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ORIGINAL ARTICLE

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Therapeutic effect of tamoxifen and energy-modulating vitamins on carbohydrate-metabolizing enzymes in breast cancer

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Abstract *Background*: Cancer cells have an abnormal energetic metabolism. One of the earliest discovered hallmarks of cancer had its roots in bioenergetics, as many tumours were found in the 1920s to exhibit a high glycolytic phenotype. An animal with cancer shows significant and progressive energy loss from the host (i.e. noncancerous) tissues, which could occur by the establishment of a systemic energy-depriving cycle involving the interaction of tumour glycolysis and host gluconeogenesis. Tamoxifen (TAM) is a nonsteroidal antioestrogen that is widely used in adjuvant therapy for all stages of breast carcinoma. To improve the therapeutic efficacy of TAM and to expand its usage in the treatment of breast cancer, it is necessary to establish an energy-enhancing programme. In order to provide sufficient energy and to prevent cancer cachexia, TAM can be supplemented with energy-modulating vitamins (EMV). In this investigation the augmentation of the efficacy of TAM by the effects of EMV supplementation on carbohydrate-metabolizing enzymes, the mitochondrial Krebs cycle and respiratory enzymes was evaluated in the mammary gland of carcinoma-bearing rats. *Methods*: Female albino Sprague-Dawley rats were selected for the investigation. The experimental set-up included one control and four experimental groups. Mammary carcinoma was induced with 7,12- dimethyl benz(*a*)anthracene (25 mg), and TAM was administered orally (10 mg/kg body weight per day) along with EMV which comprised riboflavin (45 mg/kg per day), niacin (100 mg/kg per day) and coenzyme Q_{10} (40 mg/kg per day). *Results*: Measurements were made on tumour tissue and surrounding normal tissue in all experimental groups. Tumour tissue showed significant ($P < 0.05$) increases in the glycolytic enzymes hexokinase, phosphoglucoisomerase and aldolase, and significant decreases in the gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-biphosphatase. In contrast, the surrounding tissue showed significant decreases in glycolytic enzymes and significant increases in gluconeogenic enzymes. The activities of the mitochondrial Krebs cycle enzymes isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, and respiratory chain enzymes NADH dehydrogenase and cytochrome *c* oxidase were significantly reduced in both tumour and surrounding tissue of the mammary carcinoma-bearing rats. These biochemical disturbances were effectively counteracted by supplementation with EMV, which restored the activities of all these enzyme to their respective control levels. *Conclusion*: Combination therapy of TAM with EMV not only alters carbohydrate metabolism but can also prevent body weight loss by enhancing the host energy metabolism.

Keywords Breast cancer · Cancer cachexia · Carbohydrate-metabolizing enzymes · Tamoxifen · Energy-modulating vitamins

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Introduction

Impairment of energy metabolism in cancer cells has been a recurrent finding for many years. Current thought is that proliferating cells undergo a shift from oxidative to glycolytic metabolism, where the energy requirements of the rapidly dividing cell are provided by ATP from glycolysis [1, 2]. The high glycolytic rate under aerobic conditions is important for rapidly prolif-

erating cancers not only as a major energy source, but also to provide the cells with precursors for nucleotide and lipid biosynthesis [3]. This seems to be a general property of highly malignant tumours independent of their carcinogenic origin. High rates of glucose utilization with production of lactic acid are characteristic features of the neoplastic cell [4]. Lactic acid so produced may be utilized for energy purpose by other tissues or transported to the liver for resynthesis of glucose (Cori cycle). Utilization of lactic acid by peripheral tissues would normally provide the tumour-bearing patient with maximum energy available from glucose oxidation. Gluconeogenesis from lactate, on the other hand, is an energy-requiring process that, as pointed out by Fenninger and Mider [5], may play an important role in excessive energy expenditure of the host, thus contributing to mechanisms that promote weight loss [6]. In intact glycolytic tumour cells, only 10% of the pyruvate enters a truncated Krebs cycle [7]. This results in a decrease in the overall net ATP production in the host cells due to downregulation of the activities of Krebs cycle and respiratory enzymes, resulting in turn in a loss of body weight which occurs in cancer cachexia [8].

Breast cancer is the commonest malignancy comprising 18% of all cancers in women [9]. Since 1990, death rates from breast cancer have decreased by over 25%, and this is at least in part due to adjuvant treatment with tamoxifen (TAM), a nonsteroidal antioestrogen, and chemotherapy [10]. TAM is now widely used and has led to an increase in both disease-free and overall survival of women after primary surgery [11]. It induces tumour regression in women with advanced metastatic breast cancer [12]. The antitumour activity of TAM is largely believed to be due to its occupation of the intracellular oestrogen receptor sites in the target tissue and blocking the action of biologically active oestrogen and oestradiol [13]. In addition, the antiproliferative effects of TAM may relate to its inhibition of protein kinase C [14] and its binding to calmodulin, a protein that plays a role in DNA synthesis [15]. Besides its anticarcinogenic potential, it also produces some adverse toxic side effects if taken for a long time [16, 17].

