different from control (P< 0.05). *Significantly different from control (P< 0.01). **SE: Standard Error.*

Histopathology of the Testicular

In the figure labelled 5A.1&2, H&E staining showed that the control group rats had typical testicular tissue structure, with tightly packed seminiferous tubules (ST) containing germinal epithelium (G). The spermatogenic cells in the

tubules were organised normally, with spermatogonia (bifid arrow) resting on the basement membrane with a (wavy arrow), along with spermatocytes (notch arrow), spermatids (hollow arrow), and spermatozoa (S) within the lumen. Leydig interstitial cells were also observed in the interstitium (bold arrow). In the group that received VC (Fig. 5.B1&B2), the seminiferous tubules (ST) and interstitial tissue. Each tubule was lined with stratified germinal epithelium (G) and showed healthy spermatogenic cells, including spermatogonia (bifid arrow), spermatocytes (notch arrow), spermatids (hollow arrow), and spermatozoa (S). Interstitial cells Leydig (bold arrow) were also present. However, in rats that received HU alone (Fig. 5.C1, C2, C3 and C4), the testicular tissue showed disorganised seminiferous tubules and a significant loss in the spermatogenic cell lineage (double head arrow). Some seminiferous tubules displayed considerable damage to their architecture and were aberrant with irregular basement membranes, containing only Sertoli cells (head arrow). These tubules had no spermatozoa in the lumen (L). In certain seminiferous tubules, injured spermatogenic cells (sloughed germ cells) were found falling into the lumen (**). Additionally, interstitial cells (bold arrow) and regions with degenerated Leydig cells with vacuoles (arrow) were observed in the intertubular connective tissue. Furthermore, there was a congested interstitial blood vessel (BV).

Figures 5.D1, D2, D3, and D4. show that VC, in combination with the HU group, resulted in improved outcomes. The typical appearance of testicular tissue includes some seminiferous tubules (ST) with complete spermatogenesis and the entire germinal epithelial series (G), which consists of spermatogonia, spermatocytes, spermatids, and spermatozoa. Multiple spermatozoa (S) were present inside the lumen. The inter-tubular space appeared to have a normal width and size and contained a considerable number of intact interstitial cells (bold arrow), while others revealed degenerated Leydig cells (curved arrow). Some tubules showed signs of degeneration (turn arrow), and others appeared with vacuolation (arrow) and a decrease in germinal cell numbers (double head arrow).

