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Study of Haemoglobin Level and Tumour Growth on Mouse Ascites Tumour in Response to Combination Effect of 2-Methoxyestradiol and Cyclophosphamide

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Original Research Article

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Abstract: S-180 tumour bearing mice were subjected to 2-Methoxyestradiol (2ME) and Cyclophosphamide (CP) monotherapy and 2ME and CP combination therapy on 7th day of ascetic tumour cell transplantation when the tumour growth was at log phase. Then, the effect has been studied on host's system in respect to dead cell – living cell frequency, tumour volume, haemoglobin percentage, and differential count of WBC. In 2ME and CP combination therapy, a steady increase in the dead cell or non-living cell population was noted with the steady decrease in tumour volume. Haematological studies from peripheral blood revealed a drastic depletion in neutrophil count and elevation of lymphocyte population on the 12th day and 16th day of tumour transplantation in combination therapy series. Moreover, the haemoglobin concentration is more or less stable in combination therapy treatment series. So, the 2ME and CP combination therapy provides some protective compensatory mechanisms in the body of the host.

Keywords: Combination therapy, Differential count, Viable cell, Haemoglobin Percentage, 2-Methoxyestradiol, Cyclophosphamide.

INTRODUCTION

Cancer is a complex multistage genetic disease in which a group of normal cells transforms into metastatic malignant cells. At present, surgery, radiation therapy and chemotherapy are common methods of cancer treatment. Among these, chemotherapy has become much popular due to some reasons. Firstly, it prevents cell proliferation by interfering with their ability to replicate DNA and secondly, it can induce apoptosis in cancerous cells [1-4].

But this type of treatment has some toxic side effects on normal cells. Many chemotherapeutic agents may induce cytological abnormalities (i.e. chromosomal aberrations) as well as haematological abnormalities. Use of combination treatment is a novel idea to treat cancer as combination therapies may induce less toxic side-effects at cytological and haematological level. Moreover, good combination may protect the host from some undesirable effects. In the present study, 2-Methoxyestradiol (2ME)–an anti-angiogenic, anti-neoplastic [5-10] agent has been used in combination with an alkylating anti-tumour drug cyclophosphamide (CP). CP has been used in different cancer patient as monotherapy and combination therapy [11-13]. Different types of cytological effects of 2ME and CP have been reported in different animal tumour model systems [9,10] but its effect on host's hemopoietic system during the period of treatment has not been studied yet. So, the present study has been oriented to find out the effect of monotherapy of 2ME, CP and combination therapy of 2ME and CP at haematological level during the course of treatment using Sarcoma180 tumour bearing mouse.

MATERIALS AND METHODS

Experimental animal

Swiss Albino adult mice (*Mus musculus*) with an average body weight of 20g were grouped and housed in normal laboratory condition for acclimatization at 24º - 25ºC temperatures. Mice were provided standard mice food and water ad libitum.

Selection of animal tumour model

Sarcoma 180 (S-180), a well-known transplantable tumour, was maintained intraperitoneally in Swiss albino mice (1×10^6 cells/ animal). All experiments were done in accordance to the guideline of Institutional Animal Ethics Committee (IAEC).

S-180 tumour transplantation

Freshly aspirated S-180 tumour cells were diluted with 0.9% normal saline under sterile condition and were injected intraperitoneally to normal mice for induction of ascitic tumour [14-15] for pursuing our experiments.

Preparation of drug solution

5 mg 2ME (Sigma, USA) was dissolved in 5 ml of absolute alcohol and then this solution was diluted with 5 ml of 0.9% normal saline to make the stock solution. 200 mg CP (Zydus Oncosciences, India) was dissolved in 13.4 ml of 0.9% normal saline to make the stock solution as practised in the laboratory [9,10]. A parallel positive control (vehicle) was prepared with absolute alcohol and normal saline in 1:1 (v/v) ratio. In combination therapy, both (2ME and CP) drugs were administered in the same concentration as used in monotherapy.