The current interest is to improve the chemotherapeutic effects of TAM and to expand its application in the treatment of breast cancer. In order to achieve this it is essential to introduce an energy-enhancement programme. Hence, we administered the energy-modulating vitamins (EMV) (riboflavin, niacin and coenzyme Q_{10}) along with TAM to improve the management of breast cancer. Vitamins as such show negligible/minimal anticancer effects but generally potentiate the effects of anticancer drugs and also attenuate their side effects. Formulating the right combination of vitamins is of paramount importance and needs to reflect the cancer biology and treatment. Riboflavin, in its active coenzyme forms such as flavin mononucleotide (FMN^+) and flavin adenine dinucleotide (FAD^+), participates in oxidation–reduction reactions in numerous metabolic pathways and in energy production via the respiratory chain [18]. Niacin and its cofactors, nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate ($NADP^+$), are essential in a variety of oxidation–reduction reactions involved in tissue respiration. The protective effect of nicotinamide may be due to its action as a free radical scavenger and to its effect on the inhibition of the enzyme poly ADP-ribose synthetase and on the repletion of NAD^+ levels inside the cell [19]. Coenzyme Q_{10} seems to play multiple functional roles in cells. The most well known function of coenzyme Q_{10} is, in its quinone form, the transfer of electrons in the mitochondrial electron transport chain from complexes I and II to complex III [20].

A recent novel approach to the chemotherapeutic treatment of cancer has focused on the aberrant energy metabolism of transformed cells. It may be possible to selectively inhibit tumour cell energy production, growth and survival by targeting tumour-specific enzyme systems. Improving energy production in the host cells may prevent cancer cachexia, a serious terminal complication. Hence, our current interest was to evaluate augmentation of the efficacy of TAM by the effects of EMV supplementation on carbohydrate-metabolizing, mitochondrial Krebs cycle, and respiratory chain enzymes (energy metabolism) in rats bearing DMBA-induced mammary carcinoma.

Materials and methods

Source of chemicals

DMBA (7,12-dimethyl benz[a]anthracene), TAM and co-enzyme Q_{10} were purchased from Sigma Chemical Company (St. Louis, Mo.). Riboflavin and niacin were purchased from Glaxo Laboratories, CDH Division (Mumbai, India). All other chemicals and solvents used were of the highest purity and analytical grade and were obtained from Glaxo Laboratories, CDH Division.

Animals

The studies were performed with the approval of the Institutional Animal Care and Utilization Committee. Female albino rats of Sprague-Dawley strain between 50 and 55 days of age were obtained from the National Institute of Nutrition, Hyderabad, India. During the course of the experiments, all rats were housed under a controlled environment with a 12-h light/dark cycle at a temperature of $22 \pm 2°C$, given a commercial diet and tap water ad libitum, and were weighed every week.

Experimental design

The animals were divided into five groups and each comprising six animals.

Group I: Normal healthy rats as controls. Rats at the age of 8 weeks were treated with 1 ml olive oil orally by gastric intubation. After 3 months, 0.5 ml of olive oil was given orally by gastric intubation daily for 28 days

Group II: Rats with DMBA-induced mammary carcinoma. DMBA (25 mg) dissolved in 1 ml olive oil [21] was administered to 8-week-old rats by gastric intubation. At the beginning of 2 months from DMBA administration, the rats were palpated regularly to determine the appearance of mammary tumour. The tumour was allowed to grow for one more month to attain a considerable mass and size. After 3 months, mammary carcinoma was confirmed by histological examination.

Group III: Rats with DMBA-induced mammary carcinoma treated with TAM. Rats were treated with TAM (10 mg/kg body weight) dissolved in water daily for 28 days by gastric intubation.

Group IV: Rats with DMBA-induced mammary carcinoma treated with TAM and EMV. Rats were treated with TAM (10 mg/kg body weight) and EMV comprising riboflavin (45 mg/kg) and niacin (100 mg/kg) dissolved in water, and coenzyme Q_{10} (40 mg/kg) dissolved in olive oil, daily for 28 days by gastric intubation.

Group V: Drug control rats. Rats received EMV alone at the same dosage as group IV animals. These animals were maintained on a normal diet and tap water for 3 months prior to drug ingestion.

The animals were allocated to groups at the same time. Other treatments were extended during the time as mentioned in the text. At the termination of the study (i.e. after 3 months and 28 days) the animals of all five groups were fasted over night and killed by cervical dislocation. The mammary gland (tumour and surrounding tissue) was immediately excised from the animals and weighed. Carcinomatous tissue was dissected free of grossly necrotic and hemorrhagic areas, as well as from connective tissue and normal tissue. Portions weighing approximately 500 mg were cut, placed into plastic snap-cap vials and quickly frozen in liquid nitrogen. The vials were placed in a deep-freezer at −72°C, and stored until assayed. At the time of assay, small pieces of tissue were obtained for microscopic confirmation of the diagnosis; the remainder was homogenized and used for biochemical assays.

