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ORIGINAL ARTICLE

Creatine supplementation with methylglyoxal: a potent therapy for cancer in experimental models

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Abstract The anti-cancer effect of methylglyoxal (MG) is now well established in the literature. The main aim of this study was to investigate the effect of creatine as a supplement in combination with MG both in vitro and in vivo. In case of the in vitro studies, two different cell lines, namely MCF-7 (human breast cancer cell line) and C2C12 (mouse myoblast cell line) were chosen. MG in combination with creatine showed enhanced apoptosis as well as higher cytotoxicity in the breast cancer MCF-7 cell line, compared to MG alone. Pre-treatment of well-differentiated C2C12 myotubes with cancerogenic 3-methylcholanthrene (3MC) induced a dedifferentiation of these myotubes towards cancerous cells (that mimic the effect of 3MC observed in solid fibro-sarcoma animal models) and subsequent exposure of these induced cancer cells with MG proved to be cytotoxic. Thus, creatine plus ascorbic acid enhanced the anti-cancer effects of MG. In contrast, when normal C2C12 muscle cells or myotubes (mouse normal myoblast cell line) were treated with MG or MG plus creatine and ascorbic acid, no detrimental effects were seen. This indicated that cytotoxic effects of MG are specifically limited towards cancer cells and are further enhanced when MG is used in combination with creatine and ascorbic acid. For the in vivo studies, tumors were induced by injecting Sarcoma-180 cells (2×10^6 cells/mouse) in the left hind leg. After 7 days of tumor inoculation, treatments were started with MG (20 mg/kg body wt/day, via the intravenous route), with or without creatine (150 mg/kg body wt/day, fed orally) and ascorbic acid (50 mg/kg body wt/day, fed orally) and continued for 10 consecutive days. Significant regression of tumor size was observed when Sarcoma-180 tumor-bearing mice were treated with MG and even more so with the aforesaid combination. The creatine-supplemented group demonstrated better overall survival in comparison with tumor-bearing mice without creatine. In conclusion, it may be stated that the anti-cancer effect of MG is enhanced by concomitant creatine supplementation, both in chemically transformed (by 3MC) muscle cells in vitro as well as in sarcoma animal model in vivo. These data strongly suggest that creatine supplementation may gain importance as a safe and effective supplement in therapeutic intervention with the anti-cancer agent MG.

Keywords Methylglyoxal · Creatine · Cancer · C2C12 · MCF-7

Abbreviations

CK	Creatine kinase
MCK	Muscle-specific cytosolic CK
BCK	Brain-specific cytosolic CK
MtCK	Mitochondrial CK
3MC	3-Methylcholanthrene
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
EAC	Ehrlich ascites carcinoma
MTT	(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide)
DMSO	Dimethylsulfoxide

Introduction

Treatment of cancer is almost always paralleled with, or followed by, adjuvant therapies to improve disease-related

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symptoms and overall survival of the patient. Radiotherapy and systemic therapy, which include chemotherapy, immunotherapy or hormone therapy (Mostafa et al. 2006) are the existing adjuvant therapies after surgery. Although recent research has had a substantial impact on the efficacy of cancer therapy, yet chemotherapy remains taxed with a wide array of serious side effects. The need to develop anticancer agents with minimum toxicity for cells with normal physiology is an ever increasing goal of modern day research. The fundamental property of any promising anticancer drug from a therapeutic perspective lies in its ability to distinctively affect neoplastic cells only and to spare healthy cells and organs.

Methylglyoxal (MG), a ketoaldehyde, has been known for quite some time to exert such anti-cancer and growth regulatory properties exclusively in malignant cells without seriously affecting normal cells. MG is a normal metabolite with anti-cancer properties (Együd and Szent-Györgyi 1968). The tumoricidal activity of MG is due to its efficiency in depleting the cellular ATP pool by inhibition of ATP generating enzymes, especially of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mitochondrial complex I (Biswas et al. 1997). The selective inhibitory effects of MG may be due to specific structural differences in mitochondrial complex I and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) between normal and malignant cells (Ray et al. 1997b). It is well established from various laboratories that GAPDH from normal tissue sources has a MW of 145 kDa, with four equal subunits of 36 kDa each (Ferreira-da-Silva et al. 2006). This result has been corroborated by our previous work, showing that GAPDH is indeed a homo-tetrameric protein. By contrast, previous work from our laboratory has demonstrated that GAPDH from Ehrlich Ascites carcinoma (EAC) cells is a heterodimer having two non-identical subunits of ~33 and 55 kDa MW (Bagui et al. 1999). Similar results have also been observed in the case of mouse sarcoma tissue and human leukemic leukocytes (Patra et al. 2009).

Interestingly, we have identified for the first time that purified GAPDH (33 kDa) from EAC cells remains as a heterodimer (87 $\pm$ 3 kDa) by interacting with either 55 kDa glucose-6-phosphate isomerase (GPI) or 55 kDa pyruvate kinase M2 (PKM2) subunit, both of which are glycolytic enzymes.

GAPDH can interact with both of these enzymes in EAC cells, as well as in 3-methylcholanthrene (3MC) induced tumor tissue. Moreover, it has been shown that MG glycates the PKM2 subunit of the heterodimer specifically at the R399 residue, which is located in the M2 insert of PKM2. This indicates a different molecular association of GAPDH in cancer cells as compared to normal cells, underscoring specific glycation events by MG in cancer cells (Das et al. 2016).

