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Final Report of the work done on the Major Research Project

1. Project Report No. Final

2. **UGC Reference/ letter No F.42-602/2013(SR) dated 22.03.2014 (MRP)**

Title of research project "**PRECLINICAL ASSESSMENT OF 2- METHOXYESTRADIOL (2-ME) AS ANTI-ANGIOGENIC COMPOUND: EFFECT OF 2ME ON EXPERIMENTAL TUMOUR GROWTH"**

Name of the Principal Investigator: **DR.SAMARENDRA NATH BANERJEE**

Department of Zoology,Rammohan College,102/1 Raja Rammohan Sarani,Kolkata – 700009

Tumour growth and metastatic process are angiogenesis dependent. As a tumour grows in size, it stimulates the formation of new blood vessels - a process called angiogenesis. Tumour angiogenesis supports tumour growth by allowing passage of nutrients, oxygen and waste products through an ever increasing population of cells. Different *in vitro* and *in vivo* techniques are used to elucidate the mechanisms controlling angiogenesis and to investigate how cancer therapies in particular anti-angiogenesis therapy may interfere with the angiogenic process to induce tumour regression with less toxicity. 2-Methoxyestradiol (2-ME) , the natural endogenous product , once considered an inactive end metabolite of estradiol, has recently emerged as a very promising agent for cancer treatment, is synthesized by sequential hydroxylation of the parent compounds followed by methylation in the liver (Brueggemeier, & Singh,1989 ; D Amato,1994) . 2-ME targets rapidly growing cells with relatively high specificity and does not kill non-dividing cells. The effect of different concentrations of 2ME at various time points on different cell lines (Banerjee et al 2002, 2003 ; Banerjee

and Banerjee 2005, 2008) has been studied earlier. But it is necessary to investigate the effect of 2ME on *in vivo* system. Till now little has been done on the effect of 2ME on somatic chromosome of the tumor bearing host, particularly during the period of tumor regression (Banerjee et al 2006; Mahapatra et al, 2011). So the present project has been oriented to evaluate the efficacy and safety of 2 ME on *in vivo* mouse tumour model considering mouse bone marrow toxicity, tumour growth rate or the kinetics of tumour regression and mouse survival.

Objective of the Project

Major treatment modalities (radiation and chemotherapeutic drugs) in cancer act by damaging DNA. While using a drug in cancer therapy, it is important that the drug should give significant protection to normal cells with no or minimal toxicity. Our earlier studies (Banerjee et al, 2001, 02, 03, 05) on some tumour cell lines (viz. MCF-7 and GH^3) suggested that the mechanism of action of 2ME on tumour angiogenesis and tumour growth is mediated through regulation of Vascular Endothelial Growth Factor. These studies also indicate that 2ME can reduce VEGF mediated tumour angiogenesis as well as the rate of tumour cell proliferation.

Therefore, the present study was aimed to investigate the dose dependent effect of 2 ME on *in vivo* S-180 cell line injected solid mouse tumour model considering mouse survival, tumour growth rate and bone marrow toxicity as end point.

Additionally, the efficacy and safety of 2-ME will be determined by studying the data whether 2ME can give significant protection against mutagenicity and carcinogenicity.

Three specific aims are proposed.

Specific Aim No 1 : To determine the effect of 2 ME on mouse tumour model, tumour regression and mouse survival was assessed or evaluated.

Specific Aim No 2: To evaluate the efficacy and safety of 2 ME on tumour bearing mouse, bone marrow toxicity and tumour growth rate was assessed.

Specific Aim No 3**:** To investigate the effect of antiangiogenic therapy on solid tumour, cell proliferation rate of tumours from both treated and control series will be evaluated by immunohistochemical staining of 5-bromo 2-deoxyuridine (BrdU) labeled cells.

Plan of Work and Methodology

- Selection of Animal tumour: A suitable mouse tumour model (*Mus musculus*) was selected for experiment and investigation. Inbred adult mice (*Mus musculus*) of both sexes, 4-6 weeks of age were used for experiment. Mice were maintained with food & water in the animal house.
- S-180 tumour transplantationSarcoma : 180 cell line was maintained in the inbred Swiss albino mice (inoculums 106 cells per animal) by serial intraperitoneal transplantation. S-180 tumour cells (106 cells were suspended in 0.5 ml sterile 0.9% NaCl solution) were injected intraperitoneally for development of ascitic form of tumour (Chakrabarti and Chakrabarti 1987). Tumour cells showing 85% - 90% viability as assessed by Trypan blue exclusion test were used for transplantation. Full grown ascitic form of tumour was developed within 7 days after transplantation. All experiments were done in accordance with the guidelines framed by the IAEC (Institutional Animal Ethics Committee) of Rammohan College, Kolkata (Animal House Registration No - 1795/PO/ERe/S/14 CPCSEA) for the care and use of Laboratory animals.

