

## TESTICULAR ALTERATION IN OVER DOSAGE OF AZATHIOPRINE: A HISTOLOGICAL AND HISTOCHEMICAL STUDY IN WISTAR RATS

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### ABSTRACT

**Aim:** The use of Azathioprine (AZA) in the prevention of organ rejection during transplantation has been documented for different organs of the body. In this study, we investigated the effects of azathioprine on the integrity of the testis of Wistar rats using histological and histochemical techniques.

**Methods:** Eighteen adult male Wistar rats with mean weight  $210 \pm 2.65$  g were randomly assigned into three groups. Group I (control) received 1 ml of normal saline "per os" (p.o), group II animals received 10 mg/kg AZA (p.o) while group III animals received 20 mg/kg AZA (p.o). Treatment lasted for 21 days. Twenty-four hours after treatment, animals were sacrificed; their testes excised, weighed and fixed in Bouin's fluid for histological evaluation using Haematoxylin and Eosin while histochemical studies were carried out using Gordon and Sweets, and Masson's Trichrome staining techniques. Testicular homogenate was used to assay for testosterone level.

**Results:** Treatment with Azathioprine reduced testosterone level while the histological and histochemical findings of the testicular sections revealed cyto-architectural distortions and reduced staining intensity of collagen and reticulin connective tissue fibers in AZA treated animals compared to the control group.

**Conclusion:** Results from the study reveal that the immunosuppressive drug-azathioprine when used at a concentration above 10 mg/kg disrupts the testicular integrity of adult Wistar rats.

**Keywords:** Azathioprine, Testis, Collagen, Reticulin

### INTRODUCTION

The importance of the immune system in the body's protection against harmful foreign substances is well recognized. However, this protection in certain instances can result in serious problems like elicitation of damaging immune responses causing tissue rejection following the introduction of an allograft in an individual (Richard et al., 2008). Tissue and organ transplantation have 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 (Richard et al., 2008). Azathioprine is an immunomodulatory drug often used to treat inflammatory bowel disease, autoimmune disease, prevent rejection of transplanted organs and 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 (Barbara, 2010). 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 (Maltzman and Koretzky,

2003). Azathioprine is an effective drug used alone in certain autoimmune diseases, or in combination with other immunosuppressants in organ transplantation (Maltzman and Koretzky, 2003). In vivo data indicates that inflammatory bowel disease patients treated with azathioprine have more apoptotic mononuclear cells than untreated controls; implicating apoptotic mechanism for the in vivo response to the drug in this disease (Maltzman, 2003). Immediate side-effects are uncommon with azathioprine use; but may include nausea, fatigue, hair loss, and rash. By suppressing the immune system, and lowering the number of infection-fighting white blood cells, azathioprine makes an individual more susceptible to infection (Weersma et al., 2004). Previous report shows that the oral administration of AZA significantly reduces serum testosterone in rats (Iwasaki et al., 1996). The present study was carried out to evaluate the effect of the sub-acute treatment with azathioprine on the cyto-architecture of the testis of adult male Wistar rats.

## MATERIALS & METHODS

### Drug

A commercial formulation of azathioprine (AZA) 50 mg tablets was obtained from Tuyil Pharmaceutical Industry, Ilorin, Kwara State Nigeria.

### Experimental Animals

The rats were obtained from an independent animal rearer in Ilorin, Nigeria and acclimatized for two weeks before the commencement of the experiment. They were housed in 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.

### Experimental Design

Study was performed in accordance with the ethical guidelines stipulated by the ethical committee of the College of Health Sciences, University of Ilorin, Nigeria. These guidelines were in accordance with the internationally accepted principles for laboratory animal use and care. Eighteen (18) adult male Wistar rats of mean weight of  $210 \pm 2.65$  g were used for the study. The animals were assigned to three groups of 6 animals each and treated as follows:

- Group I animals received 1 ml normal saline “per os” (p.o).
- Group II animals received 10 mg/kg AZA (p.o).
- Group III animals received 20 mg/kg AZA (p.o).

The administration of normal saline and azathioprine lasted for 21 days.

### Body Weight

The body weights of animals were taken prior to the administration of azathioprine and recorded weekly thereafter.

### Animal Sacrifice and Sample Collection

Twenty four hours post-administration, 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 (Gallenkomp FA2104A, England). The right testes were fixed quickly in Bouin’s fluid and processed for light microscopic examination using Haematoxylin and eosin (Bancroft and Steven, 1990) for general cyto-architectural demonstration of the testis, Gordon and Sweets’ silver impregnation technique (Bancroft and Steven, 1990) and Masson’s trichrome technique (Avwioro, 2002) for histochemical demonstration of reticulin and collagen fibres respectively.

### Tissue Processing for Light Microscopy

After fixation, the organs were dehydrated in ascending grades of alcohol, cleared in xylene, embedded in paraffin and sectioned at 4  $\mu$ m using the Reichert-Jung 2050 rotary microtome. Testicular sections were processed for histology and histochemistry using Haematoxylin and Eosin, Gordon and sweets’ and Masson’s trichrome staining techniques. Photomicrographs were taken with a digital camera.

### Statistical Analysis

Data were analyzed 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

The weights of the animals increased proportionally in all three groups (Table 1). The weight gained by the control animals was

significant ( $P < 0.05$ ) compared to the AZA treated groups (Table 1). The testicular weights of both treated groups reduced compared with the control group (Table 1).

Table 1: Effect of Azathioprine treatment on body and testicular weight in male Wistar rats

Group	n	Final body weight (g)	Initial body weight (g)	Weight diff (g)	Testicular weight (g)
I	6	330±10.83*	221±1.39*	+109	1.35±0.0*
II	6	219±1.49	184±1.49	+35	0.96±0.1
III	6	242±6.65	212±6.51	+30	1.16±0.6

\* $p < 0.05$  (statistically significant compared with AZA treated groups).

### Effect of azathioprine administration on testosterone levels

The effect of azathioprine administration on testosterone levels is shown in Figure 1. Azathioprine administration in rats significantly reduced ( $P < 0.05$ ) the hormonal level of testosterone compared to control group.

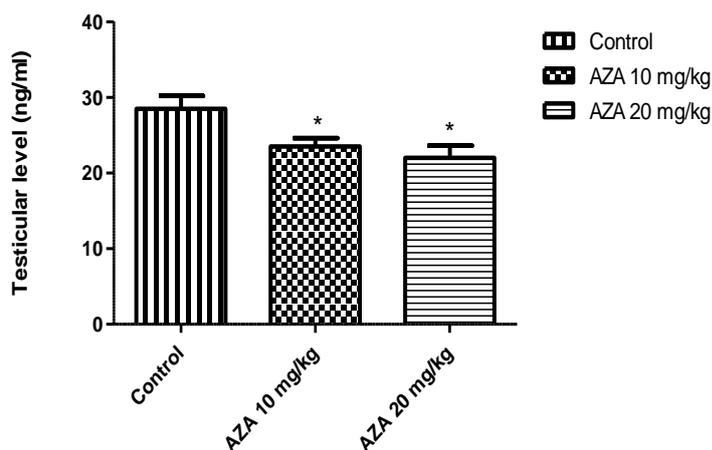


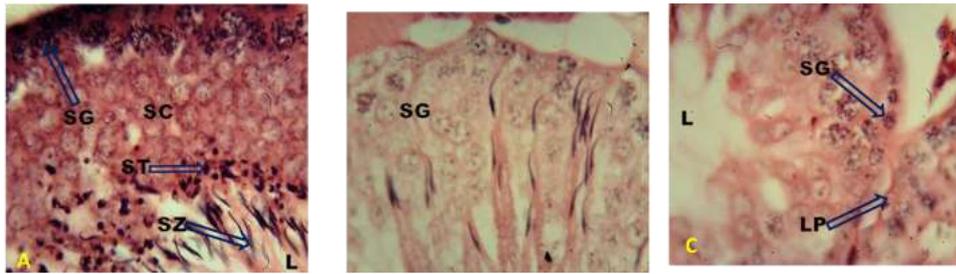
Figure 1: Chart showing testosterone level in control and azathioprine – treated animals after 21 days post-treatment \* $p < 0.05$  (statistically significant difference compared with control group).