Experimental design

Experimental animals (30 mice) were divided into five groups with 6 mice in each group (Table 1). Each group consists of two sets of experiments, i.e., one set tumour bearing mice studied on the 12^{th} day of tumour transplantation and other set studied on the 16^{th} day of tumour transplantation. Mice in all the 5 groups were fed with normal food.

Group 1 (negative control): Tumour bearing mice were maintained without any drug treatment.

Group 2 (positive control): 0.2 ml Vehicle solution (absolute alcohol and 0.9% NaCl, 1:1 ratio) was injected to tumour bearing mice intraperitoneally.

Group 3: 0.2 ml 2ME solution (6.5 mg 2ME/kg body weight) was injected once daily intraperitoneally to each mouse for five consecutive days.

Group 4: 0.2 ml CP solution was injected once daily intraperitoneally (75 mg CP/kg body weight) to each mouse for five consecutive days.

Group 5: In combination therapy group, 2ME and CP solution were given intraperitoneally as given in single therapy once daily to each mouse of this group. 2ME was given 4 h before CP to minimize the potential for drug interactions.

Table-1: Experimental design showing the total number of mice used in both 12 day and 16 day of tumour transplantation of control and treated series

		No. of Mice				
		Control	Vehicle	2ME	CP	2ME+CP
Tumour bearing mice for 12^{th} day of tumour transplantation	Set I	3	3	3	3	3
Tumour bearing mice for 16^{th} day of tumour transplantation	Set II	3	3	3	3	3

Treatment schedule

Treatment was started on the 7^{th} day of tumour transplantation, when ascitic tumour attained a measurable size. Then the treatment was continued for five consecutive days as practiced in the laboratory [9,10].

Tumour Volume

The mice were dissected and ascitic fluid was collected from the peritoneal cavity. Then ascitic fluid was measured (ml) using a graduated tube as practiced in the laboratory [10].

Tumour Cell Count

The viability and non-viability (dead) of the cell in both control and treated series were measured by Trypan Blue Exclusion test after slight modification of the technique of Chakrabarti and Chakrabarti [14]. The cells were stained with Trypan Blue (0.4% in normal saline) dye. Non-viable cells were stained whereas the viable cells did not take up the dye and remained unstained.

Tumour regression rate was estimated by analysing the change in the tumour volume and dead cell or non-viable cell count.

Haematological parameters

Collected blood was used for estimation of percentage of haemoglobin and differential count of WBC. Haematological parameters viz. haemoglobin estimation, differential count were performed by following by Sahli's method of Sood [16].

Differential count

Differential count of WBC (i.e., neutrophil, lymphocyte, monocyte etc.) in control and treated tumour bearing mice was studied by the method of Sood [16].

Statistical Analysis

The data expressed as a mean ± SE, were statistically analysed by using Student's t-test. $P < 0.05$ was considered as significant (*) and $p < 0.001$ as highly significant (**) [17].

RESULTS AND DISCUSSION

Tumour Volume and Tumour Cell Population

A steady decrease in the tumour volume with a steady increase in non-viable cell frequency was noted after the administration of 2ME and CP monotherapy and 2ME and CP combination therapy (Fig. 1 and Fig. 2a).

