AZATHIOPRINE AND METHOTREXATE IMPAIRED THE MORPHOLOGY AND FUNCTIONS OF THE KIDNEY IN ADULT WISTAR RATS.

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ABSTRACT
Azathioprine (50-150mg per day) and Methotrexate (2.5mg per week) are used in the treatment of cancer in adult Man. We evaluated the nephrotoxic effects of Azathioprine and Methotrexate in rats. Forty adult male wistar rats (150 - 230g) were used in the study. Group I was control. Experimental Groups II - V received oral administrations of 5mg/kg/bodyweight of Azathioprine per day, 15mg/kg/bodyweight of Azathioprine per day, 8mg/kg/bodyweight of Methotrexate per week and 20mg/kg/bodyweight of Methotrexate per week respectively for 35 days. Histological examinations of the kidney showed dose-dependent morphological anomalies such as irregular cyto-architecture and decreased diameters of the Urinary Space, shrunk Glomeruli and necrotic cells in Groups II – V. Statistical analyses showed dose-dependent elevated levels (P≤0.05) of superoxide dismutase and malondialdehyde in kidney homogenates of Groups II – V when compared to Group I. This implied increased oxidative stress in rats of Groups II – V. Evaluations of creatinine and urea concentrations showed dose-dependent significantly elevated levels (P≤0.05) in Groups II – V when compared to Group I. This study provided further evidence that the nephrotoxic activities of Azathioprine and Methotrexate could be due to generated increased oxidative stress, which resulted in impaired morphology and functions of the kidney in rats.

Key words: Azathioprine, methotrexate, kidney, morphology, functions.

INTRODUCTION
The final product of the glomerular filtration processes is urine (about 1500mls per day), which contains eliminated waste metabolic products (Junqueira and Carneiro, 2007). Renal diseases are implicated in a great percentage of morbidity and renal failures are of serious economic burden globally. (Kumar et al, 2004). The four basic morphologic components of the kidney: glomeruli, tubules, interstitium and blood vessels could be affected in immune-mediated disorders or exposures to toxic and infectious agents leading to impaired renal functions (Kumar et al., 2004).

Azathioprine is an immunosuppressant drug, which is used to inhibit the body’s rejection of transplanted tissues (Tripathi, 2003; British National Formulary, 2013). It interferes with the synthesis of deoxyribonucleic acid (DNA), gives rise to non-functional DNA and ribonucleic acid (RNA), inhibits the proliferation of T- and B- lymphocytes and suppresses bone marrow activities. (Tripathi, 2003; British National Formulary, 2013). Due to its antiproliferative activity, Azathioprine is used in the treatment of cancer. (Tripathi, 2003; British National Formulary, 2013). Methotrexate is a folic acid antagonist which inhibits dihydrofolate reductase and prevents the formation of tetrahydrofolic acid (THFA); thereby...

Azathioprine and Methotrexate are classified as cytotoxic drugs with reported adverse effects such as oxidative damages to the DNA/RNA and body organs (Blaisiak et al., 2002; Tripathi, 2003; Elelaimy et al., 2012). Furthermore, Ljuca et al., 2009 observed statistically significant higher serum creatinine, but lower glomerular filtration rate (GFR) and creatinine clearance in individuals treated with Azathioprine when compared with those treated with Mycophenolate Mofetil in patients that underwent kidney transplantation while Elelaimy et al., 2012 noted adverse effects of Azathioprine on the antioxidant status of kidney in rats. Similarly, impaired anomalies of renal convoluted tubules and glomeruli were observed in Methotrexate - treated mice. (Chelab and Majeed, 2009).

Genotoxic and cytotoxic agents such as Azathioprine and Methotrexate would clearly affect the kidney like other body tissues structurally and functionally. We are not aware of any previous study that evaluated the combined effects of Azathioprine or Methotrexate as cytotoxic drugs on the morphology, antioxidant and functional status of the kidney in rats. For further considerations of the nephrotoxicity profile of Azathioprine and Methotrexate as anticancer drugs, this study was undertaken to provide further evidence on the possible effects of Azathioprine and Methotrexate on the morphology, antioxidant status and functions of the kidney in adult male wistar rats.

**MATERIALS AND METHODS**

**Ethical Approval**
Ethical approval was sought and received from the Department of Anatomy of the University of Ilorin, Ilorin, Kwara State, Nigeria. The protocols for the use of animals in scientific research were strictly adhered to in compliance with World Health Organization’s provisions.

**Animal Care and Feeding**
Forty apparently healthy adult male wistar rats weighing 150 - 230g and aged 10 – 12 weeks obtained from the colony bred of the animal house of the Department of Anatomy, University of Ilorin were employed in the study. They were housed in individual cages in a well - ventilated and fumigated room with ambient temperature and good lighting. All rats were fed with standard pellet diet (Kusa Ventures Nigeria Limited, Ilorin) and received water ad libitum. The rats were acclimatized for seven days before the start of experimental procedures. The weight of each rat was taken daily using the high precision electronic analytical weighing balance (Denver, USA). Each rat was examined daily for possible behaviourial and gross morphological or physical changes.

**Chemicals, Reagents and Laboratory Equipment**
Azathioprine 50mg (Imuran) and Methotrexate 2.5mg (Neotrexate) tablets were products of GlaxoSmithKline, United Kingdom; Normal saline solution, Distilled water, Phosphate buffer, EDTA, Sulphuric (VI) acid (H_2SO_4), and Hydrochloric acid (HCl) were products of BDH Chemical Limited, Poole, England; Tris buffers, 2-thiobarbituric acid (TBA), Phosphoric acid and Pyrogallol were products of Sigma Chemicals, St. Louis USA; assay kit for Superoxide dismutase Activity was a
product of Randox Laboratories, United Kingdom; and assay kits for Creatinine and Urea Analyses were products of AGAPPE Diagnostic Limited, India. Spectrophotometer (Jenway Model 6405, UV/visible), mortar and pestle, weighing balance, centrifuge, pH meter (Rex model pHs 25), Norm-jet needles and syringes (Norm-jet Inc. Tuttlinger, Germany) and anticoagulant tubes (Sterling products, England).

Administration of Drugs
The treatment dosage of Azathioprine is 50 - 150mg daily and that of Methotrexate is 2.5mg per week in Man. Azathioprine 50mg (Imuran) and Methotrexate 2.5mg (Neotrexate) drug tablets were grinded into powdery forms using sizeable mortar and pestle in an environment free of wind and then dissolved in 10mls of distilled water. The male wistar rats employed in the study (n = 40) were divided into five groups; each comprising of eight rats. Rats of Control Group I received oral administration of distilled water daily. Rats of Experimental Groups II – V, however, received corresponding oral administrations of 5mg/kg/body weight of Azathioprine per day, 15mg/kg/body weight of Azathioprine per day, 8mg/kg/body weight of Methotrexate per week and 20mg/kg/body weight of Methotrexate per week respectively for 35 days. Doses of administered drugs were determined from previous studies that determined the toxicity profiles of Azathioprine or Methotrexate using same or similar doses in rat models. (Padmanabhan et al., 2009; Elelaimy et al., 2012). Oral administration of drugs was done with the use of a 5ml syringe and a flexible feeding tube long enough to reach the stomach through the oesophagus.

Excision and Fixation of the Kidney
The rats of Groups I - V were sacrificed on Day 35 by humane method using chloroform inhalation. The animals were kept in a dessicator with cotton wool soaked in chloroform. The kidneys were harvested after incisions were made to expose the thoracic and abdominal cavities. The kidneys were removed, taken out and fixed in 10% formal saline of at least five times its volume. (Kiernan, 1990). Blood samples were obtained through the exposed thoracic cavities from the ventricles of the heart and centrifuged immediately for biochemical analyses. (Kiernan, 1990).

Histological Analyses of the Kidney Tissues of Rats of Groups I – V
Histological examinations of the fixed kidney of rats of Groups I – V were carried out using Haematological and Eosin techniques as earlier described. (Kiernan, 1990). After complete fixation of the kidney, blocks were embedded in paraffin wax and sections cut at 5µm (micron). The tissue sections were stained with haematoxylin and eosin and mounted in Canada balsam. (Kiernan, 1990). Microscopic examination of the sections was then carried out under the Olympus light microscope to determine possible cytoarchitectural changes of the kidney following administrations of Azathioprine or Methotrexate. Photomicrographs of kidney samples were obtained with the aid of a digital camera (CANON Digital Camera, USA) attached to the Olympus light microscope.

Preparations of the Kidney and Sera samples for Biochemical Analyses.
The kidney of each rat was cut into small pieces, placed in a mortar and 0.1M phosphate buffer (extracting solution) of at least four times the volume of the organ was added. The organ was homogenized into a fine solution with the use of mortar and pestle. The homogenate was poured into a test tube and centrifuged at 10,000 revolutions per minute for 10 minutes. The supernatant was carefully removed and the residue was discarded. The supernatant
served as the sample for the estimations of Superoxide dismutase activities and Malondialdehyde concentrations. Similarly, blood samples were centrifuged to separate the serum from the red blood cells and the serum was stored away for estimation of Creatinine and Urea concentrations.