- Induction of solid tumour in mouse: The ascites form of sarcoma 180 (S-180) cells was injected subcutaneously in leg ventrally for tumour induction. The whole process was

carried out aseptically. The mean time for appearance of palpable leg tumour was 7-8 days as standardized in the laboratory (Banerjee et al 2013).

- Drug or Chemical: 2-Methoxyestradiol (Sigma, U.S.A), an anti-angiogenic end metabolite of estradiol was chosen.
- Treatment of 2ME: Treatment was started at log- phase of tumour growth, i.e. after 8th day of tumour cell injection. Solid leg tumour bearing mice was subjected to intraperitoneal injection of 2ME stock solution once daily for five consecutive days. Preparation of ME solution: 5mg ME was dissolved in 5ml absolute alcohol. Then the solution was diluted in 5ml sterilized distilled and ME stock solution was prepared. A parallel control was made to analyze the effect of the solvent.

Nontreated(NT) or Control / lDrug	Route	Dose	Average weight of Mouse
NT or Control (solution prepared with absolute alcohol and distilled water v/v 1:1 ratio	IP	0.2ml/ mouse	15 gms
2-Methoxyestradiol (2-ME) low dose	IP	0.05mg ME/Mouse	15 gms
2-Methoxyestradiol (2-ME) Medium dose	IP	0.1mg ME/Mouse	15 gms
2-Methoxyestradiol (2-ME) high dose	IP	0.5mg ME/Mouse	15 gms

Table: 1. Treatment Schedule

- Mouse survival: Mouse survival or the life span of tumour bearing mouse in both non-treated or control and treated groups was studied according to the specific protocol (Chakroborty et.al. 2004; Basu et al. 2004; Sarkar et. al. 2008).
- Tumour regression: Tumour regression in response to therapy in both non-treated or control and treated groups was studied at different time intervals by morphometric analysis of tumour size.

- Tumour volume measurement: The tumour growth or volume was measured in cubic cm using the formula: tumour volume = width x length x 0.5 width (Ray et al 2005). The volume was measured once daily for five consecutive days using Vernier calipers. Depending on the experiments, 3 to 4 mice per group were used.
- Bone marrow toxicity assessment: Bone marrow toxicity was measured by chromosomal aberration analysis. (Banerjee et. al. 2006; Banerjee & Banerjee 2008). Actually one of the pathways for the action of most antineoplastic agents is chromosomal aberrations. So observations on the effect of antineoplastic agents on the chromosome of host own body cells will provide valuable information for better monitoring of cancer therapy.

  Chromosome preparation from bone marrow cells of tumour bearing mice in both control and treated series was done according to the method as practiced by earlier workers.

  (Chakrabarti et al. 1985; Chakrabarti and Chakrabarti, 1987; Banerjee & Chakrabarti 2004).

- Study of tumour vasculature: Tumour tissues of both non-treated and treated groups were excised with sharp blade and kept in 0.9% normal saline for 1 minute. Then excised tumour tissues were observed under the Binocular Research Microscope (5 x 10 magnification) to study nature of tumour vessels according to the method as practiced in my laboratory (Mallick et al 2015).
- Cell proliferation analysis: The numbers of proliferating or viable and dead cells in control and treated series were measured by Trypan Blue Exclusion test (Mallick et al. 2017). The ascitic fluid was taken in WBC pipette and diluted 100 times with 0.9% NaCl. One drop of the Trypan blue stained (0.4%) cell suspension was kept on the Neubauer counting chamber. The proliferating or viable and dead cells were counted to study the regression pattern of the tumour. In vivo, cell proliferation of tumors from both treated and control series was also evaluated by

immunohistochemical staining of 5-bromo 2-deoxyuridine (BrdU). BrdU labeled formalin-fixed, paraffin-embedded tissue sections were used to study the rate of cell proliferation in the control and treated neoplastic tissue.

