CHANGES IN IMMUNOLOGICAL AND HAEMATOLOGICAL PARAMETERS ASSOCIATED WITH CYCLOPHOSPHAMIDE AND METHOTREXATE ADMINISTRATION IN WISTAR RATS

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ABSTRACTs

The effects of cyclophosphamide and methotrexate on CD4 count, Lymphocytes, neutrophils, White Blood Cell (WBC), Red Blood Cells (RBCs), haemoglobin, Parked Cell Volume (PCV) and platelets were studied in rats. Rats were divided into 4 groups of 6 animals each receiving single doses of cyclophosphamide (50, 75 and 100 mg/kg) or methotrexate (10, 20 and 30 mg/kg). Animals in the cyclophosphamide (100 mg/kg) and methotrexate groups were sacrificed on the eighth day while other groups were sacrificed at the end of 30 days. In another phase of the study rats were divided into 4 main groups with each group comprising of 4 sub-groups of 6 animals each and treated as already described above. Each sub-group was sacrificed weekly for 4 weeks. Blood was collected via the dorsal aorta for haematological and immunological analyses. A single administration of cyclophosphamide for eight days caused a significant (p<0.05) decrease in CD4+, PCV and body weight of the animals with no significant effect (p>0.05) on WBC, RBC, haemoglobin and total protein. Pre-treatment with single doses of 50 and 75 mg/kg of cyclophosphamide caused no significant effect on all the parameters measured at the end of 30 days except on platelets at a dose of 50 mg/kg. Lymphocytes and neutrophils were significantly (p<0.05) altered at weeks 1 and 3. Methotrexate (10 mg/kg) produced a significant reduction in all the parameters measured. It is therefore concluded that a single dose of cyclophosphamide (100 mg/kg) or methotrexate (10 mg/kg) may have a deteriorative effect on immunological and haematological parameters of Wistar rats.

Key words: Haematological parameters, Immunological indices, Cyclophosphamide, Methotrexate

INTRODUCTION

Constructing mouse models for HIV infection is faced with a lot of challenges one of which is inefficient virus binding and entry into murine cells (Potash et al., 2004). However, different methods have been adopted to mimic immunosuppression that occurs in HIV infection using laboratory animals. One of these is the use of certain drugs to reduce the activation or efficacy of the immune system in animals. Immunosuppression can be achieved with a variety of agents, including: cyclophosphamide (CYP) (Derbyshire, 1983), methotrexate (Genestier et al., 1998), corticosteroids (Martins et al., 2012), aflatoxin, (Raisuddin et al., 1993) and acrylamide (Zaidi et al., 1994; Sadek et al., 2011). Cyclophosphamide is an anticancer and immune-suppressive drug which belongs
to the nitrogen mustard subclass of alkylating agents. It is the most commonly used immunosuppressive agent with wide variations in administered doses (e.g. 2 – 100 mg/kg) (Vilquin et al., 1995; Hou et al., 2007), with or without weekly augmentation and it is believed to be one of the most potent immunosuppressive agents available for use (Kumari and Sahoo, 2005; Chighizola et al., 2011). Cyclophosphamide has been shown to suppress T-helper cell functions with prolonged reduction of B cells due to the slower rate of recovery of B lymphocytes from an alkylating agent (Berker et al., 1991). Treatment with CYP has been associated with bone marrow suppression and pancytopenia. Methotrexate is an anti-proliferative agent that affects both T-cell and B-cell immunity, and therefore may suppress antibody production (Terenishi et al., 2001). It has been reported to have effect as an immunosuppressive agent (Hsu and Katelaris, 2009) using varying doses. This study was carried out with the aim of selecting an agent with a single dose known to produce the desired degree of immunosuppressive effect on blood indices with low or no mortality in rats.