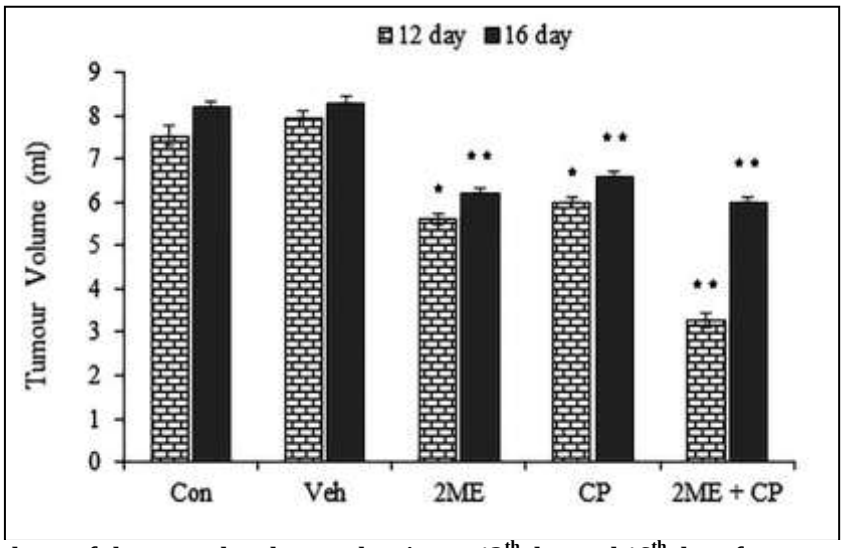

Fig.1 Tumour volume of the control and treated series on 12th day and 16th day of tumour transplantation

Graphical representation revealed that tumour volume in combination therapy of 2ME+CP treated series is decreased in comparison to control and other treated series on 12th day. But on 16th day, tumour volume in case of 2ME and CP treated combination therapy is slightly decreased in comparison to control and other treated groups. Con = Control, Veh = Vehicle, 2ME = 2-Methoxyestradiol, CP = Cyclophosphamide, 2ME+CP = 2-Methoxyestradiol + Cyclophosphamide.

Trypan Blue indicator was used to measure the frequency of viable and non-viable cells of treated series in both 12th day and 16th day of tumour cell transplantation. The tumour regression rate with a simultaneous increase in non-viable cell population was noticed in both 12th day and 16th day of tumour transplantation. (Fig. 2 a,b,c). These data were compared with the control series.

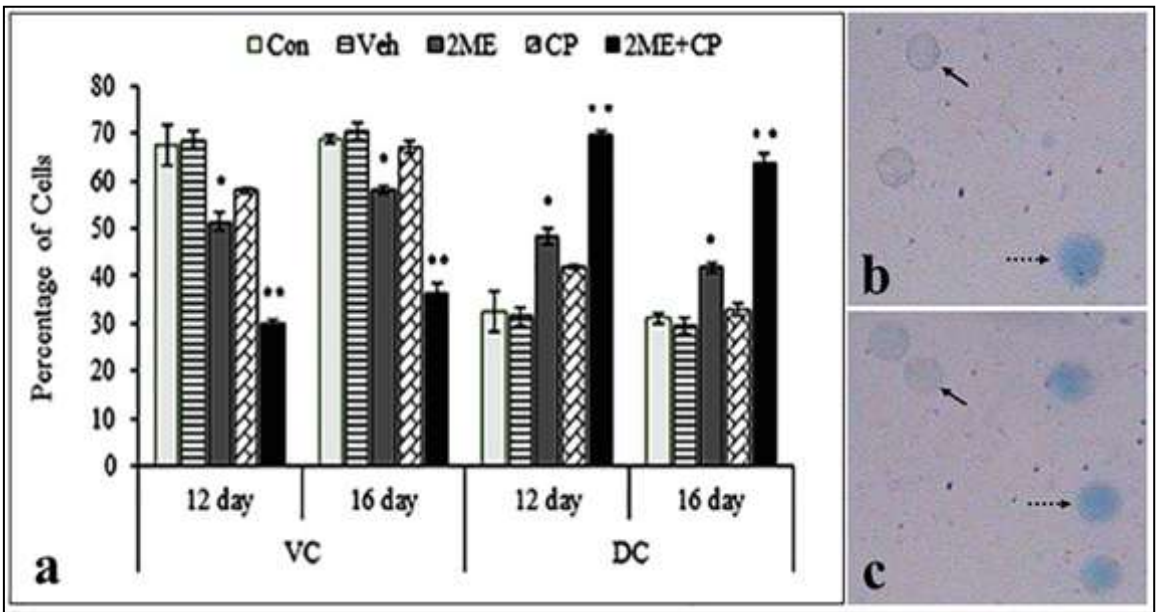

Fig 2. Trypan Blue exclusion test revealed that viable cells increased and dead cells decreased in control and vehicle.