Work done or Result

i) Solid tumour induction in mouse: The mean time for appearance of palpable leg tumour was 7-8 days.

ii) Effect of 2ME on Mouse survival: Treatment with 0.1mg ME/mouse showed considerable increase of life span of tumour bearing mouse when compared with non-treated and other treated group. The survival time was 17.2±0.58 (days, mean ± standard error, N=5) in the 0.1mgME treated group (Figure 1 Table II) The survival time of 0.1mg ME treated series was significantly longer than control and other treated groups ($P<0.001$).

iii) Tumour growth regression: Tumour growth was analysed on and from 8^{th} days to 12^{th} days after tumour cell injection. The non-treated group showed increase of tumour growth where as 0.1mg ME treatment showed regression in tumour volume. No changes in tumour volume were observed in both 0.05 mg ME and 0.5 mg ME treated groups. Interestingly, 0.5 mg ME induced toxic effect as most the specimens were expired on 8^{th} and 9^{th} day (Mallick et al 2015) [Figure: 2].

iv) Chromosomal aberration study to assess bone marrow toxicity : Bone marrow toxicity test was done through the scoring of different types of simple chromosomal aberration (SCA) and complex chromosomal aberration (SCA) to monitor the action of drug. Chromatid breaks, lesions, deletions are included within the SCA type where as centric fusion or formation of metacentric and submetacentric chromosomes, exchanges,

pulverizations are included within the CCA type. It was interesting to note that 0.5mg ME treated tumour bearing mouse revealed a large numbers of affected metaphase cells (with maximum number of chromosomal aberrations) when compared with 0.1mg ME treated mouse (Mallick et al 2015) [Figure : 3 & 4].

v) Study of tumour vasculature to analyse the anti-angiogenic effect of 2-Methoxyestradiol:

The tumour sections of control or non-treated tumour bearing mouse showed maximum number of blood vessels where as tumour sections of 0.1 mg ME treated tumour bearing mouse revealed fewer blood vessels. So 0.1 mg ME treatment induced a strong antiangiogenic response in such tumour bearing mouse by a significant decrease in tumour vasculature density (Mallick et al 2015) [Figure : 5].

vi) Cell proliferation analysis: The numbers of proliferating or viable and dead cells in control and treated series were measured by Trypan Blue Exclusion test (Mallick et al. 2017). The proliferating or viable and dead cells were counted to study the regression pattern of the tumour (Figure 6). In vivo, cell proliferation of tumors from both treated and control series was also evaluated by immunohistochemical staining of 5-bromo 2-deoxyuridine (BrdU) [Figure : 7].