Malignant cells and cardiac cells differ from other normal cells with respect to their high ATP demand. Previous studies from our group has shown that MG inhibits the respiration of mitochondria isolated from malignant, as well as from normal cardiac cells, but had no significant effect on mitochondrial respiration in a variety of other normal cells, or on cardiac tissue slices and on intact perfused heart (Biswas et al. 1997; Roy et al. 2003). MG acts at the level of complex I in cardiac mitochondria and that it inhibits electron flow specifically through complex I of the mitochondrial respiratory chain in EAC cells (Ray et al. 1997a). MG failed to affect the respiration of mitochondria (electron transport chain) isolated from various normal tissues, such as skeletal muscle, leucocytes, breast, stomach, colon, liver, gall bladder, rectum, and others (Biswas et al. 1997; Ghosh et al. 2011). It was further shown that post-mitochondrial supernatant (PMS) obtained during isolation of cardiac mitochondria was able to prevent the inhibition of respiration by MG in isolated cardiac mitochondria (Roy et al. 2003).

Creatine abrogates the inhibitory effects of MG on mitochondrial respiration

The active principle or endogenous factor in the above-mentioned post-mitochondrial supernatant (PMS) extract was identified as being creatine. Creatine seems to be the main constituent to curb the inhibitory effect of MG on mitochondrial respiration in cardiac cells but not in EAC cells (Roy et al. 2003). A concentration of 2 mM MG caused the same degree of inhibition of mitochondrial respiration (~80–85 %) with mitochondria isolated and purified from both malignant and normal cardiac cells. However, upon addition of 10 mM creatine simultaneously with MG, the inhibition was reduced by almost 90 % in mitochondria from normal cardiac cells, but in contrast, creatine totally failed to protect mitochondrial respiration of malignant cells from the inhibitory effect of MG (Roy et al. 2003) thus displaying the selectivity. However, we also tested whether MG is quenched in vitro by creatine chemically, but no quenching of MG was detected (Roy et al. 2003).

Expression of creatine kinase (CK) isoforms and their relation to cancer

Different CK isoforms are expressed in a tissue-specific manner, e.g., muscle-specific cytosolic (MCK) is expressed specifically in sarcomeric skeletal and cardiac muscles, whereas brain-specific cytosolic BCK is mainly expressed in brain, neuronal tissues photoreceptor cells and eye lens, spermatozoa, intestinal epithelia, etc. (Wallimann et al. 1992, 2007; Wyss and Kaddurah-Daouk 2000; Payne et al.

1991). Two mitochondrial (MtCK) isoforms are found: sMtCK (sarcomeric) is always co-expressed with MCK in striated skeletal and heart muscles while uMtCK (ubiquitous) is present in smooth muscle, brain, neuronal and other non-muscle tissues and is always co-expressed with BCK (Payne et al. 1991). AGAT (L-arginine:glycineamidino transferase) and GAMT (N-guanidinoacetate methyl transferase) are two enzymes that play an important role in creatine metabolism. Lower vertebrates such as fish, frogs, and birds express both these enzymes in their livers and often in kidneys (Van Pilsum et al. 1972). Mammals also show high levels of both these enzymes in pancreas, but kidneys express high amounts of AGAT and relatively low levels of GAMT. Mammalian liver displays high levels of GAMT but very low levels of Cr and CK. The status of AGAT activity is high in cow, pig, monkey, and human livers and is completely inactive in livers of rat, mouse, dog, cat, and rabbit (Wyss and Kaddurah-Daouk 2000). As the enzymes for creatine metabolism are diversely expressed among species, generalization of observations in animal models to humans is not viable. However, it was previously shown in Swiss albino mice that the levels of creatine and phosphocreatine, as well as the enzyme activities of MCK and sMtCK, decrease progressively during transformation of normal skeletal muscle tissue to sarcoma. This finding is in accordance with the status of these in post-operative human sarcoma samples where MCK and MtCK are significantly down-regulated (Patra et al. 2008). In contrast, in gastric and colonic adenocarcinoma, BCK levels are down-regulated while uMtCK is up-regulated (Patra et al. 2008). Elevated levels of BCK have been suggested as a marker for small cell lung carcinoma (Carney et al. 1984) and neuroblastoma (Ishiguro et al. 1990) and a high activity of the BCK isoform has also been reported in other malignancies (Gazdar et al. 1981; Zarghami et al. 1996; Meffert et al. 2005; Balasubramani et al. 2006). A recent study with colon cancer cells showed an up-regulation of BCK by suppression of miR-483 and miR-551a micro-RNAs (Loo et al. 2015). Metastatic cells colonizing the liver face energy deficiency as they compete for the substrates for oxidative and glycolytic metabolism with the neighboring hepatocytes in the interstitial space. BCK is up-regulated in these cancerous cells and according to a new hypothesis, the enzyme is secreted in the extracellular microenvironment where it catalyzes the reaction of creatine with ATP derived from the liver's extracellular space to generate phosphocreatine (PCr). It is then transported back into viable cancer cells to restore through BCK the intracellular ATP pool (Loo et al. 2015; Sullivan and Christofk 2015). According to this hypothesis BCK is released into the extracellular liver space by metastatic cells that are dying and PCr enters surviving cells via the creatine transporter (SLC6A8). However, it is unclear, whether the creatine transporter is able to transport charged PCr molecules and whether there is sufficient extracellular ATP in the extracellular space in the liver around metastatic cells to serve as a significant energy reserve to keep metastatic cells energetically charged. The experiments described in Loo et al. (2015) need to be confirmed and additional work is necessary to prove several steps of this hypothesis (Sullivan and Christofk 2015).