Measurements of tumour weight and tumour volume

Tumour weight was measured according to the method of Geren et al. [22] with the aid of a vernier caliper. Tumour volume was calculated according to the method of Roberto et al. [23]. After carcinogen administration, the size of the tumours was monitored by measuring externally at three dimensions.

Preparation of mitochondria from mammary gland

Mammary glands were removed and cooled in 0.3 *M* sucrose-EDTA, pH 7.4, on ice. All subsequent steps were done at 0–4°C. As much external connective tissue was removed as possible and the tissues were blotted dry, weighed and minced very finely with small scissors. The tissue was then homogenized in a Potter-Elvehjem homogenizer using two passes with a loose pestle and two passes with a tight pestle. The homogenate was strained through four layers of 10-gauge cheesecloth and the filtrate was centrifuged at 900 *g* for 5 min. The supernatant was centrifuged at 11,500 *g* for 10 min to sediment the mitochondria. The mitochondrial pellet was washed twice in 5 ml sucrose-EDTA medium. At each step, the upper fatty layer was carefully removed by aspiration and the walls of the tubes were carefully wiped clean. The final mitochondrial pellet was suspended at 10 mg mitochondrial protein per millilitre of sucrose-EDTA/BSA medium. The purity of the mitochondria was assessed by estimating succinate dehydrogenase activity. Mitochondrial protein was estimated by the method of Lowry et al. [24].

Preparation of mitochondria from tumour mammary gland

Tumour cells were identified by the appearance of a red mass under the epithelial layer of the mammary pad. Tumour mitochondria were isolated by the method of Senior et al. [25]. A portion of tumour was removed carefully from the mammary pad and placed immediately in 0.3 *M* sucrose-EDTA, pH 7.4, at 0°C. All subsequent steps were done at 0–4°C. Necrotic tissue was removed and the tumour tissue was chopped into small pieces, weighed and washed three times with 40 ml chilled sucrose-EDTA solution. The tumour tissue was then homogenized directly in five volumes of 0.3 *M* sucrose-EDTA/1% BSA, pH 7.4, in a Potter-Elvehjem homogenizer. The homogenate was then treated in the same way as the homogenate from normal mammary gland (as above). BSA is necessary to obtain functional mitochondria from tumour.

Assays of carbohydrate-metabolizing enzymes

Hexokinase activity was measured with respect to the amount of glucose utilized after the addition of ATP [26]. Phosphoglucoisomerase activity was assayed according to the method of Horrocks et al. [27]. Aldolase activity was assayed according to the method of

King [28] with fructose 1,6-bisphosphate as substrate and dinitrophenyl hydrazine as colouring reagent. The activity of glucose-6-phosphatase and fructose 1,6-bisphosphatase [29] were assayed with respect to the amount of inorganic phosphorus liberated after the addition of their respective substrate, glucose-6-phosphate or fructose 1,6-bisphosphate. Serum lactate dehydrogenase (LDH) was assayed according to the method of King [30].

Mitochondrial Krebs cycle and respiratory chain enzyme assays

The activity of isocitrate dehydrogenase was assayed by the method of King [31]. The activity of α-ketoglutarate was assayed by the colorimetric determination of ferrocyanide produced by the decarboxylation of α-ketoglutarate with ferricyanide as electron acceptor [32]. The activity of succinate dehydrogenase was assayed by the method of Slater and Bonner [33] in which the rate of reduction of potassium ferricyanide was measured in the presence of potassium cyanide. The activity of malate dehydrogenase was assayed by the method of Mehler et al. [34]. The activity of nicotinamide dinucleotide (NADH) dehydrogenase was assayed by the method of Minakami et al. [35]. The activity of cytochrome *c* oxidase was assayed by the method of Wharton and Tzagoloff [36].

Statistical analysis

All data are expressed as means ± SD from six rats. The results were analysed statistically (SPSS Software Package) using one-way analysis of variance. Post hoc testing was performed for intergroup comparisons using Tukey's HSD test. In all cases $P < 0.05$ was considered significant.

Results

Figure 1 shows the initial and final body weights of the control and experimental rats. Initially, there was no significant change in body weight of the control and experimental rats. But finally, there was a sharp drop in body weight of the mammary carcinoma-bearing rats when compared with the normal control rats. Drug-treated rats showed a gradual increase in body weight compared with untreated rats. Rats treated with TAM and EMV showed a further increase in body weight compared with rats treated with TAM only. Drug control rats showed an increase in body weight, but the increase was not significant compared with normal control rats.

Figure 2 shows the tumour weights of treated (TAM alone and TAM + EMV) and untreated rats. There was considerable tumour progression in untreated rats compared with treated rats. In drug-treated rats, tumour did not disappear totally, but a significant regression was found compared with untreated rats. Rats treated with TAM alone showed reductions in tumour weight of 42%, 50% and 61% on days 7, 14 and 28, respectively, compared with untreated disease-prone animals. Rats treated with TAM + EMV showed reductions in tumour weight of 45%, 58% and 70% on days 7, 14 and 28, respectively, compared with untreated disease-prone animals.

