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Evaluating the effect of lycopene from *Lycopersicum esculentum* on apoptosis during NDEA induced hepatocarcinogenesis

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ABSTRACT

The present study was aimed to examine the influence of lycopene from tomatoes (LycT) on apoptosis in N-nitrosodiethylamine (NDEA) induced hepatocarcinogenesis. