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The Histochemical Effects of Immunosuppressive Drug Azathioprine on the Cytoarchitecture of the Testes of Adult Wistar Rats (*Rattus norvegicus*)

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ABSTRACT: In this work, we studied the histochemical effects of ascorbic acid on immunosuppressive drug-azathioprine-induced alterations in the testis of adult Wistar rats. The rats (n=30) with mean weight 210 ± 2.65 g were assigned into four treatment (n=24) and control (n=6) groups. The rats in the treated groups II and IV received 10 and 20 mg/kg/Bwt of AZA respectively, rats in the treated groups III and V received 10 and 20 mg/kg/Bwt of AZA combined with 25 mg/kg/Bwt and 50 mg/kg/Bwt of ascorbic acid respectively, while rats in control the group received equal ml of distilled water for 21 days after which they were sacrificed; the testes were excised, weighed and fixed in Bouins' fluid for histochemical studies of reticulin and collagen. The histochemical findings of the testis sections indicated cyto-architectural distortions in the treated groups as compared with the control group. The findings show that the ascorbic acid cannot ameliorate the damages caused by azathioprine.

Keywords: Azathioprine, Ascorbic acid, Testis, Reticulin, Collagen.

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Introduction

The importance of the immune system in protecting the body against harmful foreign molecules is well recognized. However, in some instances, this protection can result in serious problems. For example, the introduction of an allograft (that is, the graft of an organ or tissue from one individual to another who is not genetically identical) can elicit damaging immune responses, causing rejection of transplanted tissue.¹ Transplantation of organ and tissue for example kidney, heart and bone marrow has become routine due to improved surgical techniques and better tissue typing. Also, drugs are now available that more selectively inhibit rejection of transplanted tissue while preventing the patient from becoming immunologically compromised.¹

Azathioprine is an immunomodulatory drug often used to treat inflammatory bowel and, autoimmune diseases, prevent rejection of transplanted organs and is also used as anticancer drug. It is an inhibitor of purine metabolism leading to DNA damage. Upon its administration, it is rapidly converted into several compounds, including the active 6-mercaptopurine.² This positive effect does not necessarily come without a price, however. By suppressing the immune system, and lowering the number of infection-fighting white blood cells, Azathioprine makes the individual more susceptible to infection while taking it. Azathioprine exists as either 25 mg or 50 mg oral tablet.

Following oral ingestion of azathioprine, it is metabolized into the active 6-mercaptopurine, which itself is a purine synthesis inhibitor. 6-Mercaptopurine impedes DNA synthesis and thus inhibits the proliferation of cells, especially the fast-growing lymphocytes. T-cells and B-cells are particularly affected by the inhibition of purine synthesis.³ Azathioprine is an effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation.³ Azathioprine blocks the downstream effects of CD28 co stimulation. 6-MP (the active metabolite) interacts directly with GTP-binding protein Rac1, thus blocking up-regulation of BCL-x1 mRNA and protein. In vivo data indicates that inflammatory bowel disease patients treated with azathioprine have more apoptotic mononuclear cells than untreated controls; this indicates that this mechanism may be responsible for the in vivo response to the drug in this disease.³ Immediate or short-term side-effects are uncommon, but these may include nausea, fatigue, hair loss, and rash. Because azathioprine suppresses the bone marrow, patients will be more susceptible to infection. Acute pancreatitis can also occur, especially in patients with Crohn's disease.⁴ As reported that Cyclosporine (Cs), Azathioprine (AZP) and MZR injured the spermatogenesis in rat. The serum level of testosterone was significantly low immediately after the oral administration of AZP, but was not changed by that of Cs and MZR.⁵

Ascorbic acid or L-ascorbic acid is an essential nutrient for humans; it functions as a vitamin. Ascorbate (an ion of ascorbic acid) is required for a range of essential metabolic reactions in all animals and plants. It is made internally by almost all organisms notable mammalian. Exceptions are most or all of the order chiroptera

(bats), and the entire suborder Anthropeidea.⁶ It is a water soluble anti-oxidant which protects the body from oxidative stress, and also serves as a co-factor in several vital enzymatic reactions.⁷ Thus, in this study, we investigated the morphologic, histochemical and oxidative changes in the testes of Wistar rats, exposed to immunosuppressive drug-azathioprine and the possible protective effects of ascorbic acid.