Figure 3 shows the tumour volumes of treated (TAM alone and TAM + EMV) and untreated rats. There was a considerable increase in tumour volume in untreated rats compared with treated rats. Rats treated with TAM alone and TAM + EMV showed a significant ($P < 0.05$) reduction in tumour volume compared with untreated disease-prone animals.

Figure 4 shows the activity of LDH in serum from control and experimental rats. LDH activity was found

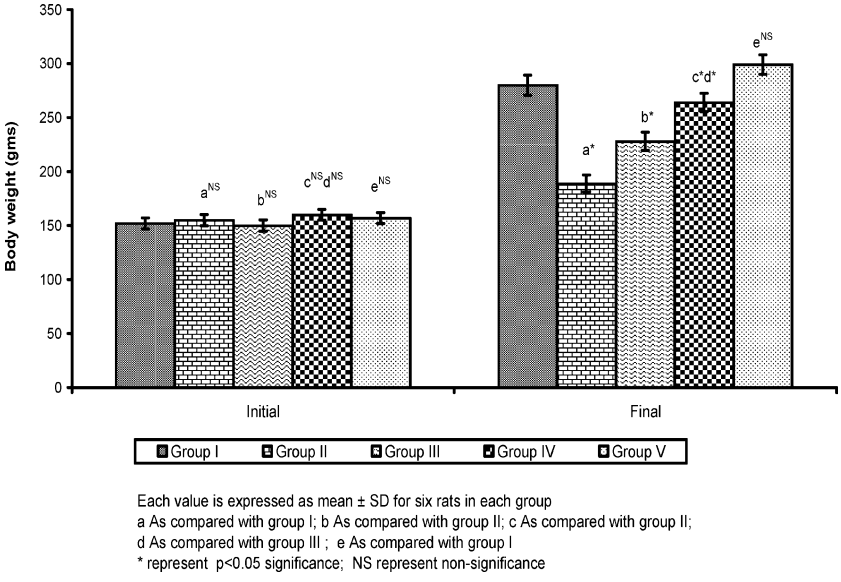
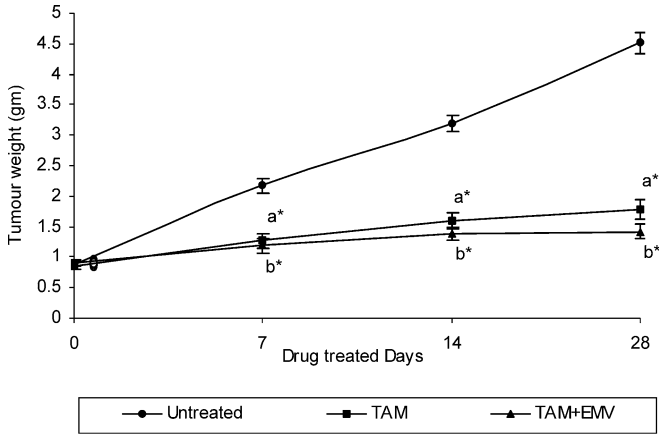


Fig. 1 Body weight changes in control and experimental animals. The data are presented as the means ± SD from six rats in each group (*a* vs group I; *b* vs group II; *c* vs group II; *d* vs group III; *e* vs group I; **P* < 0.05; *NS* not significant)

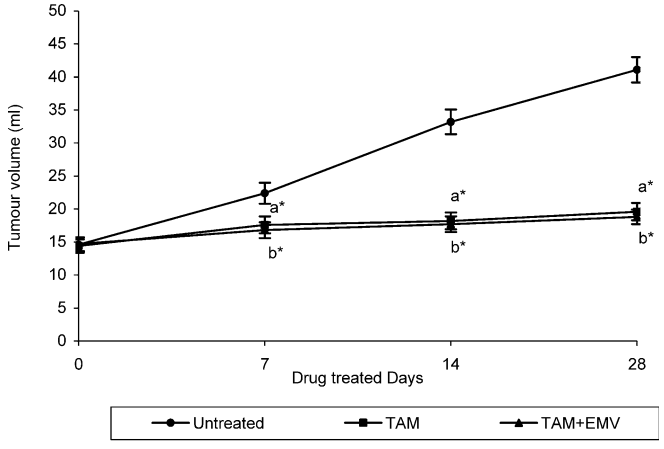


Values are expressed as mean ± SD for six rats in each group
a - comparison between untreated and TAM treated groups;
b - comparison between untreated and TAM+EMV treated groups;
* represent p<0.05 significance

Fig. 2 Effect of TAM + EMV on tumour weight. The data are presented as means ± SD from six rats in each group (*a* untreated vs TAM-treated; *b* untreated vs TAM + EMV-treated; $*P < 0.05$)