On the other hand, creatine to choline ratio is considered as a marker for some brain tumors (Kinoshita and Yokota 1997; Horska et al. 2001; Lehnhardt et al. 2005). Since this ratio is usually lower in malignancies it would suggest generally lower levels of creatine in malignant cells. The levels of different CK isoforms, as well as their substrates, creatine and phosphocreatine, and their role in malignancy are somewhat ambiguous and more detailed investigations are required. It has been observed that in EAC (Ehrlich Ascites Carcinoma) cells inoculated mice treated with MG, creatine supplementation augmented the anti-cancer activity of MG. A similar augmentation was observed in sarcoma-carrying mice induced by a sarcoma-180 cell line. During the course of the experiments, the creatine content, activity of CK and the expressions of MCK and sMtCK in sarcoma-affected skeletal muscles were restored to almost normal levels when creatine was supplemented during MG treatment (Patra et al. 2012).

Creatine and analogues as anti-cancer therapeutic intervention

The role of the creatine/creatine kinase system, together with the pleiotropic effects of creatine (Wallimann et al. 2011) is gaining importance in explaining the multiple effects of creatine in cancer biology. For example, the growth rate of tumors implanted subcutaneously in athymic nude mice and rats is significantly inhibited when the experimental animals were fed with creatine or cyclo-creatine (cCr) (Miller et al. 1993). In the case of human colon adenocarcinoma xenografts in nude mice, this anti-cancer effect has been attributed to inhibition of glycolysis and induction of acidosis (Kristensen et al. 1999). It was also reported that cyclo-creatine inhibits proliferation of Hodgkin disease-derived cell lines in an apoptosis-independent pathway and it was postulated that this creatine analogue, would therefore be advantageous in cancer therapy (Kornacker et al. 2001). There are reports in the literature that cyclo-creatine exhibits anti-cancer activity in humans and is also well tolerated by cancer patients. In addition, normal cell lines that express high CK levels were not negatively affected by cyclo-creatine (Wyss and Kaddurah-Daouk 2000).

The objectives of this study were to investigate (1) the effect of creatine and MG on different cancer cell lines in

terms of cell survival, apoptosis in vitro, and (2) to assess the tumor growth inhibition of MG in combination with creatine and ascorbic acid in Sarcoma-180-induced mice.

Materials

MG (40 % aqueous solution, w/v), creatine, 3-methylcholanthrene (3MC), MTT (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide), cell culture media, horse serum and fetal bovine serum were obtained from Sigma Chemical Co., St. Louis, Mo, USA. C2C12 mouse myogenic cells line and MCF-7 human breast cancer cell lines were obtained from American Type Culture Collection [(ATCC) Manassas, VA, USA). An Annexin V–Propidium Iodide (PI) kit was purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Other chemicals were of analytical grade and obtained from local manufacturers.

Methods

In vitro study

Cell culture

Human breast adenocarcinoma cell line (MCF-7) or normal mouse myoblast cell line (C2C12) were maintained on Dulbecco's modified Eagle's medium (DMEM), supplemented with 10 % fetal bovine serum, penicillin (100 μg/mL), streptomycin (100 μg/mL), and gentamycin (100 μg/mL) and grown in a 37 °C humidified incubator flushed by an air mix containing 5 % CO_2.

Myotube formation from C2C12 cells

C2C12 cells (mouse myoblast cell line) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10 % fetal bovine serum. Myotubes were prepared after 5 days by inducing fusion between individual C2C12 myoblast cells at 70–80 % confluent state in differentiation medium (DMEM supplemented with 2 % horse serum). Differentiated myotubes (muscle cell differentiation phase) are regarded as normal cells. These cells were treated for 48 h with 2 mM creatine as supplement with 0.5 mM MG and 1 mM ascorbic acid in different combinations.

Dedifferentiation of C2C12 myotubes by 3-methylcholanthrene (3MC)

For induction of tumorigenesis phase, differentiated myotubes were transformed with 50 nM 3MC (3-methylcholanthrene) in DMSO (dimethylsulfoxide) or with DMSO only (vehicle alone), in a fresh differentiation medium containing 2 % horse serum for another 3 days (Saha et al. 2011). In a similar fashion, dedifferentiated transformed myotubes (3MC induced) were treated for 48 h with 2 mM creatine as supplement with 0.5 mM MG and 1 mM ascorbic acid in different combinations. Phase contrast microscopy images were taken after 48 h using an Olympus IX-51 microscope.

Cell viability analysis by MTT assay on MCF-7 cells

The MTT assay (Mosmann 1983) was performed to analyze the cell viability of MCF-7 cells (breast cancer cell line) with MG supplemented with creatine. Cells in their log phase were seeded overnight in colorless DMEM in 96-well tissue culture plates such that each well contained ~10,000 cells. The cells were then incubated with increasing concentrations of MG (0.25, 0.5, 0.75, 1.0, 1.25, 1.5, and 2.0 mM) and different combinations of creatine (1, 2 and 3 mM) for 24 h at 37 °C. The medium was then replaced with 100 μL MTT (0.5 mg/mL) per well and further incubated for 4 h at 37 °C. Then the insoluble formazan was dissolved in DMSO (dimethyl sulfoxide) (100 μL/well) and absorbance was measured using a 96-well plate reader (ELx 800; Biotek, Winooski, VT, USA) at 490 nm. The percentage of cell viability was calculated compared with the untreated control (considered as 100 % viable).

Apoptosis assay by flow cytometry on MCF-7 cells

MCF-7 cells were seeded overnight in six-well tissue culture plates at a density of 5×10^5 cells/well and were treated with different concentrations of MG (0.5 and 1.0 mm) with or without creatine (1.0) for 48 h. Another set of experiment was maintained in a similar way with creatine alone (1 and 2 mM) for 48 h. Cells were trypsinized, washed and suspended in binding buffer. Finally cells were doubly stained using an annexin V–fluorescein isothiocyanate (FITC) and PI kit (Cell Signaling Technology kit no 6592S) according to the manufacturer's protocol for flow cytometric analysis of apoptotic populations. Briefly, 2 μL of annexin V–FITC and 8 μL of PI were added to the binding buffer in which the cells were suspended. Apoptosis data were acquired using a BD FACS Verse flow cytometer (BD Biosciences, San Jose, CA, USA) within 1 h and analyzed using BD FACS DIVA software.

