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

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ABSTRACT

Background

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

Methods

Fifty mice (body weight 30–35 gm) were divided into four groups as control and treatments and comprised of 12-13 animals of either sex. The animals in the control group received 4 doses of saline by injection. The animals in the CYP group received 4 doses of CYP (100 mg/kg) (intraperitoneal). The animals in the MET group received lower daily dose (30 mg/kg) in drinking water (3 mg/ml), starting 3 days prior to CYP injection and lasting until the final injection of CYP. The animals in the combination group (CYP and MET) received 4 doses of CYP (100 mg/kg) and a daily dose of MET in drinking water (3 mg/ml). 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 the rate of mortality significantly ($p < 0.01$) when compared to the control animals, while MET reduced the rate. When the combination of CYP and MET was 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 although MET possesses anti-proliferative action, it has the potential to increase the toxic effects of CYP when combined with CYP.

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

INTRODUCTION

A survival rate study is performed to understand the effect of an intervention, such as a drug, by measuring the number of subjects surviving after that intervention over a certain period of time. The time is recorded starting from a defined point to the occurrence of a predetermined event such as death; this interval is referred to as *survival time* [1]. The Kaplan-Meier survival test is routinely used to analyze the effect of intervention 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 (CYP) is a 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 an alkyl group to the Guanine base of DNA, thus producing irreversible cross-linkages in the DNA strands that leads to cell death in the 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, severe rheumatoid arthritis, multiple sclerosis, glomerulonephritis, and post organ transplantation for prevention of organ rejection [6].

CYP treatment is associated with several adverse effects, such as alopecia, thrombocytopenia, mucosal ulcerations, skin pigmentation, pulmonary fibrosis, facial abrasions, leukopenia, hematuria, hemorrhagic cystitis and petechial hemorrhage in the lungs and the small bowel [7]. These complications have been reported to impede the prognosis of the treatment; in the majority of cases, they result in mortality [8].

Metformin (MET) belongs to the class of insulin sensitizers and is frequently used for the treatment of type 2 diabetes mellitus. The drug reduces the blood glucose in several ways, such as decreasing the hepatic glucose production and activating the peripheral glucose utilization in muscle, intestine and liver [9]. The non-diabetic pharmacological properties reported for MET include reduction in the fat mass and inhibition of tumor cell proliferation [10]. Several studies in the past have indicated the beneficial action of MET when it is combined with a known anti-cancer drug [11]. However, the studies have not indicated the influence of the combination on survival rate. Hence, in this study, we aimed to evaluate the influence of MET on the survival rate when it is combined with cyclophosphamide and used as part of a chronic treatment in mice using the Kaplan-Meier survival test.

MATERIALS AND METHODS

Drugs

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

Animals

Fifty mice (11–12 weeks old) were housed individually in a 12 hr light/dark cycle environment (lights on 6:00 am). The animals had free access to food and water 24 hours a day. 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 (Approval ID 2019-CP-4).

Experimental Design

The study was done as per the previously described procedures for determining the experimental survival rate in animals [12,13]. The animals in this study were divided in to following groups comprising of 12-13 animals of either sex in each group;

- Control: Normal saline by intraperitoneal
- Treatment-1: CYP – 100 mg/kg, 4-doses on alternate days by intraperitoneal route [14]
- Treatment-2: MET – 30 mg/kg (lower dose), dissolved in drinking water (3 mg/ml) for 10 days by oral route [15]
- Treatment-4: combination of CYP + MET

In combination group, MET was administered for 10 day and on the 3rd day of MET treatment, CYP was injected in 4 doses on alternated days. Before starting the research, a pilot study was done on small group of animals to determine the safety of the selected doses of CYP and MET. The animals were observed daily to record their mortality, and their body weights were recorded every other day.

Treatment protocol

The survival rate was estimated after administering the drugs at their respective duration. MET and CYP groups received the drug for 10 day and four days, respectively. The

combination group (CYP+MET) received MET for 10 days and CYP in four dose starting from 3rd day. The mortality rate was measured daily until 35 days and the observations were tabulated to find the significance of results.