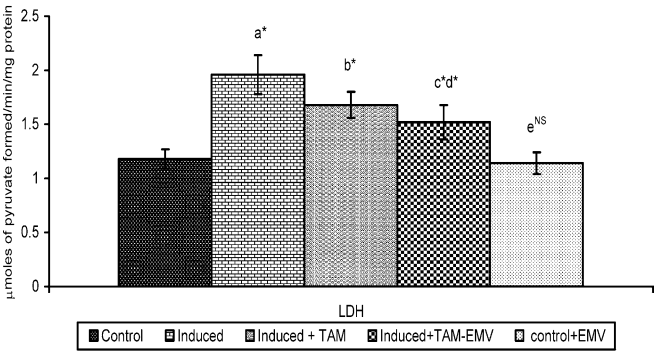
to be elevated significantly ($P < 0.05$) in tumour-bearing rats compared with control rats. The activity reverted to near-normal levels after administration of drug in groups III and IV.

The activities of the glycolytic enzymes hexokinase, phosphoglucoisomerase and aldolase, and the gluconeogenic enzymes glucose-6-phosphatase and fructose-1,6-biphosphatase in the mammary gland (tumour site and surrounding tissue) of control and experimental rats are shown in Table 1. In tumour, the activities of the glycolytic enzymes were increased (hexokinase +97%, phosphoglucoisomerase +57%, aldolase +58%), and the activities of the gluconeogenic enzymes were decreased (glucose-6-phosphatase −26%, fructose-1,6-biphosphatase −31%) compared with mammary gland of control rats. Whereas in the surrounding tissue, the glycolytic enzyme activities were markedly decreased (hexokinase −30%, phosphoglucoisomerase −29%, aldolase −37%), and the gluconeogenic enzyme activities were considerably increased (glucose-6-phosphatase +68%, fructose-1,6-biphosphatase +38%) in tumour-bearing rats compared with control rats.



Values are expressed as mean ± SD for six rats in each group
a - comparison between untreated and TAM treated groups;
b - comparison between untreated and TAM+EMV treated group;
* represent p<0.05 significance

Fig. 3 Effect of TAM + EMV on tumour volume. The data are presented as means ± SD from six rats in each group (*a* untreated vs TAM-treated; *b* untreated vs TAM + EMV-treated; $*P < 0.05$)



Each value is expressed as mean ± SD for six rats in each group
a As compared with group I; b As compared with group II; c As compared with group II;
d As compared with group III ; e As compared with group I
* represent p<0.05 significance; NS represent non-significance

Fig. 4 Activity of serum LDH in control and experimental animals. The data are presented as means ± SD from six rats in each group (*a* vs group I; *b* vs group II; *c* vs group II; *d* vs group III; *e* vs group I $*P < 0.05$; *NS* not significant)

The levels of glycolytic enzymes were significantly ($P < 0.05$) decreased and the levels of gluconeogenic enzymes significantly increased in mammary gland tumour of drug-treated compared with untreated rats. In the surrounding tissue, the glycolytic and gluconeogenic enzyme activities were nearer to their respective control activities.

The activities of the mitochondrial Krebs cycle enzymes isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinate dehydrogenase and malate dehydrogenase, and the respiratory chain enzymes NADH dehydrogenase and cytochrome *c* oxidase in the mammary gland (tumour and surrounding tissue) of control and experimental rats are shown in Table 2. The activities were significantly ($P < 0.05$) decreased in both mammary gland tumour and surrounding tissue in carcinoma-bearing rats compared with control rats. The decrease was 30% for isocitrate dehydrogenase, 37% for α-ketoglutarate, 27% for succinate dehydrogenase, 36% for malate dehydrogenase, 41% for NADH dehydrogenase and 65% for cytochrome *c* oxidase in tumour, and 23% for isocitrate dehydrogenase, 26% for α-ketoglutarate, 30% for succinate dehydrogenase, 23% for malate dehydrogenase, 32% for NADH dehydrogenase and 44% for cytochrome *c* oxidase in surrounding tissue. Enzyme activities were increased in tumour and surrounding tissue of rats treated with TAM and TAM + EMV compared to untreated carcinoma-bearing rats. These enzyme activities were in-

Table 1 Activities of glycolytic and gluconeogenic enzyme in mammary gland (tumour and surrounding tissue) of control and experimental rats. The data presented are the amounts of the following products liberated (nmol/min/mg protein, means ± SD, $n = 6$): glu-6-phosphate, fructose and glyceraldehyde for hexokinase, phosphoglucoisomerase and aldolase, respectively, and inorganic phosphorus for both glucose-6-phosphatase and fructose 1,6-bisphosphatase (*NS* not significant)