METHODS

Animals

Adult Wistar rats of either sex (180-230 g) were obtained from the Animal House of the Department of Pharmacology and Therapeutics, Ahmadu Bello University Zaria. They were allowed to acclimatise for 2 weeks prior to being used for the experiment. The research laboratory had approximately 12 hours light/dark cycle typical of this environment all year round. The rats were kept in cages and placed on standard rodent feed with free access to tap water. Rats were divided at random into 4 groups of 6 animals each. Each animal in three groups received single doses of the drugs used and the fourth group served as the control and was administered with an equivalent volume of normal saline (1ml/kg) through intraperitoneal route. The experiments were done according to the Institutional and International Ethical Standards regarding the handling and use of laboratory animals (UNDP/World Bank/WHO, 2001).

Study Design

Twenty-four rats were divided at random into 4 groups of 6 animals each. Each animal in three groups received single doses of cyclophosphamide (manufactured by Biochem Pharmaceutical Industries Ltd, India) intraperitoneally as follows: 50, 75 and 100 mg/kg. The fourth group served as the control and was administered with normal saline (1ml/kg) through intraperitoneal route. The group that received 100 mg/kg was sacrificed on the eighth day with its control group; this was done because within one week of drug treatment, mortality rate was high among 100 mg/kg CYP administered group. Other groups were left for 30 days. The study was also repeated using different doses of methotrexate (10, 20 and 30 mg/kg). Rats were divided into four groups of 8 animals per group. The test groups were given 10, 20 and 30 mg/kg methotrexate while the control group received normal saline intraperitoneally. Animals in all groups were sacrificed on the eighth day. The above method was repeated using 96 rats (males and females) divided into 4 major groups with each group further divided into 4 sub-groups of six animals each. Each of the sub-groups received 50, 75 and 100 mg/kg cyclophosphamide while the control animals received 1 ml/kg normal saline. Animals in each main group were sacrificed at one week intervals for a period of 4 weeks. Blood samples were collected from the experimental animals via the dorsal aorta into EDTA bottles. The following immunological and haematological indices were evaluated: the CD4+ count, WBCs, lymphocytes, neutrophils, RBC, PCV and Hb (using
Sysmex KX 21N Haematological Auto Analyser Machine, Japan. Weekly changes in body weights were also recorded. Other signs of immunosuppression such as roughened coat, variable degree of depression, reduction in food and water consumption were physically observed.

**Statistical Analysis**

The research results were calculated as mean ± standard error of mean (SEM). Test of significance was done using analysis of variance (ANOVA), Student t-test. Values of $P<0.05$ were considered statistically significant. Results were presented as tables and figures as appropriate.

**Results**

A single administration of cyclophosphamide (100 mg/kg) evaluated on the eighth day showed significant ($p<0.05$) decrease in CD4+, PCV, and body weight as well as increase in lymphocytes and neutrophil when compared with the control. All other parameters such as haemoglobin and WBC were lower compared with the control but the differences were not statistically significant (Table 1).

At 50 and 75 mg/kg cyclophosphamide administration, no significant difference was observed between the control and the treated rats in all immunological and haematological indices measured except platelets that showed significant difference ($p<0.05$) at 50 mg/kg but showed no significant effect at 75 mg/kg (Table 2).

Another phase of the study carried out with 96 rats showed that PCV and WBC levels were significantly different ($p<0.05$) at 75 and 100 mg/kg. At week 3, LYMP and NEUT showed significant difference ($p<0.05$) at a dose of 100 mg/kg (Table 3).

**Table 1: Effects of Single Administration of 100 mg/kg of Cyclophosphamide on Body Weight, CD4+ Count and Other Haematologic Parameters for 8 Days in Rats**

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>WTA (g)</th>
<th>WTB (g)</th>
<th>CD4+ (cells/µL)</th>
<th>PCV (%)</th>
<th>WBC ($\times10^3$/µL)</th>
<th>NEU (%)</th>
<th>LYMP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>196.3±0.3</td>
<td>199.5±4.3</td>
<td>12.0±0.4</td>
<td>40.5±1.3</td>
<td>8.25±4.1</td>
<td>26.3±3.6</td>
<td>72.8±3.7</td>
</tr>
<tr>
<td>CYP</td>
<td>195.3±3.9</td>
<td>179.3±4.1</td>
<td>3.8±0.5**</td>
<td>32.0±2.5*</td>
<td>7.8±1.4</td>
<td>15.0±3.0*</td>
<td>84.8±2.9*</td>
</tr>
</tbody>
</table>

Values are Mean ±SEM *$P < 0.05$, **$P < 0.0001$ significant different from control ($n = 8$).