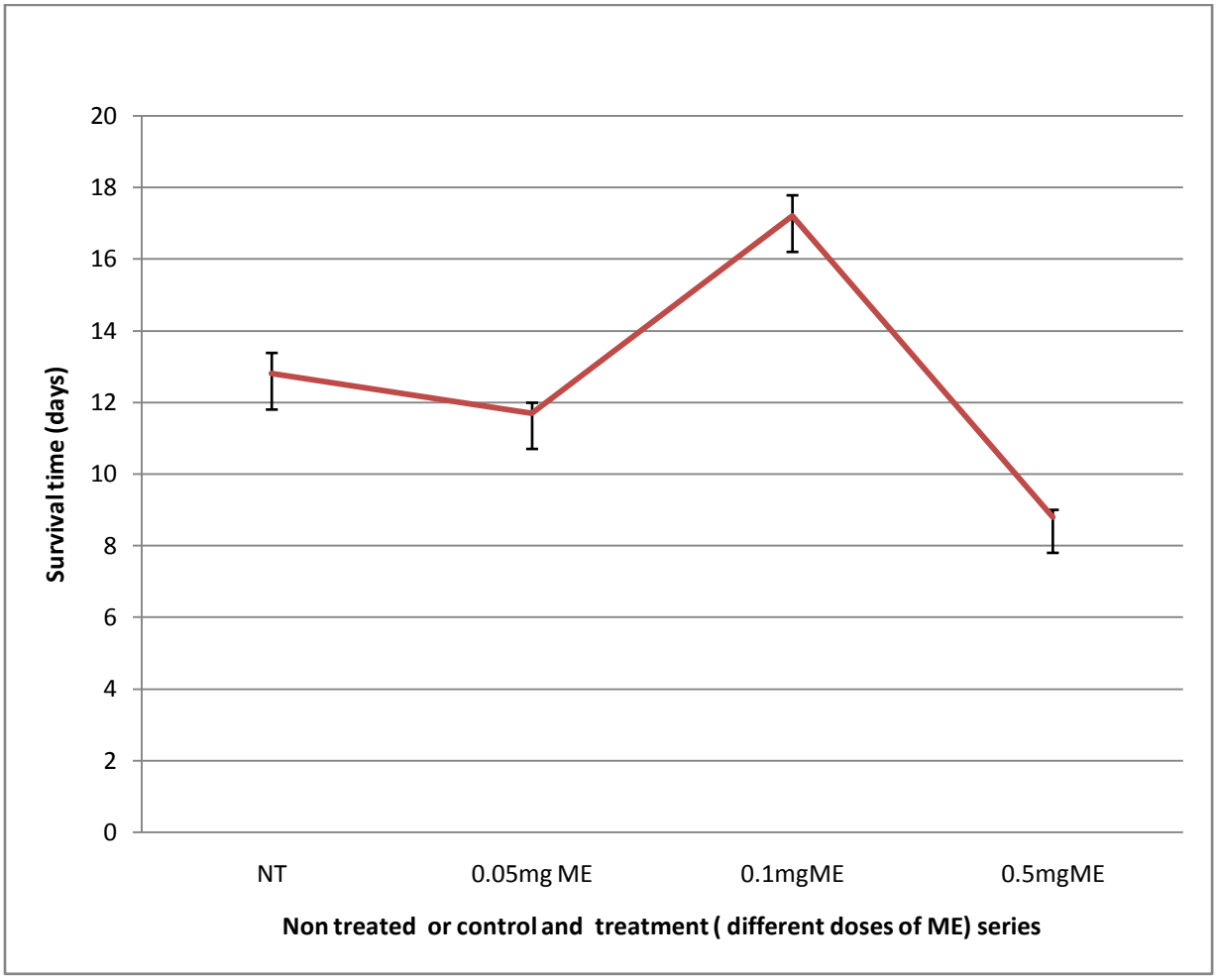


Figure 1 . The survival time (days) of non-treated or control and treated tumour bearing mouse. NT = non-treated; ME = methoxyestradiol : 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME (high dose). The survival time was 17.2±0.58 (days, mean ± standard error, N=5) in the 0.1mgME treated group. The survival time of 0.1mg ME treated series was significantly longer than control and other treated groups (P<0.001).

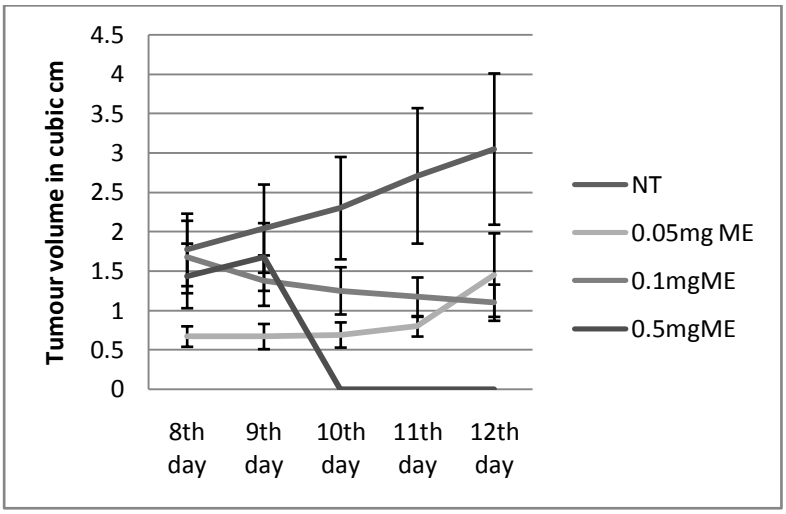


Figure 2. Morphometric analysis of tumour growth in non-treated or control and treated tumour bearing mouse. . NT = non-treated; ME = methoxyestradiol: 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME (high dose). Tumour growth was analysed on and from 8th days to 12th days after tumour cell injection. The non-treated group showed increase of tumour growth where as 0.1mg ME treatment showed regression in tumour volume. No changes in tumour volume were observed in both 0.05 mg ME and 0.5 mg ME treated groups. In addition, 0.5 mg ME induced toxic effect as most the specimens were expired on 8th and 9th day.