Enzyme	Group I (control)	Group II (tumour-induced)		Group III (tumour-induced + TAM)		Group IV (tumour-induced + TAM + EMV)		Group V (control + EMV)[a]
		Tumour[a]	Surrounding tissue[a]	Tumour[b]	Surrounding tissue[c]	Tumour[d]	Surrounding tissue[e]	
Hexokinase	8.79 ± 0.69*	17.29 ± 1.31*	6.17 ± 0.51*	11.71 ± 0.98*	7.37 ± 0.66*	13.32 ± 1.14*	8.15 ± 0.78*	8.90 ± 0.61 (NS)
Phosphoglucoisomerase	12.47 ± 1.12*	19.61 ± 1.68*	8.87 ± 0.78*	14.26 ± 1.29*	9.85 ± 0.81*	15.89 ± 1.39*	11.42 ± 0.94*	12.91 ± 1.18 (NS)
Aldolase	12.86 ± 1.17*	20.35 ± 1.81*	8.10 ± 0.61*	15.15 ± 1.47*	9.26 ± 0.87*	16.76 ± 1.49*	10.97 ± 0.91*	13.03 ± 1.22 (NS)
Glucose-6-phosphatase	9.14 ± 0.81*	6.78 ± 0.54*	15.31 ± 1.38*	7.65 ± 0.59*	13.55 ± 1.17*	8.22 ± 0.64*	10.82 ± 0.93*	9.40 ± 0.87 (NS)
Fructose-1,6-bisphosphatase	14.04 ± 1.29*	9.73 ± 0.91*	19.31 ± 1.49*	11.33 ± 1.08*	17.52 ± 1.51*	13.20 ± 1.17*	15.82 ± 1.38*	14.27 ± 1.21 (NS)

*$P < 0.05$
Comparisons with
[a]group I
[b]tumour, group II
[c]surrounding tissue, group II
[d]tumour, groups II and III
[e]surrounding tissue, groups II and III

Table 2 Activities of mitochondrial Krebs cycle and respiratory chain enzyme in the mammary gland (tumour site and surrounding tissue) of control and experimental rats. The data presented are the amounts of the following (means ± SD, $n = 6$): α-ketoglutarate liberated (nmol/min/mg protein) for isocitrate dehydrogenase; ferrocyanide liberated (μmol/min/mg protein) for α-ketoglutarate dehydrogenase; succinate oxidized (μmol/min/mg protein) for succinate dehydrogenase; NADH oxidized (nmol/min/mg protein) for malate dehydrogenase; NADH oxidized (μmol/min/mg protein) for NADH dehydrogenase; cytochrome *c* oxidase (optical density $\times 10^{-2}$/min/mg protein) (*NS* not significant)

Enzyme	Group I (control)	Group II (tumour induced)		Group III (tumour induced + TAM)		Group IV (tumour induced + TAM + EMV)		Group V (control + EMV)[a]
		Tumour[a]	Surrounding tissue[a]	Tumour[b]	Surrounding tissue[c]	Tumour[d]	Surrounding tissue[e]	
Isocitrate dehydrogenase	526.8 ± 34.1	367.1 ± 28.1*	407.4 ± 21.9*	442.1 ± 29.8*	461.3 ± 26.5*	504.2 ± 32.8*	517.1 ± 31.2*	540.9 ± 38.2 (NS)
α-Ketoglutarate	152.7 ± 12.1	96.5 ± 8.44*	112.7 ± 10.7*	119.3 ± 8.17*	126.1 ± 10.8*	136.2 ± 10.7*	141.6 ± 10.3*	167.6 ± 12.1 (NS)
Succinate dehydrogenase	221.4 ± 19.8	162.1 ± 14.5*	156.1 ± 12.3*	186.5 ± 15.1*	184.6 ± 15.9*	209.2 ± 17.0*	214.1 ± 18.4*	239.3 ± 19.5 (NS)
Malate dehydrogenase	3416 ± 92.8	2169 ± 136*	2647 ± 98.4*	2531 ± 114*	2871 ± 137*	2976 ± 109*	3136 ± 121*	3498 ± 122 (NS)
NADH–dehydrogenase (NS)	14.88 ± 0.92	8.79 ± 0.59*	10.16 ± 0.92*	9.26 ± 0.91*	10.99 ± 0.78*	10.82 ± 0.96*	13.56 ± 0.91*	15.02 ± 1.09 (NS)
Cytochrome *c* oxidase	3.56 ± 0.12	1.24 ± 0.09*	1.98 ± 0.10*	1.58 ± 0.11*	2.29 ± 0.16*	2.01 ± 0.19*	3.12 ± 0.21*	3.64 ± 0.24

*$P < 0.05$
Comparisons with
[a]group I
[b]tumour, group II
[c]surrounding tissue, group II
[d]tumour, groups II and III
[e]surrounding tissue, groups II and III

creased to a greater extent in rats treated with TAM + EMV than in rats treated with TAM alone. The activities of these enzymes were higher in the surrounding tissue than in tumour. In drug control rats, there was a slight increase in the activities of the mitochondrial enzymes but not to significant levels compared with normal rats.