### Histological Observations

The testes of the control animals demonstrated normal seminiferous tubules containing spermatogenic cells (Figure 2A) at different stages of development. The basal layer of germinal cells was supported by a basement membrane which is surrounded by a lamina propria limitans. The lumen of the seminiferous tubules contained a good population of spermatozoa. Histological changes were noticed in the treatment groups, as shown by a progressive decline in the concentration of

mature sperm cells in the lumen, and in the population of other developing spermatogenic cells in the tubules.

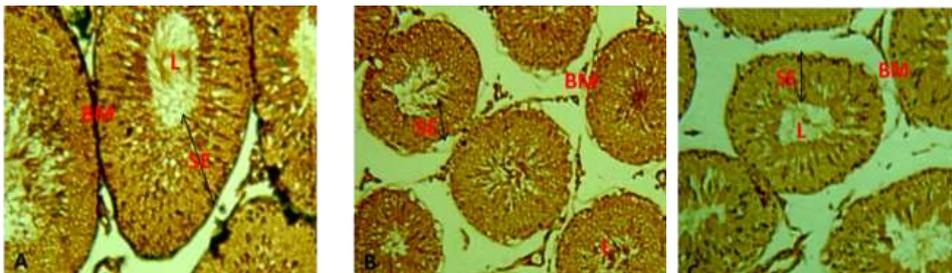
The diameter of the seminiferous tubules was also reduced compared with the control group, with the presence of wider and scanty interstitial spaces. Staining intensity also progressively reduced. These changes were more in Group C that received the higher dose of AZA (Figs. 2B-C).



**Figure 2:** Photomicrographs of part of the seminiferous tubules with spermatogenic cells at different stages of development (SG- spermatogonia, SC- spermatocytes, ST- spermatids, SZ-spermatozoa); the lumen (L) contains mature sperm cells. Compared to the Control (A), treatment groups had a reduction in the population of sperm cells and staining intensity (B-C); these changes appeared more in Group III that received the higher dose of AZA. LP= lamina propria. H&E  $\times 1000$ . (A: Control; B: 10 mg/kg AZA; C: 20 mg/kg AZA).

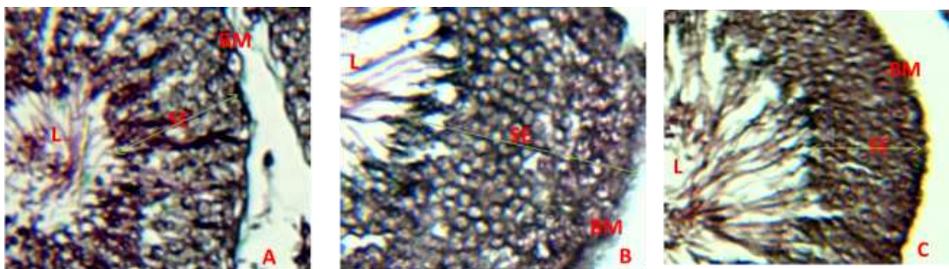
### Histochemical Observations

The testis of the control group revealed well preserved reticular connective tissue (black coloured stain) (Fig. 3A). It shows normal section of spermatogenic epithelium and basement membrane (Fig. 3A) compared with the testes of the treated groups II and III rats that shows less preserved reticular connective tissue (black coloured stain) thin spermatogenic epithelium and wide lumen (Figs. 3B-C).