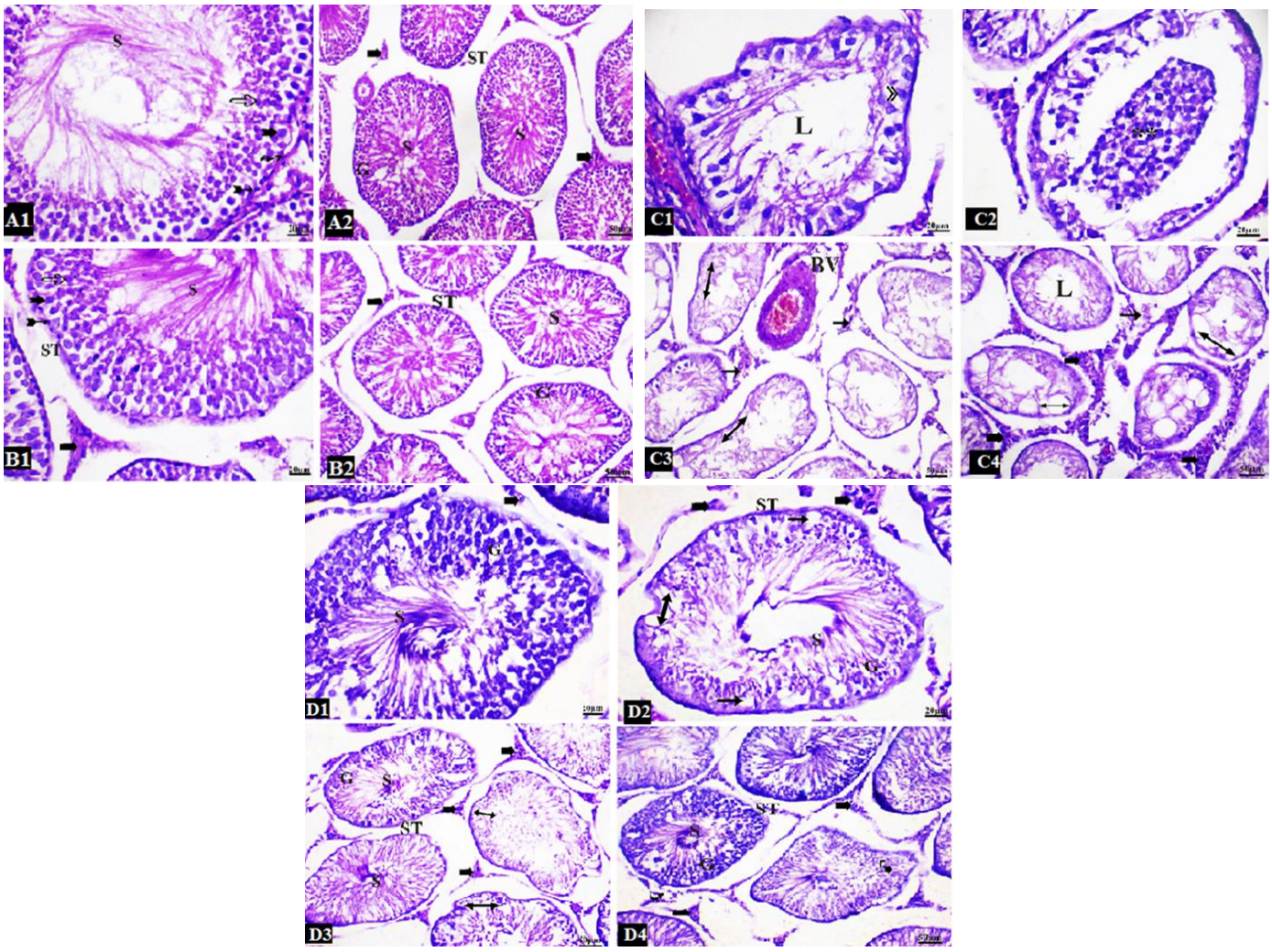


Figure 5: Light microscopic examinations of the testicular sections of the control rats (A1 & A2), VC group (B1 & B2), HU group (C1, C2, C3 & C4), and VC+HU group (D1, D2, D3 & D4).

Comet parameters

The results of comet assays using the testis cells of rats given (VC, HU, and VC+HU) are summarised in Table 4. The values for % Tailed and Tail length were significantly increased ($p < 0.05$) in the VC and VC+HU groups, and at ($p < 0.01$) in the HU group for % Tailed. Additionally, we observed a significant increase in % DNA and olive tail moment in rats treated with HU at ($p < 0.05$, $p < 0.01$), respectively, compared to the non-treated control group, as shown in Figure 6. No significant difference was observed in the tail moment in all treated groups.

Table 4. Comet parameters; % Tailed, Tail Length, % DNA in Tail, Tail Moment, and Olive tail moment of different treatment groups.

Treatments	% Tailed	Tail length	% DNA in tail	Tail moment	Olive tail moment
I	7.500±0.057	7.066±0.176	7.266±.240	.5167±0.015.	1.100±0.050
II	8.700±0.305*	8.533±0.290*	7.418±.122	.515±0.052.	1.103±0.044
III	18.633±0.240**	8.369±0.108*	8.638±.335*	.646±0.011	1.722±0.173*
IV	7.767±0.504	7.963±0.612	6.309±1.879	.561±0.051	1.081±0.097

*Values are means ± SE. Significantly different from control (P< 0.05). *Significantly different from control (P< 0.01). **SE: Standard Error.*

