Global Journal of Medical research
Volume 12 Issue 4 Version 1.0 May 2012
Type: Double Blind Peer Reviewed International Research Journal
Publisher: Global Journals Inc. (USA)
Online ISSN: 2249-4618 & Print ISSN : 0975-5888

Protective Effects of Diallyl Disulfide Against Experimentally Induced Hepatoma in Mice

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Abstract - Many herbal extracts have been reported to modify significantly, the transformation of normal cells into neoplastic cells. Garlic and its extracts are known for their hypolipidemic, hypoglycemic, antiplatelet aggregating effect as well as for its anticancer effects. Many of these health beneficial effects of garlic are attributed to its principle organosulfur compound diallyl disulfide(DADS). It was thought that DADS may be involved in anticarcinogenic & antitumorogenic effect of garlic, hence the present work was undertaken to assess the protective effects of DADS in ehrlich ascites carcinoma (EAC) cells induced hepatoma in mice. The study has three groupsnormal group (group1), the EAC cells implanted mice (group 2) & DADS-treated EAC cells implanted mice (group 3). The results indicate a significant decrease in ascitic fluid volume, ascitic fluid cell count, liver tissue amino acid nitrogen levels, liver tissue glutaminase activity & liver tissue lactate levels as well as a increase in life span observed in group 3 mice as compared to group 2 mice, suggesting that DADS gives a significant protection in group3 mice probably by decreasing the anaerobic glucose utilization as well as by interfering with protein & deoxy ribonucleotide synthesis.

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GJMR-D Classification : *NLMC Code: QY 60.R6, QY 140*

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I. Introduction

The transformation of normal cells into neoplastic cells involves at least three distinctive phases, namely- initiation, promotion and progression. Many dietary components have been reported to significantly modify each of these phases (30). Garlic (Allium sativum) a common dietary component, is known to modify the cancer process. Epidemiologic and clinical studies have shown that consumption of garlic reduced the risks of cancer incidence (5, 17, 29). A number of studies have demonstrated the chemoprotective activity of garlic by using different garlic preparations including fresh garlic extract, aged garlic, garlic oil and a couple of organosulfur compounds derived from garlic (1,18). The chemoprotective activity has been attributed to the presence of organosulphur compounds in garlic (6, 25, 31). The principle organosulphur compound present in garlic is diallyl disulphide [DADS] (3, 22). Hence it was thought DADS may be responsible for garlic's cancer protective activity. The present work was undertaken to assess the chemoprotective effects of DADS in Ehrlich ascites cells induced hepatoma in mice.

II. Materials and Methods

a) Tumor cell line & their Maintenance :

The inoculum of EAC cells was kindly provided by Amala Cancer Research Institute, Thrissur Kerala (India). EAC cells were thereafter propagated by weekly intraperitoneal injection of freshly drawn ascitic fluid (0.5 ml) from a donor mice bearing ascites tumor of 8-10 days old into healthy swiss albino male mice. Transplantation was carried out using sterile disposable syringes under aseptic conditions

b) Chemicals :

All the chemicals employed in the present study were of Analar grade (A.R). Dially disulphide (DADS) was procured from Sigma-Aldrich chemicals Pvt. Ltd. USA.

c) Animals :

In the present study, 18 Swiss male albino mice weighing 25-30g were randomly selected from animal house of Basaveshwara Medical College & Hospital, Chitradurga. The experiments were conducted according to the norms of CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals), New Delhi and Ethical clearance was obtained from IAEC (Institutional Animal Ethical Committee) of Basaveshwara Medical College.

d) Experimental design :

The mice were divided into 3 groups (6 animals per group)–normal group (group 1), control group [EAC cells implanted mice] (group 2) and protective group [DADS-treated EAC cells implanted mice] (group 3).

i. *Normal group*

This group consists of 6 swiss albino male mice that received 5.0 ml of normal saline /kg body weight orally by gastric intubation daily for a period of 10 days.

