Original Research Article

EFFECT OF CYCLOPHOSPHAMIDE AND ITS COMBINATION WITH METFORMIN ON THE SURVIVAL RATE IN MICE

ABSTRACT

Background:

Cyclophosphamide (CYP) an alkylating chemotherapeutic agent is largely used to treat several types of cancer. It is well established to cause toxic effects including hepatotoxicity, nephrotoxicity and bone marrow suppression. Metformin (MET) is an antidiabetic medication that is considered as a first-line therapy for type 2 diabetes mellitus. In this study, we aimed to investigate the effect of co-administration of MET on the CYP-induced toxicity by recording the survival rate in mice.

Methods:

Fifty mice (30-35 gm, body weight) were divided into four groups comprising both male and female. The animals in the control group received a 4 doses saline injection. The animals in the CYP group received 4 doses of CYP (100 mg/kg) (intraperitoneal) and the accumulative does is 400 mg/kg. The animals in the MET group received the drug daily in drinking water 3mg/ml started 3 days prior CYP injection and last to last injection of CYP. The animals in the combination group (CYP+MET) received 4 doses of CYP and a daily dose of MET in drinking water. The animals were observed daily to record the mortality and their body weights were recorded every alternate day. The data obtained from the study was statistically analyzed by one-way ANOVA, and $p<0.05$ was considered significant.

Results:

The data obtained from the study indicated that CYP administration increased significantly ($p<0.01$) the rate of mortality when compared to the control animals while MET reduced the rate. When combination of CYP with MET were tested, the mortality rate was found to be increased. Both CYP and MET significantly reduced the body weight compared to the control animals.

Conclusion:

The results indicated that the combination of CYP and MET reduced the survival rate of animals suggesting that though MET possess anti-proliferative action but when combined, has the potential to increase the toxic effects of CYP.

Keywords: Metformin, Cyclophosphamide, Survival rate, body weight.

INTRODUCTION

Survival rate study is used to evaluate the living subjects such as animals or human for a period of time after treatment. The study is done to understand the effect of drug by assessing the number of subjects survived after the drug was given over a period of time. The duration of the time is recorded starting from the first day of treatment to the occurrence of a predetermined event such as death and is referred as survival time [1]. Kaplan-Meier survival test is frequently used to analyze the effect of intervention such as drugs on the survival rate of subjects. The test indicates the role of treatment and its influence on the mortality rate in the experimental subjects [2].

Cyclophosphamide is synthetic anticancer drug that belongs to the nitrogen mustard group of alkylating agents [3]. CYP is a prodrug that is activated to alkylating phosphoramide mustard in the liver [4]. The drug acts by binding alkyl group to the Guanine base of DNA, thus producing irreversible cross-linkages in the DNA strands that leads to cell death in G2- and S-phases of the cell-cycle. Due to this cytotoxic property it is used extensively in a variety of carcinomas either as monotherapy or in combination with other drugs [5]. CYP is also used at lower doses for some autoimmune diseases such as systemic lupus erythematosus, multiple sclerosis, severe rheumatoid arthritis, glomerulonephritis, and post organ transplantation for prevention of rejection of the organ [6].

The treatment of CYP is associated with several adverse effects such as alopecia, thrombocytopenia, mucosal ulcerations, skin pigmentation, pulmonary fibrosis, facial abrasions, leukopenia, haematuria, haemorrhagic cystitis and petechial haemorrhage in lungs and small bowel [7]. These complications have been reported to complicate the prognosis of the treatment and in majority of cases results in mortality [8].

Metformin belongs to the class of insulin sensitizers and is frequently used as a first line for the treatment of type-2 diabetes mellitus. The drug reduces the blood glucose by multiple pathways such as decreasing the hepatic glucose production, activating the peripheral glucose utilization in muscle, intestine and liver [9]. The non-diabetic pharmacological properties reported for metformin include reduction in the fat mass and inhibition of tumor cell proliferation [10]. Several studies in the past have indicated the beneficial action of metformin when it is combined with a known anticancer drug [11]. However, the studies have not indicated the influence of the combination on the survival rate. Hence, in this study, we planned to evaluate the influence of metformin on the

survival rate when it is combined with cyclophosphamide and treated chronically in mice using Kaplan-Meier survival test.

MATERIALS AND METHODS

Drugs:

Cyclophosphamide (Endoxan®, batch 7G179C, Baxter, Germany) and metformin were produced from (Metfor®, Batch: 06285147002443, Tabuk Pharmaceuticals, Saudi Arabia). Other chemical and reagents used in the study were purchased through college regular chemical supplier.