Materials and Methods

Drugs

An available commercial formulation of azathioprine (AZA) tablets 50 mg and ascorbic acid (Vitamin C) syrup were bought from Tuyil Pharmaceutical Industry, Ilorin, Kwara State. Each ml of ascorbic acid contains 20 mg of ascorbic acid.

Experimental Animals

The rats were purchased from Home-made Research Institute in Ilorin, Nigeria. They were housed within the Animal House of the Department of Anatomy, College of Health Sciences, University of Ilorin, in different cages at room temperature, and maintained under a 12 h light/ 12 h dark cycle, with feeds and water available *ad libitum*. They were allowed to acclimatize for two weeks before the commencement of the experiment.

Experimental Design

A total of thirty (30) adult male Wistar rats of mean weight of 210 ± 2.65 g were used for the experiment. At the commencement of the experiment, the animals were assigned to five groups, each group containing six rats:

- Group I animals were given distilled water orally.
- Group II received AZA (10 mg/kg body weight) for 21 days.
- Group III received AZA (10 mg/kg body weight) simultaneously with ascorbic acid (25 mg/kg body weight) for 21 days.
- Group IV received AZA (20 mg/kg body weight) for 21 days.
- Group V received AZA (20 mg/kg body weight) simultaneously with ascorbic acid (50 mg/kg body weight) for 21 days.

The ascorbic acid, azathioprine and distilled water were administered as stated above orally, using a feeding tube (size 6), and the treatment lasted for 21 days.

Body weight

Body weights of the rats were taken prior to the administration of azathioprine and ascorbic acid and then taken weekly thereafter.

Animal Sacrifice and Sample Collection

Twenty four hours after the 21st day of treatment, the animals were sacrificed by cervical dislocation. A midline abdominal incision was made to open up the abdominal cavity and access the reproductive organs. The testes were excised and weighed using an electronic sensitive analytical balance (Gallenkamp FA2104A,

England). The right testes were excised and weighed, then fixed quickly in Bouin's fluid and processed for light microscopic examination using Gordon and Sweets⁽⁸⁾ silver impregnation technique and Masson's trichrome technique⁹ for histochemical demonstration of reticulin and collagen fibres respectively.

Tissue Processing for Light Microscopy

After fixation, paraffin embedding was done, and 4 μ m tissue sections were obtained using the Reichert-Jung 2050 rotary microtome, followed by Gordon and sweets⁸ and Masson's trichrome staining for reticulin and collagen⁹ respectively. Photomicrographs were taken with a digital camera.

Statistical Analysis

Data were analysed using SPSS 16.0 (SPSS Inc, Chicago, USA) at $p < 0.05$ and Excel 2007 (Microsoft Corporation, USA). Data were expressed as mean $\pm$ SEM. Means were compared using the students' t-test.

Results

Body and Testicular Weight Changes

Administration of 10 mg/Kg BWT and 20 mg/Kg BWT of AZA (groups II and IV), 10 mg/Kg BWT of AZA and 25 mg/Kg BWT of ascorbic acid (group III) and 20 mg/Kg BWT of AZA and 50 mg/Kg BWT ascorbic acid (group V) compared to control (group I) resulted in marked morphological changes in the testes of Wistar rats.

There was relative gain in weight, weekly recorded for both the control and treated animals. The weight gain, however, was greatest in the control group I and was statistically significant ($p < 0.05$) as shown in Table 1.

No significant difference between experimental groups II and III compared with control group I, whereas there was significant difference between experimental groups IV and V compared with control group I (Table 1).

Comparing the testis-body weight ratio, using t-test at ($P < 0.05$), there was no significant difference between the control group I and the experimental groups II, III, IV and V, i.e. low dose and high dose groups. This is shown in the table 1 below.

