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Allicin (diallylthiosulfinate) Restores the Altered Lipid Profile, Erythrocyte Fragility and Permeability in 7,12-dimethylbenz(a)anthracene-induced Hamster Buccal Pouch Carcinogenesis

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Abstract

To investigate the effect of the restoring effects of allicin (diallylthiosulfinate, *DADS*) on the altered lipid profile, osmotic fragility and membrane-bound enzymes in 7,12-dimethylbenz(a)anthracene (DMBA) – induced hamster buccal pouch carcinogenesis. 1 week after receiving *DADS* (20 mg/kg body weight) orally, the buccal pouches of hamsters were painted daily with 0.5% DMBA in liquid paraffin for 14 weeks. The experiment was terminated at the end of 16 weeks. The restoring effects of *DADS* were evaluated by measuring the lipid profile, osmotic fragility and membrane-bound enzymes were analyzed by using specific colorimetric methods. The modified levels of lipid profiles (total cholesterol, triglycerides, phospholipids, HDL, LDL and VLDL) in plasma, RBC-membrane and buccal mucosa tissues in DMBA-administered hamsters were normalized in *DADS*-treated animals. We observed an altered activity of erythrocyte membrane-bound enzyme (Na^+K^+-ATPase) and disturbed extracellular Na^+ and intracellular K^+ cation in the plasma tumor bearing animals, which suggests that the membrane permeability is affected during DMBA-induced oral carcinogenesis. The membrane stabilizing effects of *DADS* were confirmed by erythrocytes' osmotic fragility and the levels of sodium, potassium and the activity of Na^+K^+-ATPase were restored in DMBA-treated animals after treatment with the *DADS*. Increased erythrocyte fragility and permeability in cancer animals are probably due to their altered lipids, osmotic fragility and membrane-bound enzymes. Oral administration of *DADS* to cancer animals prevented the alterations in red cell fragility and the activity of membrane-bound Na^+K^+-ATPase, which indicates the role of *DADS* in maintaining the structural integrity of erythrocytes during carcinogenesis.

Keywords: Oral cancer, 7,12-dimethylbenz(a)anthracene, lipid profile, osmotic fragility, membrane-bound enzymes, allicin, diallylthiosulfinate

1. Introduction

Cancer is one of the most common causes of morbidity and mortality today (Cogliano V *et al*., 2004); it constitutes around 2.1% of all cancer (Alkhadar H *et al*., 2021). Oral cancer refers to a subset of head and neck cancers that arise in the lips, tongue, salivary glands, gingiva, mouth floor, oropharynx, buccal surfaces and other intra-oral areas (Conway DI *et al*., 2018). It is due to alcohol consumption, betel nut chewing, tobacco use, diet, lifestyle habits and infection (Sung H *et al*., 2021). Alterations in circulating lipoproteins are found to be associated with breast cancer and colorectal cancer (Forones NM *et al*., 1998). The mechanisms underlying cancer cachexia are poorly understood, but it was suggested that increased lipolysis might play a role. The changes in lipid profiles have long been associated with cancer because lipids play a key role in the maintenance of cell integrity (Glaus A, 1998). The abnormalities in lipid and lipoprotein patterns produce several pathological diseases including cancer (Manoharan S *et al*., 1995). Many cohort studies have shown in recent years that total cholesterol was associated with the risk of several different cancers (Kitahara CM *et al*., 2011; Strasak AM *et al*., 2009; Iso H *et al*., 2009). Lipoproteins are responsible for the transport of lipids through the vascular and extracellular tissue from their site of synthesis or absorption to peripheral tissues. Altered levels of HDL, VLDL and LDL have been implicated in the pathogenesis of several diseases, including cancer (Alaupovic P, 1996). Measurement of mean corpuscular fragility of erythrocyte membranes is useful to assess the alterations in the integrity of cell structure and function. Alteration in membrane fragility has been documented in hemolytic diseases, diabetes mellitus and cancer (Jain SK *et al*., 1983). The sodium pump (Na^+K^+-ATPase) has been implicated in the regulation of many cellular functions, including cell volume regulation. Na^+K^+-ATPase has an important role in regulating the osmotic balance of the red blood cells and maintains a high concentration of intracellular potassium (Mayne PD, 1994). Raman spectroscopy of serum in DMBA-induced oral carcinogenesis hamster model reveals early changes,

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suggesting serum RS (SRS) potential for early diagnosis and introducing a novel *ex vivo* sequential approach for understanding cancer (Priyanka A *et al*., 2023).