**Determination of Superoxide dismutase Activities in Kidney Homogenates**

Superoxide dismutase activities in kidney homogenates of rats of Groups I – V were determined based on the protocol described in the assay kit of Randox Laboratories Limited. (United Kingdom).

**Determination of Lipid Peroxidation Status (Malondialdehyde Levels) in Kidney Homogenates**

The thiobarbituric acid assay (TBARS assay) method was used to quantify Malondialdehyde concentrations in kidney homogenates of rats of Groups I – V as earlier described by Akinlolu et al., 2012.

**Determination of Serum Creatinine Concentrations**

Creatinine concentrations in sera samples of rats of Groups I – V were determined based on the protocol described in the assay kit of AGAPPE Diagnostic Limited, India. (4 X 50mL, Number 510009001).

**Determination of Serum Urea Concentrations**

Urea concentrations in sera samples of rats of Groups I – V were determined based on the protocol described in the assay kits of AGAPPE Diagnostic Limited, India. (4 X 50mL Number 5141101 and 4 X 100mL, Number 51411002).

**Statistical Analyses**

The Mean ± S.E.M (S.E.M. = Standard Error of Mean) value of each of the measured parameters of kidney functions (Creatinine and Urea concentrations) and antioxidant status (Superoxide dismutase and Malondialdehyde Levels) in rats of Control Group I were compared with rats of Experimental Groups II - V for any significant difference using the Student’s t-test for unpaired samples. P values of 0.05 (or less) were taken as statistically significant. Computed data were analysed using the statistical software program SPSS 15.

**RESULTS**

**Changes in Gross Morphology, Behavioural Activities and Average Bodyweight (g) of Rats of Groups I - V During Experimental Procedure.**

No anomalies of gross morphology and behavioural activities were observed in rats of Control Group I and Experimental Groups II – V. Comparison of the average bodyweights of rats of Group I (Day 1 versus Day 35) showed statistically significant increased bodyweight (P≤0.05) at the end of five weeks of experimental procedures (Table 1). In contrast, there was a statistically non-significant decreased average bodyweight (P≤0.05) in rats of Groups II – IV [Day 1 versus Day 35](Table 1). However, comparison of the average bodyweights of rats of Group V (Day 1 versus Day 35) showed statistically significant decreased bodyweight (P≤0.05) at the end of five weeks of experimental procedures (Table 1). No anomaly of gross morphology of the kidneys was observed in rats of Groups I – V when they were dissected and removed for histological and biochemical analyses.

**Histological Evaluations of the Kidneys of Rats of Control and Experimental Groups I – V.**

Examinations of the kidneys of rats of Control Group 1 showed normal histology.
Dose-dependent morphological anomalies such as disrupted cytoarchitecture of the kidney, ruptured Urinary Space of Bowman’s capsule towards the Vascular pole, shrunk Glomeruli and necrotic cells were observed in Azathioprine-treated rats of Groups II and III (Figures 2 and 3). Dose-dependent significant and non-significant decreases (P≤0.05) in glomeruli length and width respectively were observed in comparisons of Control Group I with Experimental Groups II – V (Table 2). Dose-dependent irregular cytoarchitecture and decreased diameter of Urinary Space of Bowman’s capsules were observed in Methotrexate-treated rats of Groups IV and V. (Figures 4 and 5).

Evaluations of Superoxide dismutase (SOD) Activities
Analyses of SOD activities in kidney homogenates of rats of Experimental Groups II - V showed statistically non-significant higher activities (P≤0.05) of SOD when compared to rats of Control Group I. (Table 3).

Evaluations of Lipid peroxidation Status (Malondialdehyde Concentrations)
Analyses of Malondialdehyde Concentrations in kidney homogenates of rats of Experimental Groups II - V showed statistically significant higher concentrations (P≤0.05) of Malondialdehyde when compared to rats of Control Group I. (Table 3).

Figure 1: Photomicrograph sample of the kidney of rats of Control Group I which received distilled water. (a) Haematoxylin and Eosin X 200. PCT = Proximal Convoluted Tubule, DCT = Distal Convoluted Tubule, PL = Parietal Layer of Bowman’s capsule, VL = Visceral Layer of Bowman’s Capsule, US = Urinary Space, G = Glomerulus and VP = Vascular Pole. The cytoarchitecture of the kidney appeared normal.