Statistics

All the data from the *in vivo* study was analyzed using a 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 whether MET (and its signaling mechanism) had an impact on decreasing the survival rate of the animals treated with CYP, using a Kaplan–Meier test. The survival analysis that was conducted between mice treated with CYP and mice treated with CYP and MET revealed that treatment with CYP and MET did significantly ($p = 0.01$) decrease the survival rate compared to CYP only. The protective effect of MET started on 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 groups remained flat (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 and MET group, and the CYP and MET group were significantly lower than the bodyweight of the mice in the saline-injected control group, with $p < 0.05$ (Figure 2).

DISCUSSION

The primary objective of this study was to evaluate the effects of MET on the survival rate of animals treated with CYP. Survival studies were performed 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 in the analysis of time-to-event data. The method is useful in survival analysis, as it helps researchers to determine and/or analyze the patients or participants who were lost to follow-up or dropped out of the study, those who developed the disease of interest or survived [16]. It is also applicable to compare two groups of subjects such as a control group and a placebo group or other treatment group (receiving a test drug or a standard drug). The method is useful in the fields of public health, medicine, and epidemiology as well as engineering and economics [2,16].

Our observations indicated that CYP administration significantly ($p < 0.01$) reduced the survival rate in mice when the drug was tested for 35 days (Figure 1). CYP is an alkylating agent that has important applications in the treatment of various neoplastic and inflammatory diseases [4]. Although the potential benefits of this agent have been well established, CYP has a significant toxic effects profile that has become increasingly apparent as the drug's application has increased. The drug has the potential to damage the majority of systems, thereby complicating patients' prognosis. The major components reported to contribute to the toxicity are the metabolites of CYP, such as acrolein and phosphoramide [17].

Studies conducted in the past have indicated that CYP produced toxicity to the bone marrow, cardiac cells, and lung cells, leading to the subjects' death. A 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 a significant ($p < 0.05$) reduction in body mass compared to control (Figure 2). The studies conducted in the past has indicated that anticancer drugs adversely affects the plasma leptin level and body mass [18]. The reduction in the fat mass and lean body mass is reported to enhance the rate of mortality in the subjects [19].

The data correlated to our finding, where the tested drugs not only reduced the body mass but also reduced the survival rate (Figure-2).

MET is an example of the sulphonyl urea class of anti-diabetic agents. Our observations with MET in this study indicated that chronic administration of the drug enhanced the survival rate significantly ($p < 0.01$) in mice compared to the control animals (Figure 1), in addition to 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 the ability to improve the lifespan of the tested animals because the drug activates the cellular energy sensor AMP-activated protein kinase (AMPK) in addition to enhancing the metabolic activity of beneficial intestinal microbes [20]. Furthermore, enhancements in the antioxidant status and suppression of inflammatory and stress-related processes are also linked to the beneficial actions of MET [21]. These studies suggest that MET in the present study could have exhibited a similar mechanism to increase the survival rate in the mice, and the reduction in 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 and MET on the survival rate indicated that the combination increased mortality (Graph 1), although it produced a similar effect on body weight (Figure 2). Previous studies suggested that MET has the ability to reduce the proliferation of both cancer and normal cells [22]. The mechanism has been related to AMPK activation resulting in direct phosphorylation followed by activation of tuberous sclerosis complex 2 (TSC2), leading to inhibition of the mammalian target of rapamycin (mTOR) [23]. This action could have potentiated the cytotoxic damages of CYP in the tested animals, leading to a reduction in the survival rate. However, more research in this direction is suggested to understand the precise role of the combination of CYP and 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 mice while MET improved it. Moreover, the combination of CYP and MET produced a reduction in the survival rate. The tested treatment diminished the body mass. The observations suggested that the addition of MET to CYP might result in an additive effect in the cytotoxic damages leading to more mortality in the animals. Further research is needed to understand the precise role of the combination of CYP and MET on the metabolism and physiological changes in the host system.

REFERENCE

1. Indrayan A, Surmukaddam SB. Measurement of community health and survival analysis. In: Chow SC, editor. Medical Biostatistics 2001;7:232–242.
2. Berwick V, Cheek L, Ball J. Statistics review 12: Survival analysis. Critical Care 2004;8:389–394.
3. Kemp K., et al., Alkylating chemotherapeutic agents cyclophosphamide and melphalan cause functional injury to human bone marrow-derived mesenchymal stem cells. Annals of Hematology 2011;90(7):777–789.
4. Yu Q, Nie SP, Wang JQ, Huang DF, Li WJ, Xie MY. Molecular mechanism underlying chemoprotective effects of Ganodermaatrum polysaccharide in cyclophosphamide-induced immunosuppressed mice. Journal of Functional Foods 2015;15:52–60.
5. Anders CJ, Kemp NH. Cyclophosphamide in treatment of disseminated malignant disease. British Medical Journal 1961;9:1516–1523.
6. Teles KA, Medeiros-Souza P, Lima FAC, Araújo BG, Lima RAC. Cyclophosphamide administration routine in autoimmune rheumatic diseases: a review. Revista Brasileira de Reumatologia 2017;57:596–604.
7. Watcho P, Mpeck IR, DeehDefo PB, Wankeu-Nya M, Ngadjui E, BonsouFozin GR, Kamtchouing P, Kamanyi A. Cyclophosphamide-induced reproductive toxicity: Beneficial effects of Helichrysumodoratissimum (Asteraceae) in male Wistar rats. Journal of Integrative Medicine 2019;17:366–373.
8. Wu J, Chen Y, Hageman L, Francisco L, Ness EC, Parman M, Kung M, Watson JA, Weisdorf DJ. Late mortality after bone marrow transplant for chronic myelogenous leukemia in the context of prior tyrosine kinase inhibitor exposure: A Blood or Marrow Transplant Survivor Study (BMTSS) report. Cancer 2019 Aug 14. [Epub ahead of print]
9. Bailey CJ: Metformin revisited: Its actions and indications for use. Diabetic Medicine 1988;5:315–320.
10. Suissa S, Azoulay L. Metformin and cancer: mounting evidence against an association. Diabetes Care 2014;37:1786–1788.
11. Samsuri NAB, Leech M, Marignol L. Metformin and improved treatment outcomes in radiation therapy–a review. Cancer Treatment Reviews 2017;55:150–162.
12. Hartbeit F. Survival time and mode of death in treated and untreated mice with Bergen A4 ascites carcinoma. Cancer Research 1967; 27: 95-97.

13. Campbell N, Greenaway J, Henkin J, Petrik J. ABT-898 Induces Tumor Regression and Prolongs Survival in a Mouse Model of Epithelial Ovarian Cancer. Molecular Cancer Therapeutics 2011;10:1876-1885.
14. Janelsins MC, Heckler CE, Thompson BD, Gross RA, Opanashuk LA, Cory-Slechta DA. A clinically relevant dose of cyclophosphamide chemotherapy impairs memory performance on the delayed spatial alternation task that is sustained over time as mice age. NeuroToxicology 2016;56:287–293.
15. Thangthaeng N, Rutledge M, Wong JM, Vann PH, Forster MJ, Sumien N. Metformin impairs spatial memory and visual acuity in old male mice. Aging and Disease 2017;8(1):17–30.
16. Goel MK, Khanna P, Kishore J. Understanding survival analysis: Kaplan-Meier estimate. International Journal of Ayurveda Research 2010;1: 274–278.
17. Evans BD, Smith IE, Clutterbuck RD, Millar JL. Prevention of acute deaths in mice after very high dose cyclophosphamide by divided dose schedule. British Journal of Cancer 1984; 49:43-47.
18. Krawczyk-Rybak M, Muszyńska-Rosłan K, Konstantynowicz J, Solarz E, Wołczynski S, Protas P. Effect of anticancer treatment on leptin level, fat body mass (FM) and lean body mass (LBM). Developmental Period Medicine 2004;8:297-307.
19. Lee DH, Keum N, Hu FB, Orav EJ, Rimm EB, Willett WC, Giovannucci1 EL. Predicted lean body mass, fat mass, and all cause and cause specific mortality in men: prospective US cohort study. British Medical Journal 2018;362:2575-2585.
20. Zhou G, Myers R, Li Y. Role of AMP-activated protein kinase in mechanism of metformin action. Journal of Clinical Investigation 2001;108:1167–1174.
21. Vilela DD, Peixoto LG, Teixeira RR, Baptista NB, Caixeta DC, Souza AV. The role of metformin in controlling oxidative stress in muscle of diabetic rats. Oxidative Medicine and Cellular Longevity 2016; Article ID 6978625.
22. Zhang F, Chen H, Du J, Wang B, Yang L. Anticancer activity of metformin, an antidiabetic drug, against ovarian cancer cells involves inhibition of cysteine-rich 61 (Cyr61)/Akt/Mammalian target of rapamycin (mTOR) signaling pathway. Medical Science Monitor 2018;24: 6093–6101.
23. Wang Y, Xu W, Yan Z, Zhao W, Mi J, Li J, Yan H. Metformin induces autophagy and G0/G1 phase cell cycle arrest in myeloma by targeting the AMPK/mTORC1 and