Several medicinal plants and their constituents have been reported to prevent experimentally induced squamous cell carcinomas (Dhanarasu S *et al*., 2010; Sasikumar D *et al*., 2010; Manoharan S *et al*., 2006). Our previous studies indicated that medicinal plants modulate the effect of circulatory antioxidants against oral carcinogenesis (Dhanarasu S *et al*., 2010). Various phytoconstituents such as carotenoids, vitamin C and phenolic acids present in medicinal plants possess protective effects (Samir Q *et al*., 2015). Allicin, (diallylthiosulfinate, *DADS*), is the main biologically active compound derived from garlic (*Allium sativum* L.). It became an object of interest due to its potential to confer a vast spectrum of health benefits including antimicrobial, antifungal and antiparasitic (Koch HP and Lawson LD, 199), cardioprotective (Gonen A *et al*., 2005), anti-inflammatory (Lang A *et al*., 2004) and anticancer activities (Hirsch K *et al*., 2000). However, no evidence is available on the ability of allicin to have ameliorative effects associated with altered lipid profile, osmotic fragility and membrane-bound enzymes in 7,12-dimethylbenz(a)anthracene (DMBA)-induced hamster buccal pouch carcinogenesis. Hence, the present study was designed to focus on the restorative role of allicin on the status of lipid profile, osmotic fragility and membrane-bound enzymes of DMBA-induced oral carcinogenesis.

2. 2. Materials and Methods

2.1. Chemicals

7,12-dimethylben(a)anthracene (DMBA) was obtained from Sigma Aldrich Chemical Limited (St. Louis, MO, USA) while allicin was purchased from Shanghai Harvest Pharmaceutical Co., Ltd. (Shanghai, China). All other chemicals utilized in the present study were of analytical grade.

2.2. Animals

The animals were obtained from Central Animal House, King Saud University, Riyadh, KSA. 8-10 weeks old, male golden Syrian hamsters (*Mesocricetus auratus*, 80-120 g) were used for the experiments. The animals were housed in polypropylene cages at room temperatures (22±2ºC) and relative humidity of 55±5% with a 12 hr light/dark cycle in an experimental room. They were provided with Purina chow diet pellets (Manufactured by Grain Silos and Flour Mills Organization, Riyadh, KSA) and tap water *ad libitum*. Animals were acclimatized for a week before the study. All the experimental protocols were approved by the Institutional Animal Care and Use Committee of the University of Hail, KSA.

2.3. Experimental protocol

The Golden Syrian hamsters were randomized into four groups of 10 animals each as illustrated in the experimental protocol in Figure 1. The left buccal pouches of animals allocated in group I were painted with liquid paraffin thrice a week for 14 weeks and used as negative control animals. Similarly, the left buccal pouches of animals ingroups II and III were painted with 0.5 % DMBA in liquid paraffin thrice a week for 14 weeks (Morris AL, 1961).While the animals in group II received no other treatment, those in group III received oral administration of allicin (20 mg of powder/kg body weight), starting one week before exposure to the carcinogen and continued every other day(once in 2 days), until each animal was sacrificed. Group IV animals received oral administration of allicin (20 mg/kg body weight) alone throughout the experimental period. The experiment was terminated at the end of 16weeks and all animals after being given anesthesia were euthanized by cervical dislocation. The experiments were designed according to our previous studies (Dhanarasu S *et al*., 2010; Sasikumar D *et al*., 2010; Manoharan S *et al*., 2006).