Discussion

The growth rate of mammary carcinoma cells and their carbohydrate metabolism are significantly correlated. Cancer cells generally have an elevated rate of glucose metabolism and abnormal pattern of energy metabolism compared to normal healthy cells. In the present study, we observed significantly higher activities of glycolytic enzymes in tumour of carcinoma-bearing rats. This indicates that an elevated rate of glycolysis is a characteristic of malignant cells. Through enhanced activity of key glycolytic enzymes and mitochondrial degranulation, the rate of glycolysis in tumour cells is elevated to provide high levels of intermediates for enhanced synthesis of nucleic acids and lipids in rapidly proliferating tumour cells [37].

Hexokinase is the rate-limiting enzyme which catalyses the conversion of glucose to glucose-6-phosphate in the first step of the glycolytic pathway. Hexokinase activity [38], m-RNA levels [39] and transcription rate [40] are increased markedly in rapidly growing tumours. To further potentiate the enhanced hexokinase activity achieved by overexpression, most of the enzyme is bound to the outer mitochondrial membrane, where it has direct access to mitochondrially generated ATP and is less sensitive to glucose-6-phosphate inhibition [41]. The proliferating cells undergo a shift from oxidative to glycolytic metabolism, where the energy requirements of the rapidly dividing cells are provided by ATP from glycolysis. This would direct mitochondrial ATP preferentially to glucose-6-phosphate synthesis and therefore would be expected to increase the biosynthetic pentose phosphate pathway. Hence, the glycolytic capacity of cancer cells depends totally on hexokinase activity for its metabolic fuel [38]. Fanciulli et al. [42] have demonstrated that increased hexokinase activity may not only be the consequence of altered metabolic requirements of cancer cells but may also be a modification per se to increase mitotic activity. Hence, alteration in hexokinase activity is a potential target for arresting tumour cell growth. Hennipman et al. [43] have also reported higher activity of hexokinase in breast cancer tissue. In this study, the observed increase in activity of hexokinase in tumour of animals with mammary carcinoma might have been due to the increased metabolic need of proliferating tumour cells for energy. Administration of drug to mammary carcinoma-bearing rats significantly reduced the enzyme activity and this may be a positive indication of an antitumour effect.

Phosphoglucoisomerase serves as a good index of tumour growth and is significantly elevated in cancerous animals. In agreement with our study, Campbell and King [44] have reported that phosphoglucoisomerase is an indicator of metastatic growth and is elevated in patients with neoplasms, especially after metastasis. Aldolase, another key enzyme in the glycolytic pathway, has been found to be elevated in tumour-bearing animals and in breast cancer [45]. The elevated activity of phosphoglucoisomerase and aldolase may be due to cell impairment and necrosis. The activities of gluconeogenic enzymes such as glucose-6-phosphatase and fructose-1,6-bisphosphatase are inhibited significantly in tumour of carcinoma-bearing animals. This may be due to the higher lactic acid production of neoplastic tissue, and it has been proved that tumour cells are able to utilize a large quantity of lactate for glycolysis in the form of glucose (via gluconeogenesis) and protein synthesis [46].

LDH is a tetrameric enzyme and is recognized as a potential tumour marker especially for solid tumours in assessing the progression of proliferating malignant cells [47]. Elevation of serum LDH activity is common in myocardial infarction, hepatitis and neoplastic disease [48]. The elevated activity of LDH may be due to overproduction by tumour cells, or it may be due to the release of isoenzymes from destroyed tissues [49]. Our findings are also consistent with the above reports. Lactate has been shown to progressively accumulate with increasing tumour burden [50]. The elevated LDH activity may be related to the increase in tissue lactate content with increase in tumour size. Treatment with the combination therapy caused a significant decrease in LDH activity. This clearly indicates the antitumour activity of the drug.

Drug-treated animals showed a significant drop in the activity of glycolytic enzymes and a concomitant elevation in the gluconeogenic enzymes in the mammary carcinoma cells, which indicates that the cells are converted from anaerobic to aerobic metabolism (i.e. tumour cells converted to normal cells). This modulation may be due to the antitumour activity of the drug either by inhibiting the glycolytic enzyme activities or by the suppression of tumour progression. The exact mechanism of carbohydrate metabolism altering the effect of TAM remains obscure. However, Deshpande et al. [51] have proved that TAM, by altering carbohydrate metabolism-related enzymes, retains malignant cells in the G_0/G_1 phase of the cell cycle. This process may inhibit tumour growth and hence lead to prolongation of disease-free survival.