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ii. *Control group*

This group consists of 6 swiss albino male mice with experimentally induced hepatoma. About 3x 10^6 EAC cells were injected intraperitoneally into healthy mice. These mice also received 5.0 ml of normal saline / kg body weight orally by gastric intubation daily for a period of 10 days. A well grown tumor was observed within 7-10 days.

iii. *Protective group*

This group consists of 6 swiss albino male mice, received 5.0 ml of warm aqueous solution of DADS (100mg/kg body weight) orally by gastric intubation daily for a period of 4 days. On the 4th day 3x 10^6 EAC cells were injected intraperitonially. Later 5.0 ml warm aqueous solution of DADS (100 mg)/kg body weight was given orally further for a period of 6 days.

The mice of all the three groups were maintained on standard lab feed (Amruth Rat Feed, supplied by Pranav Agro Industries, Pune, India) and tap water adlibitum throughout the study. On the 11th day, body weights of mice of all the groups were noted & abdominal circumferences were recorded. Then the mice were anaesthetized & sacrificed. The ascitic fluid was immediately collected in a clean dry graduated tube by puncturing the abdomen. The fluid volume was noted. The ascitic fluid was assayed for total proteins (11) & total cell count was assessed microscopically using Neubauer chamber. The mice were dissected & livers were procured. Blood stains of liver tissues were removed by smooth blotting & were immediately transferred into a clean pre weighed beaker. The weights of liver of individual groups were noted. Later, the liver tissues were refrigerated at 0-2°C in cold phosphate buffer pH 7.4 till further use. Each individual liver tissue procured was processed to analyze various biochemical parameters as follows:

a) To 0.2 g of liver tissue slice, 4.8 ml 10% TCA was added & allowed to stand at room temperature for 10 minutes & homogenized thoroughly in a Potter Elvehjam tissue homogenizer for 5 minutes & centrifuged at 3000 rpm for 5 minutes. The supernatant was employed for the estimation of lactate (15).

b) 0.5 g of liver tissue slice was employed for the estimation of glycogen content as explained in David Plummer – Practical manual (8).

c) To 0.3 g liver tissue, 4.7 ml of cold phosphate buffer, pH 7.4, was added & thoroughly homogenized for 5 minutes & centrifuged at 3000 rpm for 5 minutes. The supernatant was employed for the estimation of tissue total proteins (11), transaminase activity- alanine transaminase (ALT) & aspartate transaminase (AST) (13), total thiol (-SH) groups (7) & glutaminase activity (21).

d) To 0.2 g of liver tissue slice, 1.0 ml of 2/3 N H2SO4 + 1.0 ml of 10% sodium tungstate + 7.8 ml of distilled water were added, mixed well & allowed to stand for 10 minutes at room temperature. Later the contents were thoroughly homogenized & centrifuged at 3000 rpm for 5 minutes. The clear supernatant was employed for the estimation of amino acid nitrogen (AAN) (12).

e) *Data management & Statistical Evaluation :*

The data entry was carried out using MS Office Exel worksheet and statistically evaluated. The P value was calculated using 'student t' test.

III. Results

The results of the present study are given in table 1 & 2.

Table 1 narrates the bodyweight, abdominal circumference of group 1, group 2 & group 3. Also, the table gives the ascitic fluid volume, ascitic fluid total proteins, ascitic fluid cell count in group 2 & group 3. It is evident from the table that both bodyweight & abdominal circumference are significantly raised ($p< 0.001$) in group 2 as compared to group 1, whereas the same parameters are significantly lowered ($p<0.001$) in group 3 as compared to group 2. It is also evident from the table that a significant decrease ($p<0.001$) in ascitic fluid volume, ascitic fluid protein content & cell count in group 3 as compared to group 2.

Table 2 gives the liver tissue levels of glycogen, lactate, total proteins, aminoacid nitrogen, total thiol groups & activity of glutaminase, AST & ALT in group1, group 2 & group 3. It is evident from table that the levels of lactate, total proteins, amino acid nitrogen & glutaminase activity in liver tissue are significantly raised ($p<0.001$) in group 2 as compared to group 1, whereas the same parameters are significantly lowered ($p<0.001$) in group 3 as compared to group 2. It is also seen from the table that a significant decrease ($p<0.001$) in liver glycogen content & total thiol groups observed in group 2 as compared to group 1 & the same parameters are significantly raised ($p<0.001$) in group 3 as compared to group 2. However there is no significant change seen in transaminases (ALT & AST) activity in all the three groups.