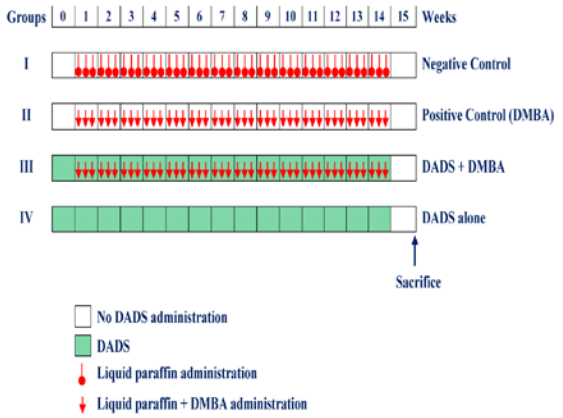

Figure 1. Experimental protocol for restoring effects of allicin against DMBA-induced carcinogenesis.

2.4. Blood and tissues samples

2.4.1. Preparation of serum and plasma

Plasma was separated from heparinized blood by centrifugation at 1000g for 15 min and stored at –20ºC for biochemical assays.

2.4.2. Preparation of Hemolysate

The erythrocytes remaining after the removal of plasma were washed three times with 310mM isotonic Tris-HCl buffer (pH 7.4). Hemolysis was carried out by pipetting out the washed erythrocyte suspension into polypropylene centrifuge tubes, which contained 20mM hypotonic Tris-HCl buffer (pH 7.2). The hemolysate was separated by centrifugation at 3500g for 15min at 20°C.

2.4.3. Isolation of Erythrocyte Membrane

The erythrocyte membrane was prepared by the method of Dodge JT *et al*. (1963) and modified by Quist EE (1980).

2.4.4. Preparation of buccal pouch tissues homogenate

Tissue samples from animals were washed with ice-cold saline and dried between folds of filter paper, weighed and homogenized using an appropriate buffer in an all-glass homogenizer with Teflon pestle. The homogenate was centrifuged at 1000g for 5 minutes and the supernatant was then used for the biochemical estimations.

2.5. Biochemical estimations

Biochemical estimations were carried out in blood and tissue samples of control and experimental animals in each group.

2.5.1. Estimation of lipid and lipoproteins

Lipid extraction was done from plasma, erythrocyte membrane and tissue by the method of Folch J *et al*., (1957). The lipid extractions were used to estimate lipid profile of plasma. Total cholesterol was estimated by the method of Parekh AC and Jung DH (1970). Phospholipids were estimated by the method of Zilversmit DB and Davis AK (1950). Triglycerides were estimated by the method of Foster LB and Dunn RT (1973). The HDL cholesterol was estimated by the heparin-manganese chloride precipitation method (Sperry WM and Webb M. 1950), whereas LDL-Cholesterol and VLDL-Cholesterol were calculated according to Friedewald WT *et al*. (1972). The *in vitro* erythrocyte osmotic fragility was evaluated in all the animals in each group using the method described by Faulknet WR and King JW (1970).

2.6. Statistical analysis

The values are expressed as mean±SD. The statistical comparisons were performed by one-way analysis of variance (ANOVA) followed by Duncan's multiple range test (DMRT), using SPSS version 16.0 for windows (SPSS Inc. Chicago; http://www.spss.com). The values are considered statistically significant if the *p*-value was less than 0.05.

3. Results

The total cholesterol, LDL-cholesterol and FFA were increased, whereas the HDL-cholesterol, phospholipids and triglycerides levels were decreased in the plasma of tumor-bearing animals as compared to negative control animals (Table 1). The total cholesterol was significantly increased whereas phospholipids were slightly decreased in the RBC membrane of tumor-bearing hamsters as compared to control animals (group I, Table 2). The level of total cholesterol was significantly increased whereas the phospholipids and FFA were decreased in buccal mucosa tissues of DMBA-painted (group II) animals as compared to control (group I) animals (Table 3). Increased c/p ratio was observed in plasma, RBC membrane and in tumor tissues. Oral administration of *DADS* normalized the altered levels of lipids and significantly prevented hyperlipidemia in tumor-bearing animals. Hamsters treated with *DADS* alone showed (group IV) no significant difference in the levels of lipid and lipoproteins as compared to control animals (group I). The fragility curve of tumor-bearing animals was shifted to the right for the control animals (group I, Fig. 2).