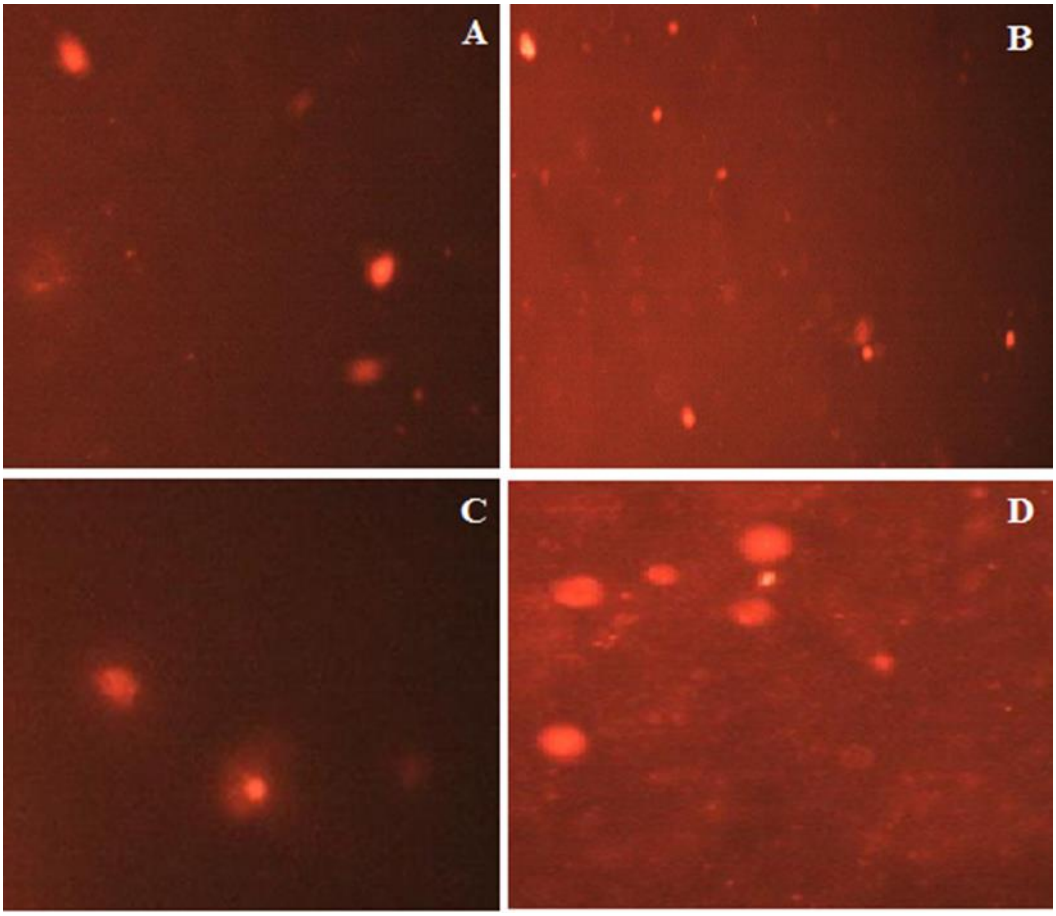


Figure 6: Photographs of cells tested using the comet assay analysis. Each spot represents the DNA of an individual cell based on the effects of oral administration of HU and VC on the comet of the Testicular tissues. (A) control group; (B) VC group; (C) HU group; (D) VC + HU group.

DISCUSSION

Our results indicate a significant decrease in testicular weight and relative weight in groups III and IV after 30 days and an increase in the absolute and relative weight of the left and right epididymis compared to the control group. Furthermore, the HU group exhibited a significant decrease in sperm motility and live sperm percentage, along with an increase in abnormal sperm, when compared to the control group. This suggests a potential adverse effect of HU on testicular health. HU treatment significantly reduced testis weight in mice, as reported by Gu [34], affecting sperm density, progressive motility, and normal morphology of spermatozoa. A significant reduction in testicular weight was

also observed with high doses of HU in mice, as was noted by [5,35,36]. Additionally, HU increased apoptosis in testicular germ cells in mice, potentially affecting alterations in testicular weight [12].