IV. Discussion

The eukaryotic cell cycle normally consists of series of events involving – growth stimulus, replication & division (3, 23, 24). It is known that many allyl sulphur compounds of herbal origin reduce the growth rate of neoplastic cells in culture as well as invivo (26), probably by blocking certain events of cell cycle. The results of the present study with 100 mg DADS/ kg bodyweight given to EAC implanted mice (refer table-1) suggests that at this dosage DADS significantly retards the development of ascites. DADS might have interfered with the cell cycle at G2/M phase as it is known that DADS arrest the cell growth at G2/M phase of cell cycle

in human colon cancer (18, 4, 19), by decreasing the kinase activity of CDK1/cyclin B complex. Further DADS is a disulphide and like any other disulphides can undergo sulphydryl exchange rections with cellular proteins & enzymes (33) as follows-

$$\text{Enz-SH} + \text{RSSR} \longleftrightarrow \text{Enz-SSR} + \text{RSH}$$

$$\text{Enz (SH)}_2 + \text{RSSR} \longleftrightarrow \text{Enz S}_2 + 2\text{RSH}$$

$$2\text{RSH} + \text{DADS} \longleftrightarrow \text{RS-SR} + \text{Allyl thiol}$$

A similar sulphydryl exchange reaction with kinases & other growth factors involved in cell cycle may suppress cell multiplication causing a reduction in tumor growth, indicating DADS has a significant chemoprotective action against EAC induced hepatoma in mice, which is evident from the results obtained in the present studies (refer table 1 & 2). Cell proliferation as well as cell multiplication requires increased DNA produ--ction (14) which means increased synthesis of deoxy-ribonucleotides. This process requires the participation of nucleotide reductase enzyme, which requires thioredoxin, a sulphydryl compound for its activity. A possible sulphydryl exchange reaction of DADS with thioredoxin as proposed above may reduce its availability hence decreases the production of deoxy ribonucleotides thus reducing the available DNA levels in cancer cell development which is evident from results depicted in table 1.

Tumor cells do act as nitrogen trappers (22) which is a required phenomenon for increased protein synthesis essential for rapid cell proliferation as well as cell multiplication. Liver tissue protein levels in group 2 shows a significant raise ($P<0.001$) as compared to group 1 (refer table 2), indicating a rise in protein synthesis, a normal requirement of increased cell multiplication. The amino acids which are essential for increased protein synthesis might have derived from an increased proteolysis of host tissue. The results given in table 2 shows a significant raise ($p<0.001$) in liver tissue amino acid nitrogen levels. This increase may partly be due to increase in glutamic acid formation through an increased activity of enzyme glutaminase (28). A significant decrease in liver tissue amino acid nitrogen levels, seen in the present studies, in group 3 mice as compared to group 2 mice suggests that DADS might have interfered with host tissue proteolysis hence causing a decrease in liver tissue amino acid nitrogen levels. This decrease in liver tissue amino acid nitrogen levels in group 3 mice, in part, may be due to decreased glutaminase activity (refer table 2) resulting in a lowered glutamic acid levels.

It is known that tumor cells prefer anaerobic glycolytic breakdown of glucose as compared to glucose oxidative pathways. The observed increase in liver tissue Lactate content in group 2 mice is clearly suggestive of the above statement whereas a significant decrease ($P<0.001$) in liver tissue lactate levels in group 3 mice as compared to group 2 mice (refer table 2) indicates probably DADS might have interfered with cellular glycolytic pathways. Many enzymes of glycolytic pathway including hexokinase, phospho fructo kinase (PFK) & pyruvate kinase (PK) are thiol enzymes (09). DADS, a disulfide might have undergone sulfhydryl exchange reaction similar to any other disulfide (27) as proposed above with glycolytic thiol enzymes hence reducing their activities which results in decreased anaerobic glycolysis thus a decrease in lactate output. This decrease in lactate level in group 3 mice as compared to group 2 mice may also be due to lowered cellular NADPH or NADH levels in group 3 mice as DADS is known to undergo reductive cleavage to its thiols using cellular NADPH or NADH, thus reducing the available NADPH or NADH causing a decrease in lactate formation.