Animal model

Female Swiss albino mice aged 4–6 weeks and weighing ~20 g were maintained under standard laboratory conditions. The animal handling and animal experimental protocols were approved by the Institutional Animal Ethics Committee of Bose Institute, Kolkata, India.

In vivo development of sarcoma tissue

Each mouse was inoculated with sarcoma-180 cells (2×10^6 cells) in one hind leg (day 0). Treatments started on day 7 and were continued up to day 16 (total 10 doses).

Animal treatment

For Sarcoma-180 inoculated groups, one group of mice remained untreated (control) and other six groups of mice were treated with MG, Cr, AA, MG + AA, MG + Cr, MG + AA + Cr, respectively. Administration of MG was intravenous and aqueous solutions of ascorbic acid and creatine were fed orally by gauge needle. Each group consisted of six mice; each mouse received one single dose/day of the compounds as indicated in the legend of Fig. 5. Each set of experiments were repeated four times.

The tumor volumes of treated and untreated mice were measured with slide calipers.

Statistical analysis

Data are presented as mean ± standard deviation (SD) unless mentioned otherwise. Graphs are plotted using software GRAPHPAD Prism 5. ANOVA (one way) was performed to analyze the data followed by *Dunnett* post hoc test (to compare between control versus all the treated groups) and/or *Tukey* (to compare between all the groups with each other) post hoc test (unless mentioned otherwise). Statistical significant difference was set at $P < 0.05$. Survival rate was determined by a Kaplan–Meier analysis using the software GRAPHPAD 5.0.

Results

Effects of MG supplemented with Cr on myotubes

Differentiated (untransformed) and dedifferentiated (transformed) myotubes (formed by fusion of C2C12 cells) were treated with MG (0.5 mM) or MG (0.5 mM) + AA (1 mM), or MG (0.5 mM) + AA (1 mM) +Cr (2 mM) for 48 h (Fig. 1). The changes in morphology were observed under a phase contrast light microscope at 200× and 400× magnification. Control cells (untransformed myotubes) appeared as normal fibrillar shape with firm attachment to the culture dishes, which is a normal cell growth phenomenon with this myogenic cell line after fusion (Fig. 1A). Transformed cells had a rounded-up appearance, but still adhered to the matrix (data not shown) after 24 h of treatment for all cases. Treatment for 48 h with combinations of MG, MG + AA, MG + AA + Cr resulted in more loss of cell attachment to the matrix, many cells were rounded up, and many floating cells in the culture plate were observed, indicative of cell death (Fig. 1B). More floating cells were observed in case of MG + AA + Cr treated cells than in MG + AA and in MG alone compared to untreated one. The number of dead cells could not be quantified; however, the observation is presented in a qualitative manner depending on cell morphology.

Effect of MG supplemented with Cr on cell viability

To evaluate the effect of creatine, varying doses of MG and Cr were tested on MCF-7 cells by MTT assay. Methylglyoxal affected cell viability of MCF-7 cells in a dose-dependent manner (Fig. 2A). MG at a concentration of 1.25 mM was able to reduce cell viability to almost 60 % (Fig. 2E). The viability was further reduced to almost 80 % with the addition of 2 mM creatine (Fig. 2E). Creatine alone has no significant effect on cell viability at 2 mM. However, creatine at a concentration of 3 mM could cause a slight but significant decrease in cell number (Fig. 2B). These results clearly demonstrate that MG, if combined with creatine, significantly reduced cancer cell viability compared to MG alone.

Creatine synergistically enhanced apoptosis of MG

MG-induced apoptosis was detected by phosphatidyl serine externalization. Treatment with 0.5 and 1.0 mM MG (Fig. 3B, D) showed that 17.9 and 44.8 % early apoptotic (annexin V–FITC$^+$/PI$^-$) cells and 2.87 and 23.4 % of cells were late apoptotic (annexin V–FITC$^+$/PI$^+$), respectively. Furthermore, apoptosis was increased after combination treatment with MG (0.5 mM) plus creatine (1 mM) resulting in 49.6 % of early apoptotic cells (annexin V–FITC$^+$/PI) and 25.4 % of late apoptotic cells (annexin V–FITC$^+$/PI$^+$) compared to 17.9 % early apoptotic and 2.87 % late apoptotic cells with MG (0.5 mM) alone (Fig. 3C). For MCF-7 cells, there was nearly a threefold increment in the early apoptotic cell population when 0.5 mM MG was supplemented with 1 mM creatine compared to 0.5 mM MG only. Creatine alone at a concentration of either 1 or 2 mM had no significant effect on apoptosis of MCF-7 cells (Fig. 4B, C). These results indicate the synergistic effect of Cr with MG.