WBC = White Blood Cell, NEU = Neutrophil, LYMP = Lymphocyte, PCV = Packed cell volume, CYP = Cyclophosphamide, WTA = Initial weight, WTB = weights after one week.

Single administration of methotrexate (10, 20 and 30 mg/kg) for eight days showed significant ($p<0.05$) changes in $CD4^+$ WBC, lymphocytes, neutrophils, RBCs, PCV and Hb at 10 mg/kg (Figures 1 and 2). The weights of the animals also show some changes, although not significantly different ($p>0.05$) from the control (Figure 3).
Table 2: Effects of Single Exposure of Cyclophosphamide on Body Weight, CD4+ Count and Haematological Parameters after 30 days in Rats

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>WTA (g)</th>
<th>WTB (g)</th>
<th>CD4+ (cells/µl)</th>
<th>PCV (%)</th>
<th>WBC (×10³/µL)</th>
<th>NEU (%)</th>
<th>LYMP (%)</th>
<th>PLT (×10³/µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>202.3±5.3</td>
<td>207.2±5.0</td>
<td>10.2±1.7</td>
<td>45.5±1.5</td>
<td>4.9±1.0</td>
<td>23.7±2.9</td>
<td>76.3±2.9</td>
<td>2775.0±37.4</td>
</tr>
<tr>
<td>50</td>
<td>199.5±5.4</td>
<td>199.2±5.2</td>
<td>6.2±0.8</td>
<td>46.0±2.5</td>
<td>8.0±1.7</td>
<td>20.5±2.6</td>
<td>76.7±3.9</td>
<td>177.5±15.8*</td>
</tr>
<tr>
<td>75</td>
<td>199.8±4.9</td>
<td>199.0±5.2</td>
<td>6.7±0.9</td>
<td>45.0±1.5</td>
<td>13.1±5.5</td>
<td>23.0±6.2</td>
<td>76.3±6.5</td>
<td>214.7±30.3</td>
</tr>
</tbody>
</table>

Values are Mean ±SEM, *P<0.05 significantly different from control (n = 8).
PCV = Packed Cell Volume, WBCs = White Blood Cells, NEU = Neutrophils, LYMP = Lymphocytes, PLT = Platelets, WTA = Initial weight, WTB = weights after one week.

Table 3: Effects of Single Administration of 100 mg/kg of Cyclophosphamide on Body Weight, CD4+ Count and Other Haematological Parameters for Four Weeks in Rats