Table 1. Shows the levels of plasma lipid profiles in control and experimental animals in each group.

Parameters	Group I Negative Control	Group II Positive Control (DMBA)	Group III *DADS* + DMBA	Group IV DADS alone
Total Cholesterol (mg / dl)	81.61±5.64^{a}	138.5±12.86^{b}	91.73±6.92^{c}	81.01±6.20^{a}
HDL-Cholesterol (mg/dL)	21.55±1.69^{a}	16.52±1.33^{b}	23.40±1.72^{ac}	21.03±1.54^{d}
LDL-Cholesterol (mg/dL)	49.7±3.81^{a}	136.52±11.32^{b}	57.00±4.30^{c}	51.10±3.78^{ac}
VLDL-Cholesterol (mg/dL)	9.68±0.75^{a}	16.41±1.34^{b}	11.74±0.84^{c}	7.71±0.68^{a}
Phospholipids (mg/dL)	91.61±7.51^{a}	81.22±5.41^{b}	87.82±7.42^{ab}	91.42±6.88^{a}
Triglycerides (mg/dL)	48.47±3.68^{a}	83.41±6.34^{b}	53.61±4.10^{c}	41.61±3.14^{d}
Free Fatty Acids (mg/dL)	6.10±0.46^{a}	11.30±0.82^{b}	6.66±0.51^{a}	5.84±0.44^{a}
C/p ratio	0.89±0.07^{a}	1.71±0.12^{b}	1.05±0.09^{c}	0.89±0.06^{a}

Values are expressed as mean ± SD for 10 animals in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Table 2. The table shows the levels of RBC membrane lipid profiles in control and experimental animals in each group.

Parameters	Group I Negative Control	Group II Positive Control (DMBA)	Group III *DADS*+DMBA	Group IV DADS alone
Total Cholesterol (μg / mg protein)	128.42±9.75^{a}	198.01±13.236^{b}	137.41±11.43^{a}	127.02 ±9.67^{a}
Phospholipids (μg / mg protein)	277.62±21.12^{a}	241.35±18.40^{b}	266.75±21.14^{abc}	274.75±21.90^{ac}
C/p ratio	0.46±0.03^{a}	0.82±0.07^{b}	0.52±0.03^{a}	0.46±0.03^{a}

Values are expressed as mean ± SD for 10 animals in each group.
Values not sharing a common superscript letter differ significantly at $p<0.05$ (DMRT).

Table 3. The table shows the lipid profile in the buccal mucosa tissues of control and experimental animals in each group.

Parameters	Group I Negative Control	Group II Positive Control (DMBA)	Group III *DADS* + DMBA	Group IV DADS alone
Total Cholesterol (mg / g tissue)	3.88±0.28[a]	8.61±0.66[e]	4.21±0.33[a]	4.01±0.32[a]
Phospholipids (mg / g tissue)	12.60±0.94[ab]	7.91±0.61[e]	12.30±0.87[bc]	13.65±0.95[a]
Free Fatty Acids (mg / g tissue)	8.91±0.66[a]	5.51±0.42[e]	8.44±0.65[ab]	8.73±0.67[ab]
C/p ratio	0.31±0.01[a]	1.09±0.07[e]	0.34±0.04[a]	0.29±0.03[a]

Values are expressed as mean±SD for 10 animals in each group.
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).