A reliable criteria for assessing the potential use of any anticancer agent is the prolongation of life span of animals (16). Andreani et al (2) has suggested that an increase in lifespan of ascites bearing animals by 25% is considered as indicative of significant drug activity. An increase in life span by 50% i.e. 25.6 ± 0.81 days in group 3 against 16 ±0.89 days in group 2 (refer table 1) in present study suggesting that DADS has a significant protective effect on EAC induced hepatoma bearing mice. Further, a decrease in tumor volume & viable tumor cell count observed in present study (refer table 1) can also be considered as an important marker of reduced tumor burden & enhanced life span of EAC bearing mice. Increased life span % [ILS] & inhibitory growth rate % [IRT%] was calculated as given by Gupta et al (20) to evaluate the effect of DADS on life span of hepatoma induced mice. The percent increase in life span was found to be 50% and inhibitory growth rate percent was found to be 45.43% suggesting that DADS has a significant inhibitory effect on tumor development.

In conclusion, DADS by interfering with protein synthesis as well as with the glucose breakdown in cancer cells, results in reduced cancer cell proliferation & multiplication. Thus shows significant protection against EAC induced hepatoma bearing mice.

Table 1 : The table showing the body weight, abdominal circumference, ascitic fluid volume, cell count, ascitic fluid total proteins & mean survival days of - Group 1, Group 2 & Group 3 mice.

Group	*Body weight(g)*	*Abdominal circumference (cm)*	*Ascitic fluid volume (ml)*	*Ascitic fluid Cell count (Cells/mm³)*	*Ascitic fluid total proteins(mg/ml)*	*Mean survival time(days)*
Group 1 n=6	23.11 ± 2.02	7.75 ± 0.41	--	--	--	--
Group 2 n=6	32.48*** ± 1.99	12.83*** ± 0.5	10.08 ± 0.97	220,815.00 ± 44,645.32	187.5 ± 22.36	16.00 ± 0.89
Group 3 n=6	26.50*** ± 2.09	9.08*** ± 0.39	5.50*** ± 1.18	70,158.83*** ± 14,990.15	108.33*** ± 17.07	25.66*** ± 0.81

Note :

- Number in parenthesis indicate the number of liver specimen
- The values are expressed as their mean ± SD
- Statistical evaluation : probability level - * $p<0.05$, ** $p< 0.01$, *** $p<0.001$

Table 2 : The table showing the liver tissue levels of glycogen, lactate, total proteins, aminoacid nitrogen, total –SH groups, glutaminase activity & transaminases activity (ALT, AST) in - Group 1, Group 2 & Group 3 mice.

Group	*Glycogen Content (mg/g)*	*Lactate Content (mg/g)*	*Total Proteins (mg/g)*	*Aminoacid nitrogen (µgAAN/g)*	*Total SH groups (mg SH/g)*	*Glutaminase units*	*ALT (IU)*	*AST (IU)*
Group 1 n=6	12.29 ± 2.01	1.81 ± 0.27	144.09 ± 12.17	550.0 ± 32.86	2.24 ± 0.20	18.37 ± 1.17	21.1 ± 0.4	28.8 ± 1.17
Group 2 n=6	1.17*** ± 0.24	2.91*** ± 0.10	201.28*** ± 8.50	680.0*** ± 17.88	1.28*** ± 0.15	31.12*** ± 1.52	19.14 ± 0.45	30.05 ± 1.04
Group 3 n=6	3.14*** ± 0.43	2.20*** ± 0.10	164.92*** ± 7.84	586.6*** ± 27.32	1.78*** ± 0.17	22.11*** ± 0.95	20.02 ± 0.74	28.04 ± 0.65

Note :

- Number in parenthesis indicate the number of liver specimen
- The values are expressed as their mean ± SD
- Statistical evaluation : probability level - * $p<0.05$, ** $p< 0.01$, *** $p<0.001$
- Glutaminase : 1 unit = µg NH4 liberated /g liver/hr.

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