The use of HU in patients with sickle cell disease may lead to reduced total sperm count and unpredictable spermatogenesis [37, 38, 39, 40]. Mice treated with HU experienced a reduction in testosterone levels and sperm production, as well as a decrease in sperm motility and density, along with an increase in abnormal sperm morphology [6]. Linked a decrease in the number of sperm entering the epididymis after spermatogenesis to a decrease in epididymal weight and stored sperm density [41, 42]. Reduced plasma testosterone concentrations, due to HU treatment, caused rapid regression in the epididymal epithelium, impacting sperm maturation and survival [43]. Additionally, individuals treated with HU showed shrinkage in the epididymis and a decrease in stored sperm density and progressive motility, as observed in patients with SCD [5].

Several studies have demonstrated the positive effects of VC on male reproductive health. VC has been found to reduce testicular damage in rats, improve semen quality, and decrease abnormal sperm. Furthermore, it enhances testicular histology and sperm morphology [44]. Consumption of VC is linked to increased testosterone levels, improved sperm count, and reduced sperm abnormalities [45]. VC also reduces abnormalities, enhances sperm count, and improves motility [46]. Furthermore, VC enhances the weight, volume, structural integrity, and function of the testis and epididymis, promoting healthy spermatogenic cells and improving sperm morphology [47].

Our research findings revealed that administering VC to rats significantly increased FSH and testosterone levels. However, we observed no significant changes in LH levels in any of the treated groups compared to the control group. Previous studies have demonstrated that treatment with ascorbic acid elevated FSH and testosterone levels, improved sperm quality in rats [48], and increased testosterone levels, sperm count, and sperm morphology [45, 49]. increased FSH, LH, and testosterone levels [50], which aligns with the results of our study. These hormones are considered essential in male infertility [51]. However, decreased testosterone levels in a dose-dependent manner [52], contradicting our findings.

Exposure to HU led to a significant decrease in testicular SOD, GSH, and CAT, while there was a significant increase in MDA. However, when combined with VC, there was a significant improvement in these parameters. According to Parlak et al. (2017) [53], Schiff bases, a derivative of HU, exhibit antioxidant activity and an increase in MDA levels. HU alone led to decreased levels of antioxidant vitamins, SOD, GSH, and CAT activities, and increased MDA activity [54], which coincides with the study's findings. Additionally, Karagozoglu et al. (2013) found that HU increased SOD activity [55]. However, treatment with HU resulted in induced testicular damage, including decreased MDA levels and increased levels of antioxidant enzymes [52] [56], which contradicts the study's findings.