Effect of Cr on Sarcoma-180 inoculated mice in combination with MG

To investigate the anti-cancer effect of MG in combination with creatine and ascorbic acid in vivo, seven groups of mice were inoculated with sarcoma-180 cells in the left hind leg and treated in various combinations. Group 1-untreated, i.e., control, group 2, 3, 4, 5, 6, and 7

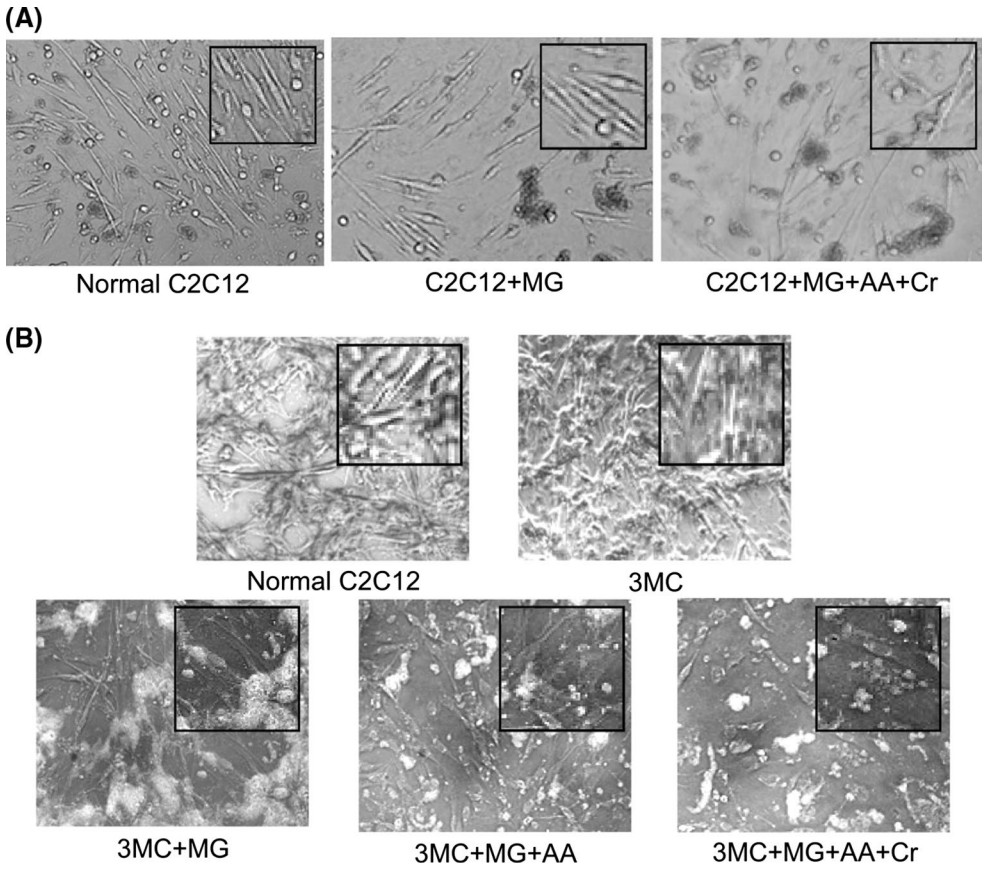


Fig. 1 **A** In vitro viability studies of normal C2C12 cell line after differentiation to myotubes, untreated (*left*), treated with 0.5 mM MG alone (*middle*) and treatment with 0.5 mM MG plus 1 mM ascorbic acid and 2 mM creatine (*right*). **B** Cytotoxic anti-cancer effects of MG and MG in combination with ascorbic acid and creatine at different concentrations on C2C12 cell line previously transformed with 50 nM 3MC. *Upper panel*, untreated normal C2C12 cells (*left*) and after transformation with 50 nM 3MC (*right*). *Lower panel*, such 3MC-transformed cells treated with 0.5 mM MG alone (*left*), 0.5 mM MG plus 1 mM ascorbic acid (*middle*) and 0.5 mM MG plus 1 mM ascorbic acid and 2 mM creatine (*right*). Morphological changes were viewed by phase contrast microscopy at 200× magnification. *Inset* magnified images of cells (400×)

are treated with MG, AA, Cr, MG + AA, MG + Cr and MG + AA + Cr, respectively. There was a regression in volume of tumor induced by sarcoma-180 when treated with MG and a further reduction was observed when MG was administered in combination with ascorbic acid and creatine ($P < 0.05$) (Fig. 5A).

Creatine at a concentration of 150 mg/kg body wt/day has no significant effect on the reduction of tumor volume (Fig. 5A). MG alone at a concentration of 20 mg/kg body wt/day could reduce the tumor volume by almost 30–35 % and in combination with AA (50 mg/kg body wt/day), the reduction was almost 50–55 %. Volume of the tumor was reduced significantly by almost 80–85 % when the tumor-bearing mice were treated with MG + AA + Cr compared to untreated control or treated with creatine alone (Fig. 5A).

It was also evident that the combination treatment of MG and creatine increased the survival of mice (Fig. 5B, C). Mean survival time of untreated control (sarcoma bearing mice) group was determined to be 46 days and MG treated 55 days. When MG was supplemented with AA alone the mean survival time increased to 77 days. MG in combination with AA and creatine could increase the mean survival time to 98 days. Compared to control, treatment with MG + AA + Cr or MG + AA showed significant increase in survival time ($P < 0.0001$). When MG + AA + Cr treated group was compared with MG + AA treated group, the P value was determined to be <0.001 (by *Tukey* post hoc test).

Neither creatine (150 mg/kg body wt/day) nor ascorbic acid (50 mg/kg body wt/day) alone has any significant effect on cancer cells (Du et al. 2012; Ray and Ray 1998).