mTORC2 pathways. Journal of Experimental and Clinical Cancer Research 2018; 37: 63.

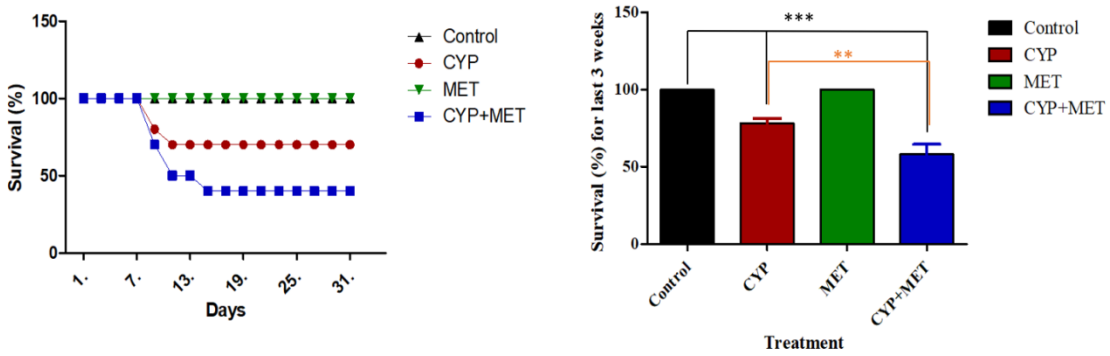

Figure 1: MET reduces the survival rate of the CYP-treated mice. The effect of MET on survival was determined in a mouse model. Treatments were 4 doses of CYP (100 mg/kg), and MET was continuously administered orally by dissolving it into the drinking water.

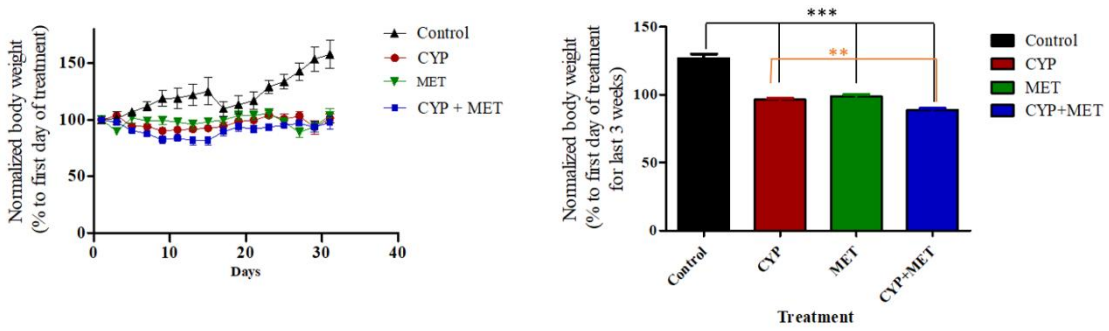

Figure 2*:* Effect of CYP and MET on the body weight of the animals in the experimental mouse model. The effect of the CYP, MET, and CYP and MET treatments on the animals' body weight compared to controls. The mice were injected with 4 doses of CYP 100 mg/kg (i.p.). Their body weight was monitored every two days. Data are presented as means ± SEM (n = 10–14), and were normalized to the average body weight on day one, which was the first day of the treatments.