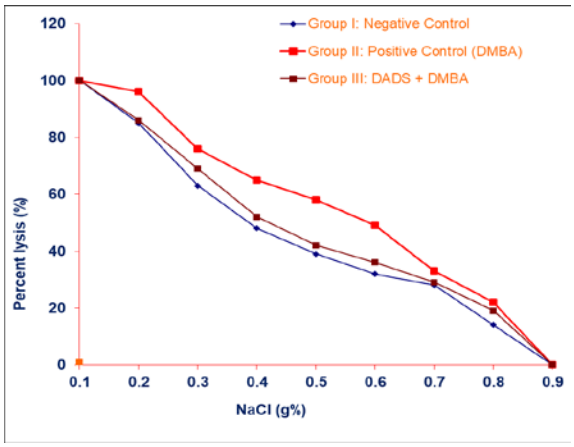

Figure 2. Shows the osmotic fragility curves for control and experimental animals in each group.

The mean corpuscular fragility was also significantly higher in cancer (group II) animals as compared to controls (Table 4). The administration of DADS to tumor-bearing animals led to a leftward shift in the osmotic fragility curve, particularly evident in animals with cancer within group III. Mean corpuscular fragility values did not differ significantly in animals treated with *DADS* alone (group IV) as compared to control animals (group I). In table 4, the activity of membrane-bound Na^+K^+-ATPase was significantly decreased in the RBC membrane of DMBA-induced buccal mucosa cancer animals (group II) as compared to control group animals (group I). The plasma sodium level was decreased whereas potassium level was increased in DMBA-induced oral carcinogenesis. The levels of sodium, potassium and the activity of Na^+K^+-ATPase were restored in DMBA-treated animals after treatment with the *DADS*. No significant differences were noticed between the control and animals treated with the *DADS* alone (group IV).

Table 4. The table shows the levels of plasma sodium and potassium and the activity of erythrocyte membrane Na^+K^+-ATPase and mean corpuscular fragility in control and experimental animals in each group.

Parameters	Group I Negative Control	Group II Positive Control (DMBA)	Group III *DADS* + DMBA	Group IV DADS alone
Plasma Sodium (meq / l)	121.81±9.19[a]	104.93±8.15[b]	116.42±8.71[ab]	121.62±8.51[a]
Plasma Potassium (meq / l)	3.64±0.35[a]	5.39±0.35[b]	3.96±0.31[a]	3.65±0.28[a]
Erythrocyte membrane Na^+K^+-ATPase (Units*)	0.42±0.03[a]	0.24±0.02[b]	0.45±0.02[c]	0.40±0.04[ac]
Mean Corpuscular Fragility**	0.35±0.04[a]	0.56±0.05[b]	0.41±0.03[a]	0.39±0.03[a#]

Values are expressed as mean ± SD for 10 animals in each group.
The graphical representation for these values is not shown in figure 2.
Values not sharing a common superscript letter differ significantly at p<0.05 (DMRT).
* μ moles of inorganic phosphorus liberated/hour/mg protein.
** Concentration of NaCl solution (g %) at 50 % hemolysis.

4. Discussion

Lipids are important for various biological functions in the cell membrane of the human body including maintaining cell integrity, cell growth and division of normal and malignant tissues (Chawda JG *et al*., 2011). Changes in the lipid profiles have been observed in group II DMBA-induced oral cancer hamsters (Neerupakam M *et al*., 2014). The increased levels of plasma triglycerides (TGs), LDL-Cholesterol and VLDL-Cholesterol (Laisupasin P *et al*., 2013, Kapil U *et al*., 2013) were observed in cancer animals compared with group I animals. Cholesterol can facilitate metastasis via the induction of estrogen-receptor-positive cancer cells (Cedó L *et al*., 2019). However, the conflicting findings regarding dyslipidemia and cancer, as documented by Ma HQ *et al* (2016) and Li X *et al* (2018), underscore the intricate and multifaceted nature of this disease relationship. The levels of total cholesterol, free fatty acids and LDL-cholesterol were significantly increased whereas phospholipids and HDL-cholesterol were decreased in the plasma of DMBA-treated animals. The level of total cholesterol was increased whereas phospholipids were moderately decreased in the erythrocyte membrane of DMBA-painted animals. The level of cholesterol was increased whereas phospholipids and free fatty acids were decreased in tumor tissues of cancer-bearing animals as compared to control animals. Oral administration of *DADS* brought back the values to near normal range in DMBA-painted hamsters. It has been reported that cancer subjects showed weight loss accompanied by hyperlipidemia at advanced stages of the tumor. The increase in plasma cholesterol in cancer animals can be related to increased circulatory free fatty acids, which in turn leads to increased VLDL-Cholesterol secretion by the liver, resulting in an increase in cholesterol output into circulation. Increased plasma FFA is attributed to the hypermetabolic state of