Discussion

It is evident from the results presented here that creatine augments the anti-cancer effect of MG. This is supported by experiments performed both in vitro and in vivo. In our

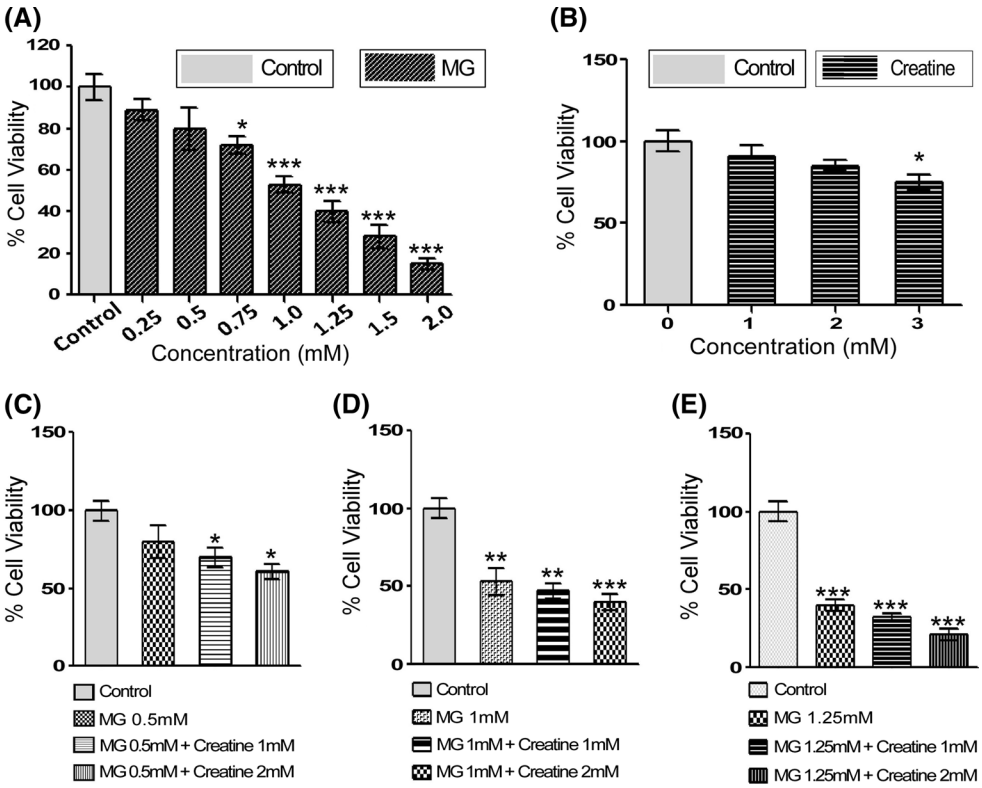
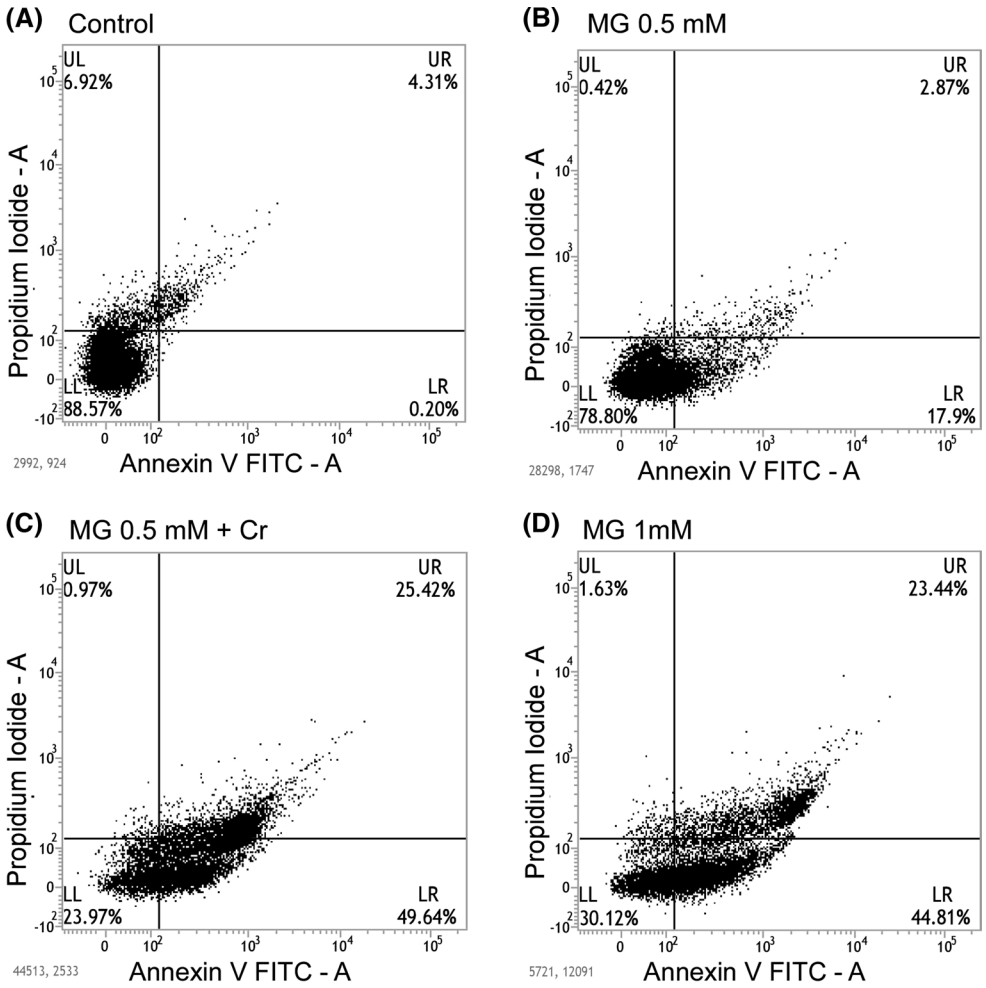