Figure 2: Photomicrograph sample of the kidney of rats of Experimental Group II which received 5mg/kg/bodyweight of Azathioprine. Haematoxylin and Eosin X 200. PCT = Proximal Convoluted Tubule, DCT = Distal Convoluted Tubule, PL = Parietal Layer of Bowman’s capsule, VL = Visceral Layer of Bowman’s Capsule, US = Urinary Space, G = Glomerulus and VP = Vascular Pole. The cytoarchitecture of the kidney appeared disrupted and some necrotic cells could be observed in the kidney tissue.
Figure 3: Photomicrograph sample of the kidney of rats of Experimental Group III which received 15mg/kg/bodyweight of Azathioprine. Haematoxylin and Eosin X 200. PCT = Proximal Convoluted Tubule, DCT = Distal Convoluted Tubule, PL = Parietal Layer of Bowman’s Capsule, VL = Visceral Layer of Bowman’s Capsule, US = Urinary Space, G = Glomerulus and VP = Vascular Pole. The cytoarchitecture of the kidney appeared disrupted. The Glomerulus appeared shrunk and some necrotic cells were observed in the kidney tissue.

Figure 4: Photomicrograph samples of the kidney of rats of Experimental Group IV which received 8mg/kg/bodyweight of Methotrexate. (a) Haematoxylin and Eosin X 200. PCT = Proximal Convoluted Tubule, DCT = Distal Convoluted Tubule, PL = Parietal Layer of Bowman’s Capsule, VL = Visceral Layer of Bowman’s Capsule, US = Urinary Space, G = Glomerulus and VP = Vascular Pole. The diameter of the Urinary Space was reduced while the Bowman’s capsule appeared irregular.

Figure 5: Photomicrograph samples of the kidney of rats of Experimental Group V which received 20mg/kg/bodyweight of Methotrexate. (a) Haematoxylin and Eosin X 200. PCT = Proximal Convoluted Tubule, DCT = Distal Convoluted Tubule, PL = Parietal Layer of Bowman’s Capsule, VL = Visceral Layer of Bowman’s Capsule, US = Urinary Space, G = Glomerulus and VP = Vascular Pole. The diameter of Urinary Space of Bowman’s capsule was markedly reduced.
Table 1: Changes in Bodyweight (g) of Rats of Control and Experimental Groups I – V During Experimental Procedure.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Dose of Drugs</th>
<th>Day 1</th>
<th>Day 35</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Distilled water</td>
<td>152.50g±5.0</td>
<td>180.00g±9.6</td>
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<tr>
<td>II</td>
<td>5mg/kg/bodyweight Azathioprine</td>
<td>197.25g±1.1</td>
<td>194.38g±1.3</td>
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<tr>
<td>III</td>
<td>15mg/kg/bodyweight Azathioprine</td>
<td>212.50g±7.0</td>
<td>191.88g±2.5</td>
</tr>
<tr>
<td>IV</td>
<td>8mg/kg/bodyweight Methotrexate</td>
<td>207.50g±3.6</td>
<td>196.57g±0.3</td>
</tr>
<tr>
<td>V</td>
<td>20mg/kg/bodyweight Methotrexate</td>
<td>202.00g±9.0</td>
<td>176.15g±3.1</td>
</tr>
</tbody>
</table>

Figure 6: Creatinine Concentrations (mg/dl) in Sera Samples of Rats of Control and Experimental Groups I - V. B.W. = Bodyweight, SEM = Standard Error of Mean.
Table 2: SOD Activities and Malondialdehyde Concentrations in Kidney Homogenates of Rats of Control and Experimental Groups I, II and IV.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Dose of Drugs</th>
<th>SOD Levels (U/ml)</th>
<th>Malondialdehyde Concentrations (µMol/Litre)</th>
<th>Statistical Significance at P≤0.05. (Group I vs. Group II or IV)</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Distilled water</td>
<td>2.6±0.1</td>
<td>30.0±0.1</td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>5mg/kg/bodyweight Azathioprine</td>
<td>2.91±0.2</td>
<td>39.5±1.5</td>
<td>YES</td>
</tr>
<tr>
<td>IV</td>
<td>8mg/kg/bodyweight Methotrexate</td>
<td>2.56±0.1</td>
<td>36.0±1.0</td>
<td>YES</td>
</tr>
</tbody>
</table>

Figure 7: Urea Concentrations (mg/dl) in Sera Samples of Rats of Control and Experimental Groups I - V. B.W. = Bodyweight, SEM = Standard Error of Mean.
Table 3: SOD Activities and Malondialdehyde Concentrations in Kidney Homogenates of Rats of Control and Experimental Groups I, II and IV.