Somatic Metaphase complement from Non-treated and ME treated tumour bearing Mouse

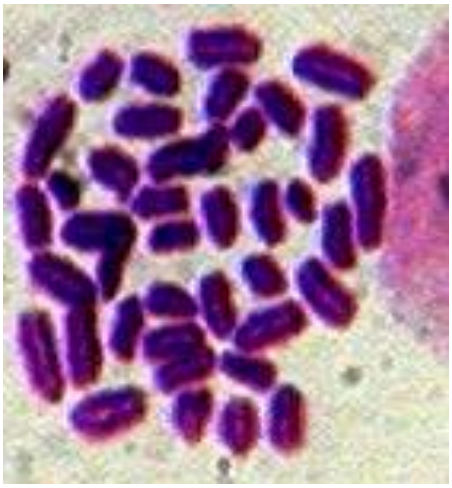


Figure: 3a Metaphase chromosome prepared from bone marrow cell of Non- treated tumour bearing mouse (2n= 40: all chromosomes are normal)

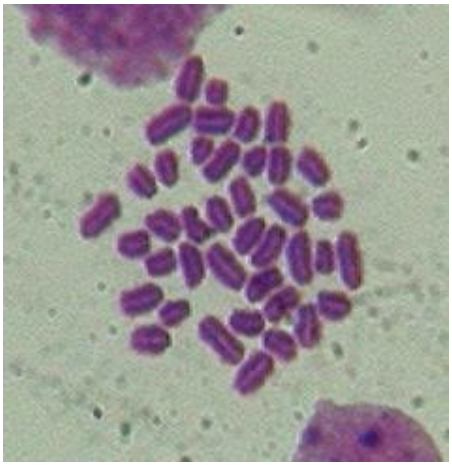


Figure : 3b Metaphase chromosome prepared from bone marrow cell of 0.1mg ME treated tumour bearing mouse showing normal chromosomes(2n= 40)

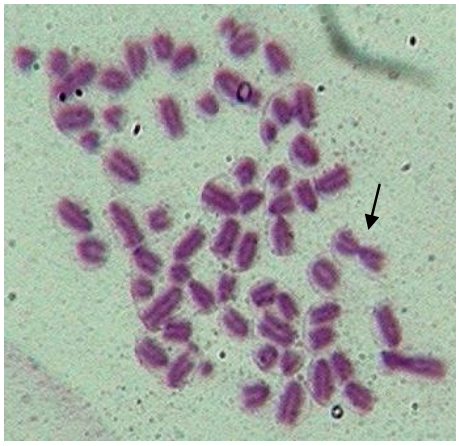


Figure: 3c Metaphase chromosome prepared from bone marrow cell of ME treated tumour bearing mouse showing centric fusion (arrowed).

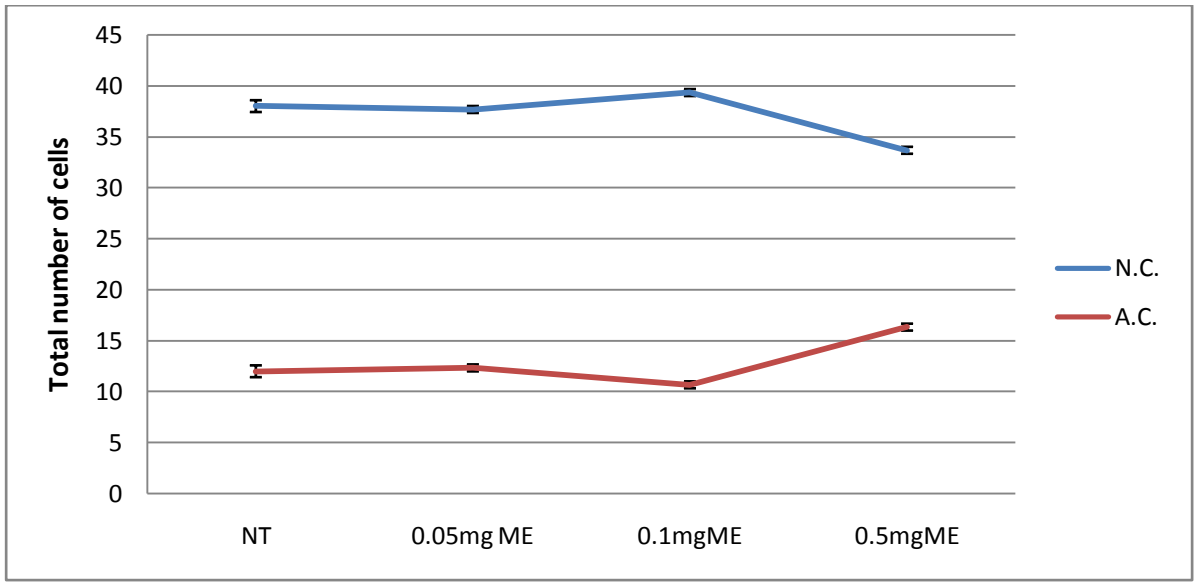


Figure 4a Distribution of normal metaphase cells (N.C.) and affected metaphase cells (A.C.) in non-treated or control and treated tumour bearing mouse. NT = non-treated; ME = methoxyestradiol : 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME(high dose). 0.5mg ME treated tumour bearing mouse revealed large numbers of affected metaphase cells when compared with 0.1mg ME treated mouse.