Histochemical Observations

The testes of the control group I show well preserved reticular connective tissue or reticulin fibres (black coloured stain) (Fig. 1A). They show normal section of spermatogenic epithelium and basement membrane (Fig. 1A) compared with the testes of the treated groups II, III, IV and V rats that show less preserved reticular connective tissue or reticulin fibres (black coloured stain) thin spermatogenic epithelium and wide lumen (Figs. 1B-E).

The testes of the control group I show well preserved collagen fibres (blue/black coloured stain) (Fig. 2A). They show normal section of spermatogenic epithelium

and basement membrane (Fig. 2A) compared with the testes of the treated groups II, III, IV AND V rats that show less preserved collagen fibres (blue/black coloured stain) with thin spermatogenic epithelium and wide lumen (Figs. 2B-E).

Discussion

The findings of the present study show that azathioprine is deleterious to the testes and administration ascorbic acid did not ameliorate the damages resulting from administration of azathioprine.

Previous study has shown that consumption of AZA has some beneficial and therapeutic effects on the body. AZA has been used in the management and prevention of rejection following organ transplantation.

Azathioprine is also used to treat a vast array of autoimmune diseases, such as inflammatory bowel diseases, for example, Crohn's disease and ulcerative colitis, multiple sclerosis, atopic dermatitis, autoimmune hepatitis, myasthenia gravis and restrictive lung disease, rheumatoid arthritis and pemphigus⁽³⁾.

As reported that cyclosporine, azathioprine and methotrexate injured spermatogenesis in rat. The serum level of testosterone was significantly lower immediately after the oral administration of AZA.⁵ The testes secrete testosterone which enhances spermatogenesis under the influence of gonadotropic hormones secreted by the pituitary gland. The testes and pituitary gland are linked together as the gonado-pituitary axis in endocrine and reproductive biology.

The current study shows that consumption of AZA at dose of 10 mg/kg body weight and 20 mg/kg body weight led to impairment in the quantity and quality of connective tissues in the testes of adult Wistar rats. The weight gain being highest in the control group is consistent with expected findings considering possible toxic effects of the treatment agent.

The reticulin fibres reaction is well demonstrated in the spermatogenic epithelium and basement membrane by Gordon and Sweet silver impregnation. The intensity of the black stain was greater in the control group compared to the treatment groups. The black stain was more in the 10 mg/kg body weight of AZA and 25 mg/kg body weight of ascorbic acid groups compared to the 20 mg/kg body weight of AZA and ascorbic acid 50 mg/kg body weight of ascorbic acid groups. The higher intensity of the stain, positivity of the argilophilic-black, means the presence of more reticulin fibres, which are fine branching fibres which give a supporting framework to the richly cellular tissue of the testes. Reticular fibres enhance spermatogenesis and are the basis for the good outcome of the testicular morphology. This observation is in agreement with the work of Iwasaki⁵ who reported that the seminiferous tubules were affected in a dose dependent manner following azathioprine administration. It has been similarly reported that the seminiferous tubules were distorted and atrophied as a result of the loss of their structure following azathioprine administration.¹⁰

The collagen fibres' reaction is well demonstrated in the connective framework by Masson's trichrome technique. The intensity of the blue stain is greater in the control group compared to the treatment groups. The blue stain is more in the 10

mg/kg body weight of AZA group compared to the 20 mg/kg body weight of AZA. The high intensity of the stain i.e. positivity of the blue colour, means the presence of more collagen fibres which consists of fine branching fibres which give a supporting framework to the richly cellular tissue of the testes. Collagen fibres enhance spermatogenesis. Also, in the azathioprine-treated animals, there was widening of the interstitial spaces. This was mainly observed in rats that received 20 mg/kg body weight of azathioprine. This observation is in agreement with that of Karawya and El-Nahas who reported widening of the interstitial spaces due to edema in azathioprine-treated rats. The spermatogenic series cells were also reduced in size and number.¹⁰ This could lead to low sperm counts which is in agreement with the work of⁽¹¹⁾.

Conclusion

The current study revealed that the consumption of the azathioprine, particularly when consumed at 20 mg/kg body weight, has deleterious effect on the quality and quantity of the connective tissues characteristics of the adult male Wistar rats that is reticular and collagen fibres, hence on the integrity of the rat testis. These damages were not ameliorated with the administration of ascorbic acid.

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