<table>
<thead>
<tr>
<th>Week Sacrifice</th>
<th>Dose (kg/kg)</th>
<th>WTA (g)</th>
<th>WTB (g)</th>
<th>CD4+ (cells/µl)</th>
<th>PCV (%)</th>
<th>WBC (×10³/µL)</th>
<th>NEU (%)</th>
<th>LYMP (%)</th>
<th>Hb (g/dL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wk 1</td>
<td>0</td>
<td>199.0±0.01</td>
<td>203.0±0.01</td>
<td>5.3±1.2</td>
<td>43.0±2.0</td>
<td>9.3±0.3</td>
<td>33.8±1.9</td>
<td>62.3±2.6</td>
<td>14.3±0.7</td>
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<tr>
<td>50</td>
<td>202.4±4.8</td>
<td>207.8±6.1</td>
<td>4.0±3.7</td>
<td>33.6±2.2</td>
<td>7.8±0.5</td>
<td>25.8±2.4</td>
<td>70.5±2.5</td>
<td>11.1±0.7</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>202.8±5.5</td>
<td>194.0±4.7</td>
<td>4.0±1.7</td>
<td>31.4±2.8</td>
<td>7.1±0.2</td>
<td>26.8±1.5</td>
<td>71.7±1.2</td>
<td>11.7±0.3</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>201.7±7.3</td>
<td>188.0±4.8</td>
<td>5.0±1.7</td>
<td>33.8±2.1</td>
<td>7.3±0.4</td>
<td>30.3±4.1</td>
<td>63.8±4.6</td>
<td>11.4±0.8</td>
<td></td>
</tr>
<tr>
<td>Wk 2</td>
<td>0</td>
<td>204.3±9.1</td>
<td>209.7±6.9</td>
<td>9.8±0.9</td>
<td>44.3±1.8</td>
<td>9.4±0.6</td>
<td>27.8±1.3</td>
<td>66.2±1.7</td>
<td>14.8±0.6</td>
</tr>
<tr>
<td>50</td>
<td>199.8±7.1</td>
<td>198.8±7.3</td>
<td>10.0±0.6</td>
<td>41.8±1.0</td>
<td>9.1±0.6</td>
<td>28.2±1.0</td>
<td>67.5±0.9</td>
<td>13.9±0.3</td>
<td></td>
</tr>
<tr>
<td>75</td>
<td>199.2±7.3</td>
<td>192.0±6.1</td>
<td>9.5±0.6</td>
<td>42.5±1.2</td>
<td>8.8±0.7</td>
<td>28.7±3.4</td>
<td>66.7±3.6</td>
<td>14.1±0.4</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>206.7±11.5</td>
<td>180.0±5.8</td>
<td>8.7±0.3</td>
<td>39.7±0.7</td>
<td>8.8±0.7</td>
<td>33.3±0.7</td>
<td>59.3±0.7</td>
<td>13.2±0.2</td>
<td></td>
</tr>
<tr>
<td>Wk 3</td>
<td>0</td>
<td>200.3±6.0</td>
<td>206.2±6.4</td>
<td>11.2±0.8</td>
<td>49.2±0.6</td>
<td>9.9±0.6</td>
<td>29.8±3.6</td>
<td>65.6±4.1</td>
<td>16.4±0.2</td>
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<tr>
<td>50</td>
<td>203.8±7.9</td>
<td>196.8±7.1</td>
<td>11.2±1.1</td>
<td>48.8±0.7</td>
<td>9.9±0.3</td>
<td>28.4±2.5</td>
<td>66.0±2.9</td>
<td>16.2±0.2</td>
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<tr>
<td>75</td>
<td>198.3±8.4</td>
<td>185.8±8.3</td>
<td>12.3±1.3</td>
<td>48.0±1.2</td>
<td>9.7±0.6</td>
<td>20.8±9.2</td>
<td>65.7±1.5</td>
<td>15.9±0.4</td>
<td></td>
</tr>
<tr>
<td>100</td>
<td>202.1±4.2</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are Mean ±SEM, *p<0.05, **p<0.02 and ***p<0.001 versus control (n = 8); PCV = Packed Cell Volume, WBC = White Blood Cell, LYMP = Lymphocyte, NEU = Neutrophil, Hb = Haemoglobin, WTA = Initial weights, WTB = weights after one week; NS = No survival.
Figure 1: CD4 Count and Other Immunological Parameters Alteration Associated with Methotrexate Dosing in Rats; Values are Mean ±SEM, *p<0.05, versus control (n = 6). WBC = White Blood Cell, LYMP = Lymphocytes, NEU = Neutrophils, no. of deaths = 11.

Figure 2: Haematological Parameters Alteration Associated with Methotrexate Dosing as an Immunosuppressive Agent in Rats; Values are Mean ±SEM, *p<0.05, versus control (n = 6); RBC = Red blood cell, PCV = Packed Cell Volume, Hb = Haemoglobin, No. of deaths = 11.