But 2ME+CP combination therapy treated series exhibits inhibitory effect of viable cell population in both 12th day and 16th day of tumour transplantation (a). In both cases, i.e., 12th and 16th day of tumour transplantation, the inhibitory effect of viable cell population is less in 2ME and CP monotherapy treatment in comparison to combination therapy, b,c: Trypan Blue exclusion test of control and 2ME + CP combination therapy series, respectively, where black arrow denotes viable cell and dotted arrow indicates dead cell. VC = Viable Cell, DC = Dead Cell, Con = Control, Veh = Vehicle, 2ME = 2-Methoxyestradiol,

CP = Cyclophosphamide, 2ME + CP = 2-Methoxyestradiol + Cyclophosphamide.

Haematological Parameters

The histographical representation of haemoglobin content (Fig. 3) and differential count (Fig. 4 A) in S-180 tumour bearing control specimens revealed no marked alterations in both 12th day and 16th day of tumour transplantation. Interestingly, an elevation in the percentage of haemoglobin on 12th day of tumour transplantation was noted in combination therapy treated series. The trend was followed upto 16 day of tumour transplantation (Fig. 3). It should be mentioned here that, elevation in the percentage of haemoglobin was also noted in 2ME and CP monotherapy treated series in both 12th and 16th day of tumour transplantation.

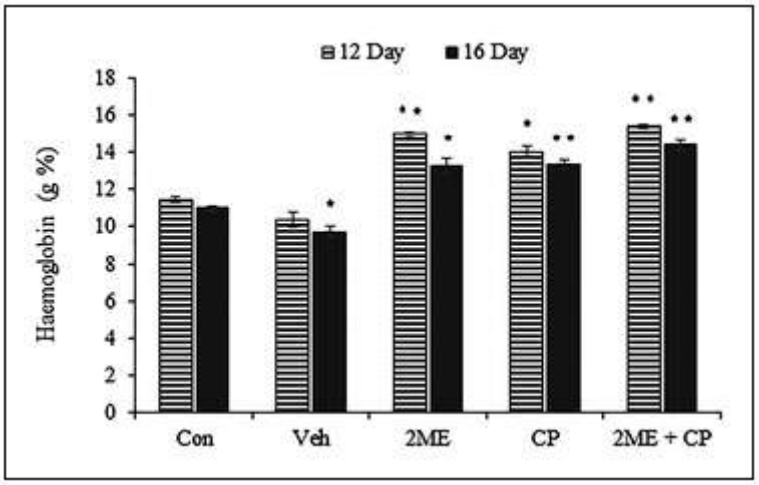

Fig-3: The histographical representation of haemoglobin content

Fig 3. Graphical representation revealed that percentage of haemoglobin restored in combination therapy as well as in monotherapy whereas it is low in control and vehicle on both 12th and 16th day of tumour transplantation. Con = Control, Veh = Vehicle, 2ME = 2-Methoxyestradiol, CP = Cyclophosphamide, 2ME + CP = 2-Methoxyestradiol + Cyclophosphamide.

Differential Count

Differential counts of leukocytes are noticed by a shift from the frequency of neutrophil to the frequency of lymphocytes. In control series, for the set up with 12th day of tumour transplantation, an elevation in neutrophil count was noted whereas a slight fall in lymphocyte count was observed. For 16th day of tumour transplantation of control series, there is a tendency of elevation of lymphocytes whereas neutrophil population is unaffected (Fig. 4 A,B,C). But in 2ME + CP combination therapy treated series, the lymphocyte population is increased in both 12th day and 16th day of tumour transplantation set up in comparison to the frequency of neutrophil population. On the basis of differential count of WBC, in relation to neutrophil and lymphocyte population, it has been noted that with gradual decrease of tumour volume and viable cell population in 2ME and CP treated series the neutrophil population decreased but the lymphocyte population increased. Parallel studies on differential count were performed on 2ME and CP monotherapy treated groups. But these studies did not show any remarkable change even on the 16th day of tumour transplantation.