In the present study, mammary carcinoma-bearing animals showed decreased activities of mitochondrial Krebs cycle and respiratory chain enzymes in both tumour and surrounding tissue of the mammary gland compared with normal control rats. Reductions in the activities of Krebs cycle and respiratory chain enzymes prove a defect in aerobic oxidation of pyruvate which might cause the low production of ATP molecules. Cancer cells have been reported to cause tissue hypoxia

where there is increased oxygen demand. In hypoxia, mitochondrial Krebs cycle and respiratory chain enzyme activities are expected to be low [52]. Decreased activity of these enzymes might be due to alteration in the morphology and ultrastructure of cancer cells and the ability of mitochondria to undergo metabolic changes compared with normal cells, and furthermore the number of mitochondria is drastically reduced in tumour cells [3]. The decrease in mitochondrial content might be due to the marked deficiency in one or more electron transport chain compounds. After treatment with TAM alone and TAM + EMV, these enzymes reverted to near-normal levels, and the combination therapy was more effective than TAM-only therapy. The major mechanism by which cancer cells revert back to aerobic metabolism and thus normal cells has three basic stages: enhancement of the Krebs cycle, enhancement of the respiratory chain enzyme activities and enhancement of oxygen transport to the cells. On treatment with the combination therapy, the activities of these enzyme were significantly increased, which clearly indicates the reversal of cancer cells to normal ones. One would expect the anaerobic metabolism to cause the breast cancer due to the lack of coenzyme Q_{10}, and when it is replenished, aerobic metabolism to restart and the cancer cells to turn back into normal cells [53].

Mitochondria are involved in a variety of processes of which oxidative phosphorylation is the most important. Detoxification of oxygen via its reduction to H_2O by the cytochrome oxidase system takes place in the mitochondria. Cytochrome *c* oxidase and NADH dehydrogenase are the enzymes involved in the electron transport chain and are located in the inner mitochondrial membrane. The process is ultimately linked to the production of useful energy-rich compounds such as ATP [54]. In this study, we observed a decrease in the activities of mitochondrial respiratory chain enzymes in mammary gland tumour of rats, and these activities were significantly enhanced upon treatment with the combination therapy. This effect was due to the presence of cofactors in EMV (FMN, FAD, NAD^+, NADH, coenzyme Q), which participate in oxidation–reduction reactions in numerous metabolic pathways and enhance the activity of Krebs cycle and respiratory chain enzymes [55, 56].

The surrounding normal tissue of the mammary glands showed lower activities of glycolytic, Krebs cycle and respiratory chain enzymes and higher activities of gluconeogenic enzymes. This may have been due to an inadequate supply of glucose to the normal surrounding tissues. Usually malignancies that commonly display a high rate of glycolysis exhibit a low rate of gluconeogenesis, Krebs cycle and respiratory chain enzymes. The metabolic complications of this situation are sufficient to provide a cachexic mechanism [57]; in the anaerobic breakdown of glucose to lactic acid in tumours, two ATP molecules per glucose molecule are yielded net to the tumour, but synthesis of glucose from the resulting lactic acid (or from any other precursor) via gluconeogenesis requires the utilization of the equivalent of at least six ATP molecules derived from normal host sources. In the Cori cycle per se, the equivalent of at least 14 ATP molecules are, therefore, lost from the body economy with each specific recycling (based on two equivalents of 'lactate' being recycled to glucose), 2 ATP molecules to the cancer cell and 12 ATP molecules from normal host tissues. As the tumour enlarges, consuming ever-increasing amounts of glucose, vast energy reserves from the host can be depleted in maintaining the circuit of glucose presentation to the malignant cell, reconversion of lactic acid and other precursors to glucose in host cells, and representation of the resulting glucose to the cancer cells. This systemic ''metabolic circuit'', which is characterized by a malignancy's utilization of glucose to produce relatively small amounts of energy for its own needs at the expense of relatively large amounts of energy from the host, gives every indication of being an operational biochemical mechanism for the production of cancer cachexia [57, 58].

The functional significance of glycolysis in cancer appears therefore to be twofold: as a source of energy production (growth) for the tumour and as a source of lactate that initiates a progressive energy loss (cachexia) in the host through marked stimulation of gluconeogenesis. Inhibition of gluconeogenesis would not only cause inhibition of energy loss leading to cachexia, but also, if tumour energy gain and host energy loss are functionally interrelated, as seems probable, provide a possible means of inhibiting tumour growth itself. The above-mentioned changes returned to their respective control limits in drug-treated animals. This may be assumed as the combination therapy prevented host body weight loss by enhancing ATP production via the electron transport chain.

In conclusion, the overall projected mechanism of action being an inhibition of host energy loss (cachexia) caused by a directly augmented pathway of gluconeogenesis in cancer, the drug-treated animals showed a significant increase in body weight compared to the untreated tumour-induced animals. This modulation can be attributed to supplementation with EMV, and mainly due to the cofactors of these vitamins (FMN, FAD, NAD^+, NADH, coenzyme Q, etc.) which participate in oxidation–reduction reactions in numerous metabolic pathways and enhance glycolysis, the Krebs cycle and ATP production via an electron transport chain in the host tissue. On the other hand, in tumour the anticarcinogenic and apoptotic effects of TAM inhibited cancer cell proliferation and led to apoptosis. No significant changes were observed in drug control animals, clearly indicating that the drug does not show any deleterious side effects. It may be assumed that the combination therapy not only suppressed tumour growth, but also enhanced ATP production in the host, thereby preventing cancer cachexia. Hence, this combination therapy could be of major therapeutic value.

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