Fig. 2 Determination of cytotoxicity (by MTT assay) on MCF-7 cells at 24 h, **A** with varying doses of MG; **B** varying dose of creatine, **C–E** in different combination of MG and creatine as indicated in the figure. MCF-7 cells were seeded at a density of 10^4 cells per well and treated with various concentrations of MG and Creatine. Concentrations of all the mentioned compounds are indicated in the X axis of the figure. Each datum is the mean ± standard deviation of three experiments ($n = 3$). P value of <0.05 was considered to be significant (by one-way ANOVA followed by Tukey (to compare between all the groups with each other) and Dunnett (to compare between control versus all the treated groups) post-test). $^*P < 0.05$, $^{**}P < 0.001$, and $^{***}P < 0.0001$ compared to the control group (i.e., without any treatment)

previous work with skeletal muscle sarcoma, it was established that a decrease both in creatine content and in CK activity of the affected muscles occurred as malignancy progressed. This was always accompanied by a progressive loss of muscle phenotype, accompanied by specific CK-based changes in muscle energy metabolism with dedifferentiation of the muscle tissue towards sarcoma (Patra et al. 2008). It has also been demonstrated that with the regression of malignancy, the creatine content, CK activity, as well as expression of the MCK and sMtCK isoforms, are restored to almost normal levels by MG treatment and dedifferentiated sarcoma tissue in the affected muscles is gradually replaced by healthy, fully differentiated muscle tissue. Ascorbic acid is cytotoxic toward cancer cells and is believed to act as a carrier for MG (Szent-Györgi 1978). It facilitates the formation of protein adducts (Tuma et al. 1984), acts synergistically with MG (Ray et al. 1991) and augments the anti-cancer activity of MG (Ghosh et al. 2006).There are reports in literature that ascorbic acid at a low concentration has no effect on cancer cells (Ray and Ray 1998), but it shows significant cytotoxicity towards cancer cells at very high concentration (Chen et al. 2005; Du et al. 2012). Though ascorbic acid is synthesized in different rodent groups, yet the plasma level remains very low to attain its anti-cancer activity (Du et al. 2012). Therefore, in the experimental in vivo studies, a non-cytotoxic dose of ascorbic acid (50 mg/kg body wt/day) was used in combination with MG. As creatine and its analogues have been indicated to show anti-cancer activity (Kornacker et al. 2001), the role of creatine supplementation as a therapeutic intervention in MG-based anti-cancer treatment was investigated in this present study. Both in vitro and in vivo results indicate that the combination of MG with creatine and ascorbic acid significantly enhanced the anti-cancer activity of MG.

Creatine and cyclo-creatine display anti-cancer effects and are well tolerated by patients (Wyss and Kaddurah-Daouk 2000). These compounds may inhibit glycolysis and, parallel with this, cancer growth (Miller et al. 1993). MG itself has been shown to affect cancer cell growth by inhibiting glycolysis (Halder et al. 1993). Though clinical evidence for anti-cancer effect of methylglyoxal is under study, in this recent work we have tried to correlate the actions of creatine and MG in a combined anti-cancer therapeutic approach. Previously the effect of creatine and methylglyoxal in in vivo model bearing carcinoma (Ehrlich Ascites Carcinoma) had been demonstrated (Ghosh et al. 2006). In this paper, the efficacy of this combination was studied in human breast carcinoma cell line MCF-7 which was shown to undergo apoptosis. The underlying mechanism for such phenomenon has to be elucidated further. As there is rarely any drug for treating sarcoma, the effect of creatine-supplemented methylglyoxal was studied on in vivo sarcoma model and also in transformed C2C12 (muscle myoblast) cell line. Whether these transformed C2C12 cell line undergo apoptotic death needs to be studied further.



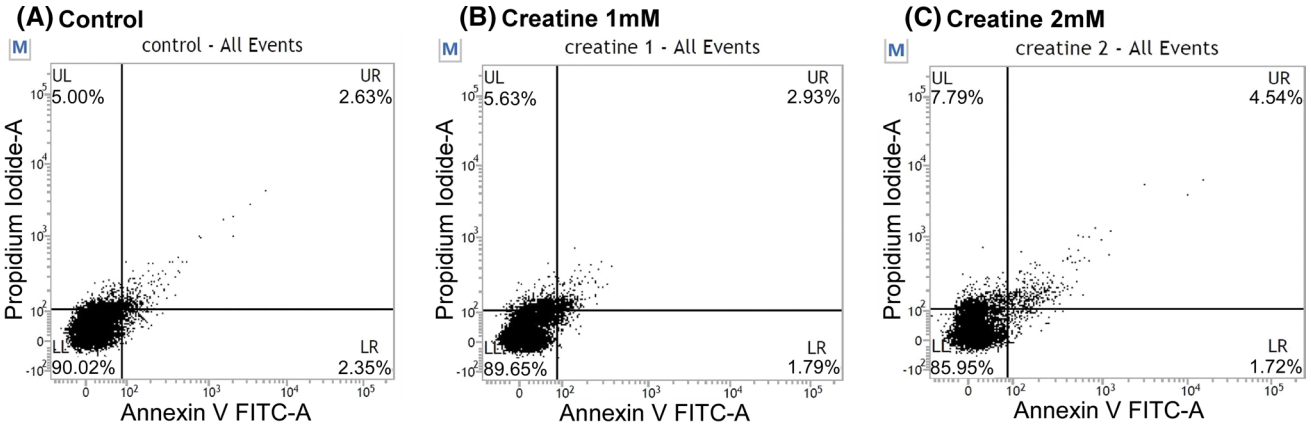


Fig. 3 Analysis of apoptosis in MCF-7 cells by Flow Cytometry. Effect of MG supplemented with creatine. **A** Control, **B** MG (0.5 mM), **C** MG (0.5 mM) + creatine (2 mM), **D** MG (1 mM)

Fig. 4 Effect of creatine: **A** control, **B** creatine (1 mM), **C** creatine (2 mM). In order to quantify the apoptotic rate, different subpopulations were distinguishable: UL, Annexin V-negative but PI-positive, i.e., necrotic cells; UR, Annexin V/PI-double positive, i.e., late apoptotic cells; LL, Annexin V/PI-double negative, i.e., live cells; LR, Annexin V-positive but PI-negative, i.e., early apoptotic cells. The percentage of each cell population is mentioned in the corresponding figures. These are typical data from one experiment; the experiment has been performed four times and similar results were obtained

The cytotoxicity of MG was increased with a higher degree of apoptosis in the case of MCF-7 cells (Figs. 2, 3). Similar cytotoxicity was found for 3MC-induced dedifferentiated C2C12 cells, but not in case of normal cells. These results are in agreement with our previous finding that creatine acts as a supplement in MG treatment (Patra et al. 2012).