Figure 3: Body Weight Alteration Associated with Methotrexate Dosing in Rats; Values are Mean ±SEM, not significant compared to control, (n = 6); Final Weight = weights after one week.
DISCUSSION

This study showed that a single dose (100 mg/kg) of cyclophosphamide was associated with lowered CD4+ count, PCV and lymphocyte count as well as body weight. These may be attributed to the immunosuppressive effect of cyclophosphamide which was reported in the literature (Dubuy et al., 1971; McCormick et al., 1987; Carter et al., 1988) using varying doses of 2 mg/kg (Vilquin et al., 1995), 10 mg/kg (Hou et al., 2007), 30 mg/kg (Rubenstein et al., 1985), 75 mg/kg (Vahlsing et al., 1975), 100 mg/kg (Kishimoto et al., 1990) and 250 mg/kg (Guttmann, 1974). Cyclophosphamide displays either immunosuppressive or immunopotentiating effects, depending on the dosage and the timing of drug administration (Nowak et al., 2006).

In this study, the ability of 100 mg/kg cyclophosphamide to cause anaemia in rats based on its effect on PCV has been demonstrated. Okwuosa et al. (2012) has reported the depleting effect of cyclophosphamide on some haematological parameters in laboratory animals. Effects of CYP at 100 mg/kg on all the parameters measured indicate its effectiveness in suppressing the immunity. In contrast, mortality rate was high probably as a result of toxic effect of CYP at that dose. Furthermore, the activity of CYP further evaluated through day 30 showed no activity on all the parameters suggesting that CYP did not cause immunosuppression at these doses. This may be attributed to the metabolism of CYP which may be rapid. Tasso et al., (1992) had earlier reported that CYP was nearly cleared from plasma by 24 hours after its administration in paediatric patients. This explains why there was no effect in many of the parameters evaluated in CYP administered animals after 8 days.

Methotrexate significantly affected all measured haematological parameters at 10 mg/kg. The effects of methotrexate observed in this study may be attributed to its immunosuppressive effect on blood chemistry. Methotrexate has been reported to have immunosuppressive effects (Kozub and Simaljakova, 2011); leading to lowered number of WBCs and other immune system cells (neutrophil) as observed in this study. Herman et al., (2004) also reported a reduction in CD4+ CD28+ in patients on methotrexate; in contrast methotrexate caused an increase in CD4+ in this study. It has also been reported that infections occur more often in patients treated with methotrexate compared with other drugs used in rheumatoid arthritis (Colleoni et al., 2002). These provided the impetus for methotrexate to be used as an immunosuppressive agent in this study. The key to the success of using methotrexate is due to the fact that it is efficacious at a low dose with minimal side effects (Rau and Herborn, 2004). This statement is in line with the work of Herman et al., (2004) who reported a higher effectiveness of low dose methotrexate therapy in rheumatoid arthritis patients. This explains the effectiveness of 10 mg/kg of methotrexate used in this study as an immunosuppressive agent aside other dosages used. The most feared side effect of methotrexate is myelosuppression which normally occurs at the beginning of the treatment and results in attenuation of bone marrow as well as pancytopenia (Kozub and Simaljakova, 2011).

Moreover, decrease in weight was observed in rats administered with methotrexate in this study. This reduction in weight may be caused by reduction in food intake observed during the study. Weight loss has been reported in rats administered with a single dose of methotrexat as a result of decrease in food intake (Jahovic et al., 2004). Methotrexate
also causes changes in bowel motility as well as an increase in sodium and potassium secretion in rats in which a study had earlier reported (Carneiro-Filho et al., 2004) which may be a significant reason for the decrease in weight observed in this study.

CONCLUSION

It can be concluded that a single dose of cyclophosphamide at 100 mg/kg and methotrexate at 10 mg/kg for eight days induce severe changes in immunological and haematological profiles of Wistar rats as reflected by significant decrease in CD4, WBCs, lymphocytes, neutrophils, PCV and body weight of these animals. Meanwhile the toxicity of these drugs leads to high mortality rate at effective dose.

References