Fig. 4. A: graphical representation of differential count of WBC in S-180 tumour bearing mice revealed a number of neutrophils and less number of lymphocytes in comparison to combination therapy on 12th day of tumour transplantation and increase in lymphocyte and decrease in neutrophil number in control series on 16th day of tumour transplantation, B,C: Differential count of WBC of control and treated series, where black arrow indicates neutrophil (B) and dotted arrow indicates lymphocyte (C). N = Neutrophil, L = Lymphocyte, Con = Control, Veh = Vehicle, 2ME = 2-Methoxyestradiol, CP = Cyclophosphamide, 2ME+CP = 2-Methoxyestradiol + Cyclophosphamide.

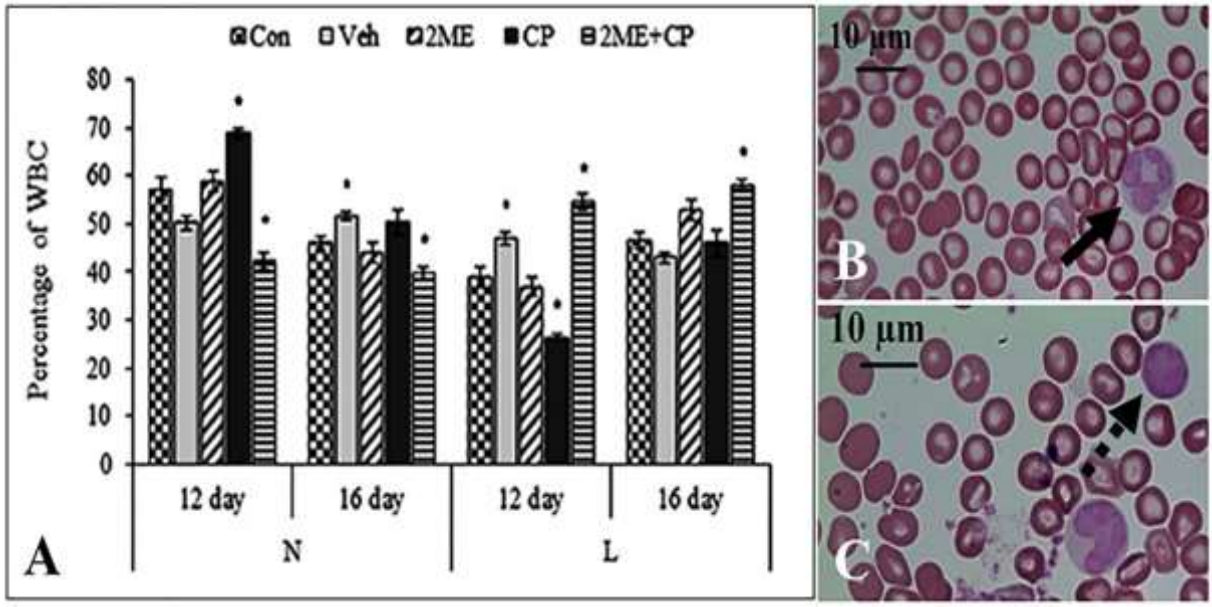

Fig-4: The histographical representation of differential WBC count and picture of blood cell population

Haematological studies revealed that some protective mechanisms do exist in the body of the host system which prevent the animals to be severely anaemic during the period of combination therapy treatment. It is a well-known fact that advanced stage of malignancy is characterized by a severe depression in the haemoglobin level [18]. Such condition may induce different types of infections. It is interesting to note that the haemoglobin concentration remained stable or normal during the treatment of combination therapy of 2ME+CP. Moreover, the rise in the lymphocyte population on 12^{th} day as well as on 16^{th} day of tumour transplantation in 2ME+CP combination therapy also produces compensatory protective mechanism in the body of host of S-180 tumour bearing mice.

CONCLUSION

The present findings indicate that, combination therapy of 2ME and CP is a potent therapeutic measure for rapid regression of ascitic tumour in S-180 tumour bearing mice. Analysis of hematological parameters showed a minimum toxic effect in combination therapy group. Further studies in relation to erythrocyte count, platelet count and WBC count may throw new light in this field.

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