Animals:

Fifty mice (11–12 weeks-old) were housed individually in a 12h light/dark cycle environment (lights on 6:00 am). The animals had free access to food and water at 24 hours' time. The animals were monitored daily, and their body weight was measured every two days. The studies were done after obtaining the Institutional Ethical Clearance for Animal experimentation.

Experimental design:

The animals were separated into four groups; each group was treated in different drug. The animals in the control group had received a 4 injections of saline, whereas the animals in the CYP group had received a four doses of 100 mg/kg and accumulative dose was 400 mg/kg [12]. The mice in the MET group received MET daily in drinking water 3 mg/ml started 3 days prior CYP injection and last to last injection of CYP [13]. Mice in the combination group (CYP+MET) received a four doses of CYP and a daily administration of MET in drinking water. The animals were observed daily to record the mortality and their body weights were recorded every alternate day.

Drug Administration:

The mice were injected with CYP (100 mg/kg in a four doses given every other day, i.p). Metformin hydrochloride (MET) was dissolved into drinking water at a concentration of 3mg/ml. This was obtained by calculating the daily consumption of water by the animals and the dose. MET was administered daily and given one days prior to the CYP injection.

Statistics:

All the data from *in vivo* study was analyzed using one-way ANOVA test and represented as mean ± SEM. The data was individually compared by a two-tailed Student's *t*-test with $p<0.05$ considered statistically significant.

RESULTS

A. Effect of CYP and MET on the survival rate in mice.

A parallel mouse model was developed to better understand if MET (and its signaling mechanism) had an impact on decreasing the survival rate of the animals treated with CYP using Kaplan–Meier test. The survival analysis that was conducted between mice treated with CYP and mice treated with CYP+MET revealed that treatment with CYP+MET did significantly (P=0.01) decrease the survival rate compared to CYP only. The effect of protectant effect of MET has started in day five. Thus, the study revealed that adding MET to CYP would potentially induce the toxic effect of CYP. The survival rate of the control and MET only remained flat in their groups (Figure-1).

B. Effect of CYP and MET on the body weight of mice

As shown in Figure 2, the bodyweight of the mice in the CYP group, the CYP+MET group and CYP+MET group were significantly lower than the bodyweight of the mice in the saline-injected control group and the *p*-value <0.05 (Figure-2).

DISCUSSION

The primary objective of this study is to assess the effects of MET on the survival rate of animals treated with cyclophosphamide (CYP). Survival studies were done in the research to evaluate the influence of an intervention on the appearance of a specific 'event', which could be appearance of a side effect, response to a treatment, development of a disease or death [2].

Kaplan-Meier is one of the commonly employed statistical methods used to analyze time to event data. This method is useful in survival analysis, as it helps researchers determine and analyze the patients, participants, or animals that is lost to follow up or not complete of the study by death or any other factor [14]. The test is also applicable to compare two groups of subjects such as a control group and treated group (test drug or standard drug). The method is widely used in the fields of the public health, medicine and epidemiological studies and economics [2,14].

Our observations indicated that CYP administration reduced significantly ($p<0.01$) the survival rate in mice when the drug was tested for 35 days (Figure-1). CYP is an alkylating agent that has vital clinical applications in the treatment of several neoplastic and inflammatory diseases [4]. Although the potential benefits of this chemotherapeutic agent have been well established, CYP has a significant toxic effects profile that become increasingly obvious, as the drug application increased. The drug has potential to damage majority of systems thus complicating the prognosis of the treatment in the patients. The major components reported to contribute in the toxicity are the metabolites of CYP such as acrolein and phorphoramide [15].

The studies conducted in the past have indicated that CYP produced toxicity to the bone marrow, cardiac and lung cells, leading to death of the subjects. Similar type of toxicity could have occurred in our study when CYP was chronically administered to the animals. The possibility of cross-linkages with the DNA leading to inhibition in the cell growth, mitotic activity can be linked from the observation on the body weight where CYP-treated mice showed significant ($p<0.05$) reduction in the body mass compared to control (Figure-2).

MET is an example of sulphonyl urea class of antidiabetic agents. Our observations with MET in this study indicated that chronic administration of the drug enhanced the survival rate significantly ($p<0.01$) in the mice compared to the control animals (Figure-1), besides reducing ($p<0.001$) the body mass (Figure-2). Earlier studies indicated that MET has the ability to improve the functioning of mitochondria and positively regulates the hepatic gluconeogenesis [9]. Studies suggested that MET has an ability to improve the lifespan in the tested animals because the drug activates the cellular energy sensor AMP-activated protein kinase (AMPK) besides enhancing the metabolic activity of beneficial intestinal microbes [16]. Further, enhancement in the antioxidant status and suppression of inflammatory and stress-related processes are also linked to beneficial actions of MET [17]. These studies suggest that MET in the present study could have exhibited similar mechanism to increase the survival rate in the mice and the reduction in the body weight could be due to better glucose metabolism and reduction in the fat mass in the animals.