cancers (Legaspi A *et al*., 1987). Cholesterol is an essential constituent of lipoprotein fractions like LDL, HDL and VLDL. Seventy-five percent of the plasma cholesterol is transported in the form of LDL cholesterol. Body cells sequester cholesterol from the LDL fraction of lipoproteins (Kesaniemi YA *et al*., 1983). In the present study, a significant decrease in plasma HDL-cholesterol and an increase in LDL-cholesterol were observed in cancer animals (Budd D and Ginsberg H, 1986, Dessi S *et al*., 1992). The increase in plasma cholesterol in cancer subjects can therefore be related to a decrease in HDL fraction or increase in LDL-cholesterol. Cholesterol is essential for the maintenance of the structural and functional integrity of biological membranes. It is also involved in the activity of membrane-bound enzymes (Sabine JR, 1977). The observed increase in cholesterol and c/p ratio (group II animals) indicates the loss of membrane fluidity in cervical cancer patients (Cooper RA, 1977). Alterations in the erythrocyte lipid composition may be a reflection of altered plasma lipid, due to an ineffective exchange mechanism with plasma. An increase in cholesterol in tumor tissues is due to the sequestration of cholesterol from circulation for the biogenesis of new bio-membrane in tumor tissues. Lowered fatty acids in tumor tissues are responsible for decreased phospholipids levels and are partly responsible for lowered lipid peroxidation in tumor tissues. Oral administration of *DADS* to DMBA painted animals reversed the lipids levels to near normal range, which indicates their lipids regulatory effects in tumor-bearing animals.

Erythrocytes of tumor-bearing animals were more fragile than those from control animals (Abou-Seif MA *et al*., 2000). Erythrocytes and erythrocyte membranes are more vulnerable to lipid peroxidation due to constant exposure to high oxygen tension and richness in polyunsaturated fatty acids respectively (Eritsland J, 2000). Increased osmotic fragility in cancer animals can be due to the increased oxidative stress in erythrocytes (Dhanarasu S *et al*., 2010, Sasikumar D *et al*., 2010and Manoharan S *et al*., 2006). Overproduction of reactive oxygen species has been implicated in the alterations of membrane structure and function. Increased lipid peroxidation is therefore responsible for the increase in osmotic fragility (Hebbel RP, 1986). The decline in red blood cell reduced glutathione observed in tumor-bearing animals is partly responsible for the increased osmotic fragility of erythrocytes (McLellan LI and Wolf CR, 1999).We observed an altered activity of erythrocyte membrane-bound enzyme (Na^+K^+-ATPase) and disturbed extracellular (Na^+) and intracellular (K^+) cation in the plasma tumor-bearing animals, which suggests that the membrane permeability was affected during DMBA-induced oral carcinogenesis. Free radical-induced oxidative damage to membrane ATPase has been assumed to be crucial for cell lysis (Brovelli A *et al*., 1977). Increased erythrocyte fragility and permeability in cancer animals are probably due to their altered lipids, lipid peroxidation and antioxidant status. Oral administration of *DADS* to these cancer animals prevented the alterations in red cell fragility and the activity of membrane-bound Na^+K^+-ATPase, which indicates the role of *DADS* in maintaining the structural integrity of erythrocytes during carcinogenesis.

5. Conclusion

The *DADS* protected the alteration seen in membrane fragility and permeability. It also reversed the lipids levels to near normal range, which indicates their lipids regulatory effects in tumor-bearing animals. The present study thus concludes that allicin has potent restoring effects on altered lipids and red cell fragility in DMBA-induced oral carcinogenesis.

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