Histopathological analysis revealed that the control group and the group treated with VC showed a typical testicular structure. On the other hand, the group treated with HU displayed disorganised seminiferous tubules, loss of spermatogenic cell lineage, and damaged tubular architecture. Some tubules exhibited Sertoli cells without spermatozoa, injured spermatogenic cells, degenerated Leydig cells with vacuoles, and congested interstitial blood vessels. Combining VC and HU improved testicular structure, spermatogenesis, and germinal epithelial series, but some areas still showed degeneration. The administration of VC improved testicular histomorphometric changes in rats [47]. Conversely, the exposure to HU caused a reduction in spermatogonia count and other germ cells in mice [57]. However, researchers researched the testicular toxicity of HU in mice and its role in apoptosis in germ cell death but noted improvement with VC administration in rats [12]. A systematic review by Cilio et al. (2024) [58] indicated that HU improved testicular structure in patients with haematological disorders when combined with VC. However, it was also associated with the worsening of semen parameters, potentially resulting in azoospermia in patients with SCD, essential thrombocythemia, and polycythaemia vera. Additionally, effect on spermatogenesis in SCD patients [40]. There is no evidence of irreversible effects on sperm production in young males with severe SCD genotypes [8]. The comet assays conducted on rat testis cells revealed significant increases in % Tailed and tail length in rats treated with VC and VC+HU groups, as well as in the HU group for % Tailed. Additionally, HU treatment led to an increase in % DNA and olive tail moment. However, no significant differences were observed in the tail moment in all treated groups. HU arrested DNA replication without exhausting the levels of dNTPs [59]. Our findings align with this to a certain extent, as we observed a notable increase in % DNA and olive tail moment in rats treated with HU. The HU negatively affects the elongation and initiation phases of replication, resulting in a slow progression through the S phase [60]. Prolonged treatment with low doses can cause cell death due to DNA damage [61], which aligns with the observation of significant increases in % Tailed and Tail length in the HU group. A metabolic response to dNTP pool depletion was determined as the primary mechanism rather than direct DNA damage [62]. Additionally, HU increased mutation rates, aneuploidy, and specific nucleotide transversion rates in yeast, consistent with previous observations [63].

CONCLUSION

In conclusion, HU reduces testicular weight and the levels of SOD, CAT, and GSH while increasing FSH and MDA levels, leading to sperm abnormalities. Additionally, HU causes noticeable changes in embryonic tissue and comet assay parameters. When VC is added, there is a significant increase in both absolute and relative epididymis weight. Rats receiving VC showed a reversal of the effects of HU, indicating that low levels of VC may help protect against testicular damage.

Conflicts of Interest

There are no conflicts of interest.

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تأثير فيتامين سي والهيدروكسي يوريا على صحة الجهاز التكاثري في ذكور الفئران

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المستخلص

الهيدروكسي يوريا هو دواء يستخدم لعلاج أمراض مختلفة ويتم دراسته أيضًا لتأثيراته على تكوين الحيوانات المنوية. وثبت أن فيتامين سي، أحد مضادات الأكسدة يحمي خلايا الحيوانات المنوية من الإجهاد التأكسدي، مما قد يؤدي إلى تحسين الخصوبة وجودة الحيوانات المنوية. في دراستنا شملت أربعة وعشرين من ذكور الفئران البيضاء البالغة مقسمة إلى أربع مجموعات، كانت المجموعة الأولى بمثابة المجموعة الضابطة، وتلقت المجموعة الثانية 5 ملغ من فيتامين سي، وتلقت المجموعة الثالثة 100 ملغ من هيدروكسي يوريا، بينما تلقت المجموعة الرابعة مزيجًا من فيتامين سي و هيدروكسي يوريا لتقييم الوظائف الأساسية للجهاز التناسلي، بما في ذلك مستويات الهرمونات، وعلامات مضادات الأكسدة، ومؤشرات الإجهاد التأكسدي، والتشريح النسيجي المرضي، وتحديد تلف الحمض النووي. وأظهرت الدراسة أن هيدروكسي يوريا سبب في انخفاض بشكل كبير في وزن الخصية، مع زيادة في مستويات (SOD ، CAT، GSH، FSH، MDA) وتسبب في تشوهات في شكل الحيوانات المنوية. أيضًا تسبب الهيدروكسي يوريا في حدوث تغيرات واضحة في التركيب النسيجي للخصيتين والحمض النووي DNA بينما أظهرت الفئران المعالجة بفيتامين سي زيادة معنوية في وزن البربخ المطلق والنسبي مقارنة بالمجموعة الضابطة. علاوة على ذلك، أدى تدخل فيتامين سي إلى عكس الآثار الضارة التي لوحظت في الفئران التي تغذت على هيدروكسي يوريا، مما يشير إلى أن الجرعات المنخفضة من فيتامين سي يمكن أن تحمي من تلف الخصية.

الكلمات المفتاحية: هيدروكسي يوريا، فيتامين سي، أنسجة الخصية، تلف الحمض النووي.