**Figure 3:** Photomicrographs of the testis of a control animal (A) showing normal SE-seminiferous epithelium, BM-basement membrane, L-Lumen, more positive for reticulin fibres compares with sections of treated rats (B-C). (Gordon & Sweet's  $\times 100$ ). (A: Control; B: 10 mg/kg AZA; C: 20 mg/kg AZA)

The testis of the control group also demonstrate well preserved collagen fibres (blue/black coloured stain) (Fig. 4A). It reveals normal section of spermatogenic epithelium and basement membrane (Fig. 4A) compared with the testes of the treated groups II and III rats that show less preserved collagen fibres (blue/black coloured stain) with thin spermatogenic epithelium and wide lumen (Figs. 4B-C).



**Figure 4:** Photomicrographs of the testis of the control animal (A) showing normal SE-seminiferous epithelium, BM-basement membrane, L-Lumen, more positive for collagen fiber compared with treated rats (B-C). (Masson's trichrome  $\times 100$ ). (A: Control; B: 10 mg/kg AZA; C: 20 mg/kg AZA)

## DISCUSSION

The maximum licensed dose for oral AZA is 5 mg/kg/day in individuals (Gregoriano et al., 2014). Tolerance of large single doses of azathioprine have been reported as the patient showed no relevant symptoms apart from vomiting, slight decrease in white blood cell count and marginal changes in liver function parameters (Carney et al., 1974, Chow et al., 2004). This study was carried out to evaluate the sub-acute administration of an over dosage of azathioprine on the cyto-architecture of the testis in adult male Wistar rats. Findings from this study revealed that the oral administration of azathioprine was associated with increase in body weight and decrease in testicular weight and testosterone in comparison with control animals throughout the three weeks experimental period. Azathioprine has been used in the management and prevention of rejection following organ transplant (Maltzman and Koretzky, 2003). Previous results have shown that azathioprine injured spermatogenesis in rat by reducing the serum level of testosterone following its oral administration (Iwasaki et al., 1996). Result from the current study was in line with this as azathioprine treatment dose dependently reduced the level of testosterone in animals when compared to the control. Alterations in the histology of the liver and kidneys, reduction in tissue antioxidant capacity have been reported following azathioprine administration (Onanuga et al., 2013). In the current study, azathioprine could be responsible for the changes observed in the testicular histology of the treated animals, with progressive decline in the population and size of the developing spermatogenic cells and the mature sperm cells. These findings are in consonance with earlier studies which revealed the occurrence of distortion and atrophy of the seminiferous tubules in a dose-dependent pattern following AZA administration (Karawya and El-Nahas, 2005). The interstitial spaces were also noticed to be wider in the treated animals. The current study also shows that the administration of AZA at doses 10 and 20 mg/kg 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 are well demonstrated in the spermatogenic epithelium

and basement membrane by Gordon and Sweet's silver impregnation technique. The intensity of the black stain was greater in the control compared to the treatment groups. The higher intensity of the stain, positivity of the argyrophilic-black, means the presence of more reticulin fibres, which consists of fine branching fibres which give a supporting framework to the richly cellular tissue of the testis. Reticular fibres enhance spermatogenesis and basis for the good outcome of the testicular morphology. This observation is in agreement with the work of Iwasaki et al., (1996) who reported that the seminiferous tubules were affected in a dose dependent manner following azathioprine administration. Similarly reports have revealed seminiferous tubules distortion and atrophy as a result of the loss of their structure following azathioprine administration (Karawya and El-Nahas, 2005). The reaction of collagen fibres was well demonstrated in the connective framework by Masson's trichrome staining technique. The intensity of the blue stain was greater in the control compared to the treatment groups. The collagen fibre staining was more in the 10 mg/kg AZA group compared to the 20 mg/kg of AZA group. The more staining intensity and 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 testis. 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 (2005) who reported widening of the interstitial spaces due to edema and reduction in size and number of spermatogenic series cells in azathioprine-treated rats. This could lead to low sperm counts which is in agreement with the reports of Iwasaki et al., (1996) and Ramirez et al., (1991).

## Conclusion

We conclude that the administration of azathioprine in excess of 10mg/kg is detrimental to the architecture of the testes. Care should be taken when prescribing it as it may affect male reproductive function.

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