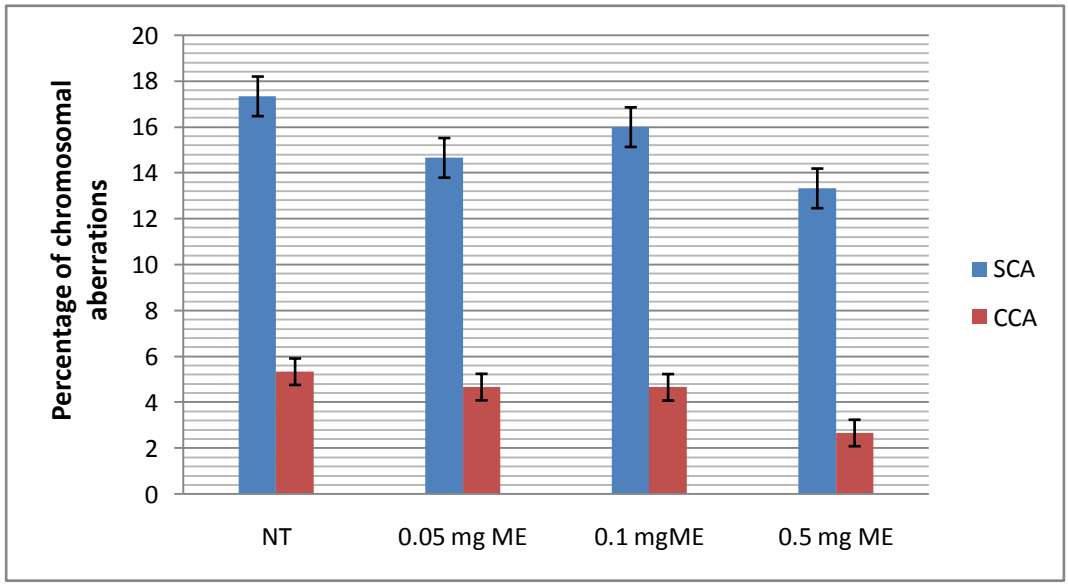
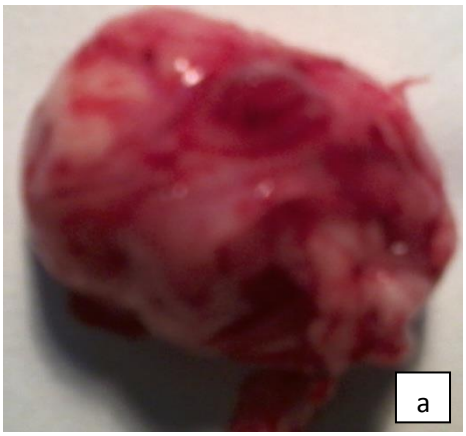


Figure 4b Percentage of different types of chromosomal aberrations in control and treatment groups. SCA = simple chromosomal aberrations includes chromatid break, lesion, deletion etc.CCA = complex chromosomal aberrations includes centric fusion, exchanges etc. NT = non-treated; ME = methoxyestradiol : 0.05mg ME (low dose); 0.1mg ME (medium dose) and 0.5mgME(high dose).