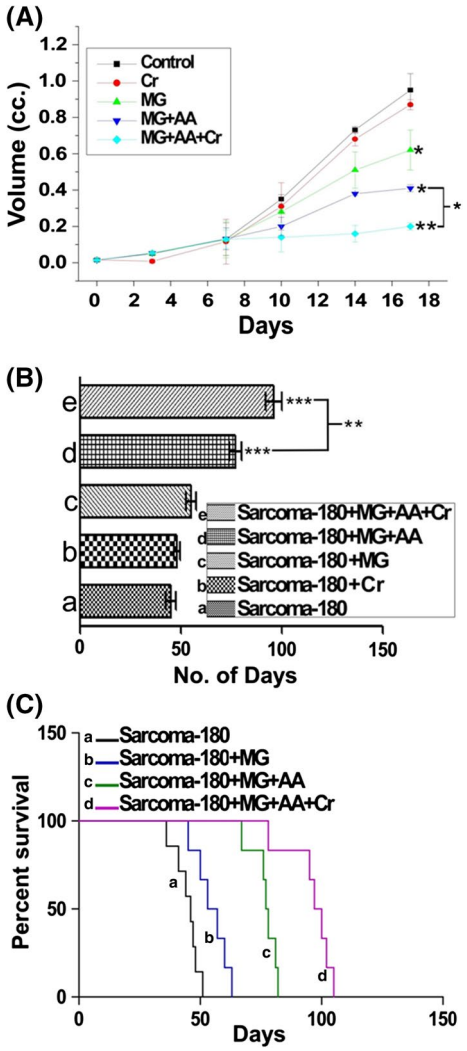


Fig. 5 Regression of tumor volume and survival analysis in sarcoma-180-inoculated mice treated with various combinations of MG, AA and Cr. **A** Reduction in tumor volume at different combinations of MG, AA and Cr. 2×10^6 cells were inoculated in left hind leg of each mouse ($n = 6$ in each group/experiment) to develop solid tumor. Treatment was started after 7 days of inoculation and was continued up to day 16. Day of inoculation was considered as day 0. These experiments were repeated for four times. Each point represents the mean ± standard deviation of four experiments. *P* value of <0.05 was considered to be significant in all cases (by one-way ANOVA followed by Tukey (Tukey's multiple comparison) post hoc test). $*P < 0.05$ and $**P < 0.001$ compared to the control group (i.e., without any treatment). Statistical significance between MG + AA and MG + AA + Cr is denoted by $*(P < 0.05)$. *Doses* mg/kg body weight/day. MG = 20 (treated intravenously) AA = 50 (fed orally). Cr = 150 (fed orally). *MG* methylglyoxal, *AA* ascorbic acid, *Cr* creatine. **B** Survival plot of different groups of mice ($n = 6$/group). Values are represented as mean ± SEM. ($P < 0.05$ was considered significant by one-way ANOVA followed by Tukey post-test). $***P < 0.0001$ compared to control (i.e., without any treatment) group (a). The statistical significance between the groups (d) and (e) is $** P < 0.001$. **C** Kaplan–Meier survival curve for untreated (*a*), MG treated (*b*), MG and AA treated (*c*) and MG, AA treated and supplemented with Cr (*d*) tumor-bearing mice. $n = 6$/group. Values are mean ± SEM ($p < 0.001$)

There are several reports in literature that have described the cardiotoxicity of specific classes of cancer therapeutic agents and cardiovascular toxic effects as important complications during cancer chemotherapy (Albini et al. 2010). Anthracyclines like doxorubicin interfere strongly with cardiac energetics (Tokarska-Schlattner et al. 2006), and can lead to congestive heart failure (CHF). As major chemotherapeutic agents used in the treatment of cancer are associated with strong adverse effects on the cardiovascular system, so a further goal of cancer therapeutics is the identification of new compounds or cardiomyocyte-protective agents that can prevent cardiotoxicity. It is evident from our previous work, creatine successfully abrogates the inhibitory effect of MG on normal cardiac mitochondria, but not of malignant cells. Creatine supplementation has recently been shown to reduce doxorubicin-induced cardiomyocellular injury (Santacruz et al. 2015). Furthermore, recent studies have demonstrated a protective effect of creatine due to its direct oxygen-radical scavenging capacity (Lawler et al. 2002) and indirect antioxidant effects (Sestili et al. 2011). The role of creatine as a cardioprotective agent needs to be elucidated further.

In conclusion, creatine supplementation to tumor-bearing mice, in combination with MG, resulted in a marked reduction of tumor growth and a significantly better animal survival rate (Fig. 5). It appears from the studies that anticancer effect of methylglyoxal is augmented in presence of ascorbic acid and further improved when creatine is added in combination. The role of MG and potential additive effect by creatine supplementation needs to be evaluated further.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest in this work.

Ethical approval All procedures performed in studies involving animals were in accordance with the ethical standards of the Institutional Animal Ethics Committee of Bose Institute, Kolkata, India.

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