<table>
<thead>
<tr>
<th>Groups of rats</th>
<th>Dose of Drugs</th>
<th>SOD Levels (U/ml)</th>
<th>Malondialdehyde Concentrations (µMol/Litre)</th>
<th>Statistical Significance at P≤0.05. (Group I vs. Group II or IV)</th>
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<td>IV</td>
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<td>36.0±1.0</td>
<td>YES</td>
</tr>
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</table>

**Evaluations of Kidney Functions Tests (Creatinine Concentrations)**

Evaluations of serum Creatinine levels in rats of Experimental Groups II - V showed statistically significant higher levels (P≤0.05) when compared to rats of Control Group I (Figure 6).

**Evaluations of Kidney Functions Tests (Urea Concentrations)**

Evaluations of serum Urea levels in rats of Experimental Groups II - V showed statistically significant higher levels (P≤0.05) when compared to rats of Control Group I (Figure 7).

**DISCUSSION**

Our findings showed no anomalies of gross morphology and behavioural activities in rats of Control Group I and Experimental Groups II – V. However, statistically non-significant decreased average bodyweights (Groups II – IV) and significant decreased average bodyweights (Group V) were observed in rats of experimental groups in comparison of average bodyweights (Day 1 versus Day 35) at P≤0.05. (Table 1). The observed weight loss in rats of Groups II – V could possibly be due to inhibition of DNA syntheses and increased oxidative stress with consequent cellular damage of body organs in affected rats. This agreed with the works of Rusell and Rusell, 1991 which reported weight loss in rats exposed to cytotoxic agents such as Azathioprine and Methotrexate.

Histological examinations of the kidney showed dose-dependent morphological anomalies such as irregular cytoarchitecture and decreased diameters of the Urinary Space, shrunk Glomeruli and necrotic cells in Groups II – V. Cellular necrosis results from compromise of the structural and functional integrity of membranes and is induced by sudden environmental insults with accompanied deranged physiological conditions. (Kumar et al., 2004). Our results possibly implied kidney anomalies via DNA or chromosomal damage following administrations of Azathioprine and Methotrexate which probably resulted in cellular necrosis and impaired glomerular membranes. Our observations are in agreement with previous studies which reported impaired anomalies of renal convoluted tubules and glomeruli in Methotrexate - treated mice. (Chelab and Majeed, 2009).

Evaluations of the antioxidant status of the kidney showed significant higher activities (P≤0.05) of SOD and malondialdehyde levels (Table 3) in renal tissues of rats of Experimental Groups II - V when compared to rats of Control Group I. This implied increased oxidative stress in renal tissues of rats of Groups II – V. Increased lipid peroxidation is associated with oxidative...
stress, compromised cell membranes and cellular damage in organisms. (Akinlolu et al., 2012). The observed morphological anomalies of the kidneys of rats of Groups II – V could, therefore, have been due to Azathioprine or Methotrexate – induced oxidative stress. This is in agreement with previous studies which reported increased levels of superoxide dismutase and lipid peroxidation in Methotrexate – treated rats (Asvadi et al., 2011) or Azathioprine-treated rats (Elelaimy et al, 2012).

We observed statistically significant higher levels of creatinine and urea (P≤0.05) in rats of Experimental Groups II – V when compared to rats of Control Group I. (Figures 6 and 7). Creatinine and urea levels provide one of the direct measurements of glomerular filtration rate and when elevated are indicative of kidney damage. (Nwangwu et al., 2011; Nwangwa, 2012). Our findings, therefore, suggest possible compromise of the integrity of glomerular membranes and impaired functional status of the kidneys in Azathioprine or Methotrexate-treated rats resulting in poor glomerular filtration activities of the kidney. (Junqueira and Carneiro, 2007; Guyton and Hall, 2011).

This is in agreement with previous studies which reported statistically significant higher serum creatinine, but lower glomerular filtration rate (GFR) and creatinine clearance in individuals treated with Azathioprine when compared with those treated with Mycophenolate Mofetil in patients that underwent kidney transplantation (Ljuca et al, 2009); and statistically significant higher serum creatinine and urea in Methotrexate-treated rats (Asvadi et al., 2011).

Our observations on the combined effects of Azathioprine or Methotrexate as cytotoxic drugs on the morphology, antioxidant and functional status of the kidney in rats have not been previously reported. This study, therefore, provided further evidence that the nephrotoxic profiles of Azathioprine and Methotrexate could be due to generation of reactive oxygen species with consequent adverse effects on the cytoarchitectural components and functions of the kidney leading to poor glomerular filtration. We concluded that Azathioprine and Methotrexate administrations impaired the morphology and functions of the kidney in adult male wistar rats.

Conflicts of Interest: We declare that there was no conflict of interest whatsoever in undertaking this study; and no funding was received from any agency or institutions.

REFERENCES