Morphology of Leg Tumour in Mouse induced by S-180 tumour cell



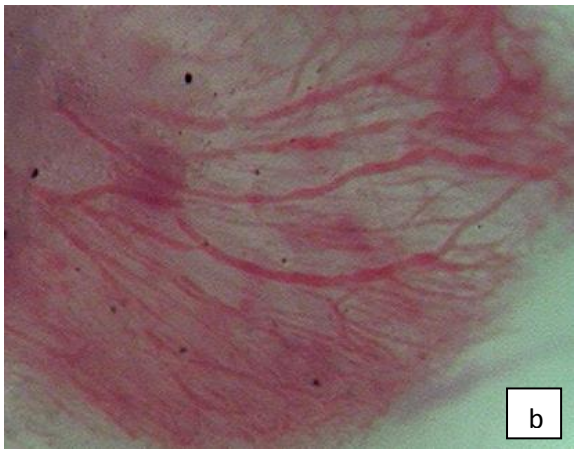
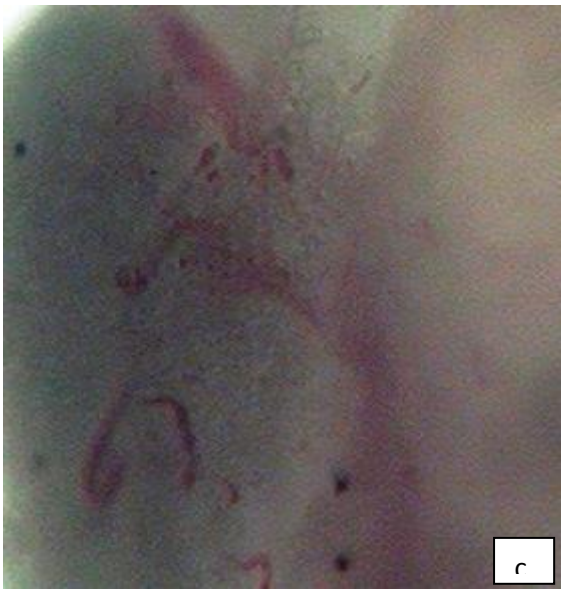


Figure 5 a. Morphology of Tumour in non-treated group b. Section of tumour in non treated group showing vascularisation



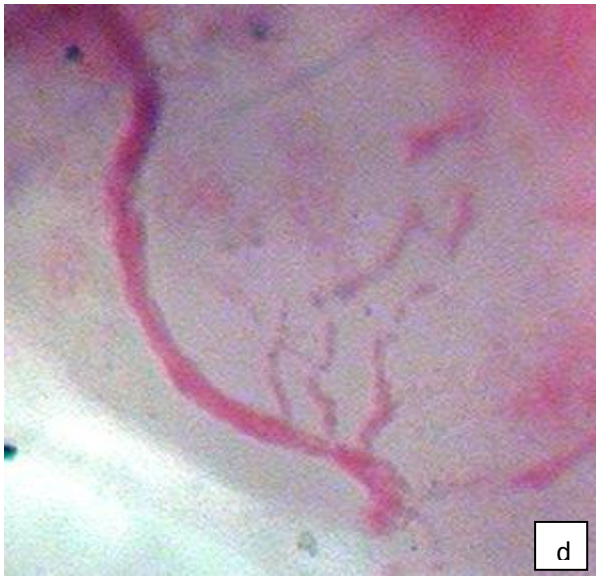
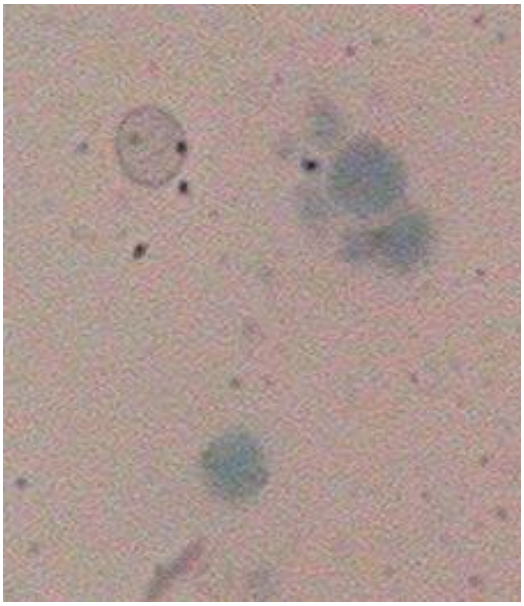
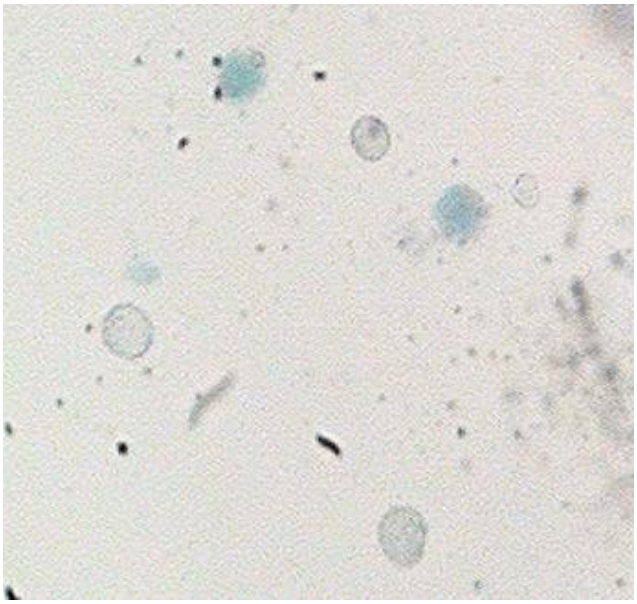


Figure 5 c and d. Section of tumour with fewer blood vessels in ME treated tumour bearing mouse showing anti-angiogenic effect.