The studies conducted to evaluate the combination of CYP+MET on the survival rate indicated that the combination increased the mortality (Fig-1) besides producing similar effect on body weight (Figure-2). Previous studies suggest that MET has the

ability to reduce the proliferation of both cancer and normal cells [18]. This action could have potentiated the cytotoxic damages of CYP in the tested animals, leading to reduction in the survival rate. However, more research in this direction is suggested to understand the precise role of combination of CYP with MET on the mechanism of metabolism and physiology in the host cells.

CONCLUSION

The results from the present study indicated that CYP decreased the survival rate in the mice while MET improved it. Moreover, the combination of CYP+MET produced reduction in the survival rate. The tested treatment diminished the body mass. The observations suggested that addition of MET to CYP might produce additive effect in the cytotoxic damages leading to more mortality in the animals. Further research is needed to understand the precise role of combination of CYP and MET on the metabolism and physiological changes in the host system.

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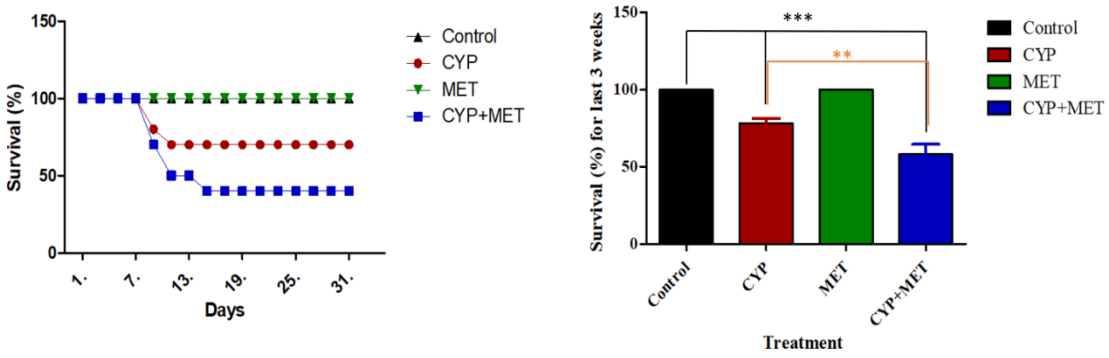

Figure 1: MET reduces the survival rate of the CYP-treated mice. The effect of MET on survival was determined in a mouse model. Treatments were a four doses of CYP (100 mg/kg), and MET was continuously administered orally by dissolving it into the drinking water.

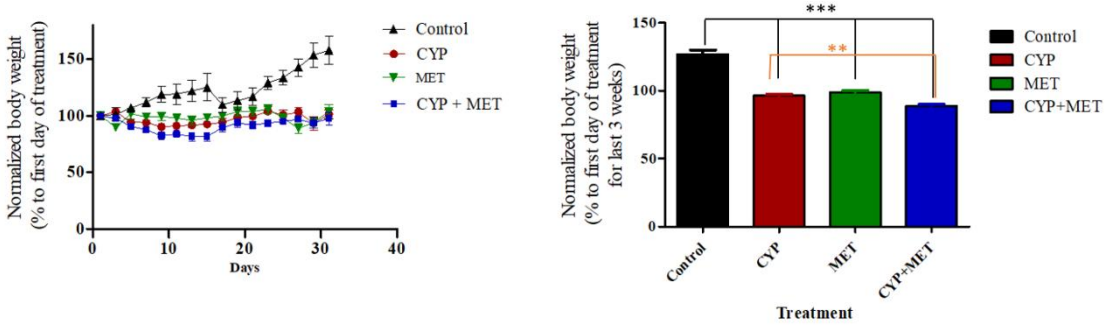

Figure 2: Effect of CYP and MET on the body weight of the animals in the experimental mouse model. The effect of the CYP, MET, and CYP+MET treatments on the animals' body weight compared to controls. The mice were injected with a four doses of CYP100mg/kg (i.p.). Their body weight was monitored every two days. Data are presented as means ± SEM (n = 10–14), and were normalized to the average body weight on day one, which is the first day of the treatments.