Cell Proliferation Study by Dead and Living cell count





a **b**

Figure: 6 a = ME treated Tumour cell showing maximum number of dead cell (blue coloured) ; b= Control or non-treated cell showing maximum number of living cell(transparent or light coloured)

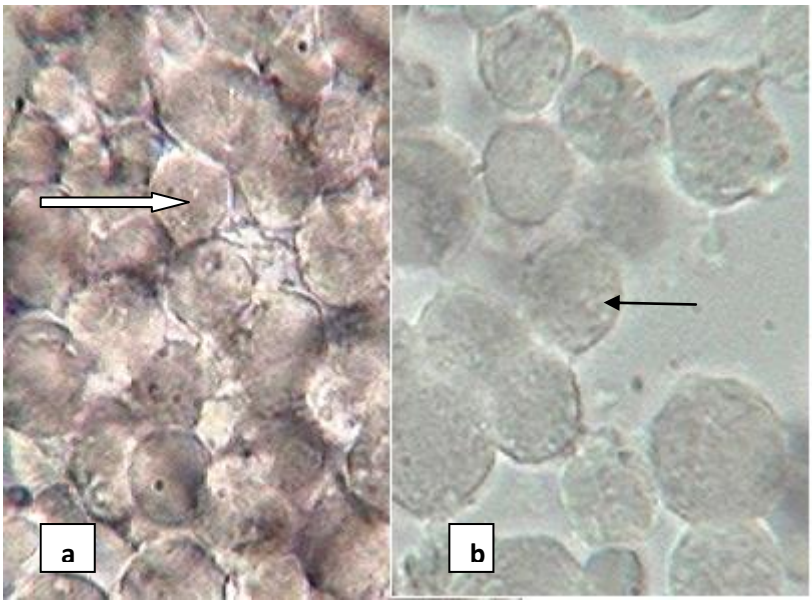


Figure : 7. Cell proliferation study by BrdU labeling method.

a = BrdU labeled proliferated cell (white block arrow) in control condition and b = normal cell (black line arrow) population in ME treated BrdU labeled tumour cells

Executive summary

The results indicate that 2ME (0.1mg ME concentration) is highly potent antineoplastic and antiangiogenic drug. The regression of the tumour during the course of therapy was determined which is correlated with the gradual increase of mouse survival or life span of 0.1mg ME treated tumour bearing mouse. Daily 2ME treatment (0.1mg ME for 5 consecutive days) induced a strong anti-angiogenic response as shown by a decrease in tumour vascular density or by a decline in the number of blood vessels in tumour. Analysis of bone marrow metaphases clearly points out that 0.1 mg ME can protect bone marrow as it inhibits different types of chromosomal aberrations.

No significant changes in the survival rate of mouse or life span of tumour bearing mouse and tumour volume were observed in NT or control, 0.05mg and 0.5mg ME treated groups. Interestingly, 0.5mg dose has exerted toxic effect as most of the tumour bearing mice were expired within 2nd or 3rd day of the treatment.

So, the present result is in agreement with the previous findings as it was studied on some in vitro tumour cell lines i.e. MCF-7 and GH3 (Banerjee et al., 2002; 2003). Overall, we conclude that 0.1mg ME is more effective than 0.05mg and 0.5 mg ME not only in controlling the tumour regression with blood vessels proliferation but also in prolonging survival time or life span of tumour bearing mouse. Moreover, 0.1mg ME treated bone marrow cells revealed a significantly less number of affected metaphase cells with minimum number of chromosomal aberrations when compared with 0.5mg ME treated mouse.

So 2ME may be applied as a novel therapeutic drug for cancer.

I have also extended the study to *in vivo* mouse model to evaluate the efficacy and safety of 2ME in combination with cyclophosphamide (antineoplastic drug). But the effect of low dose of combination therapy of Cyclophosphamide and 2-Methoxyestradiol has not been studied yet. In

the present experiment, combination therapy of 2ME and CP significantly enhanced the therapeutic efficacy than 2ME and CP alone as evident from increased dead or non-viable tumour cell number and survivability of the tumor bearing hosts following combination treatment. Regression spectrum of the tumour was also determined by studying the metaphase index which was noticed during the end of the combination therapy.

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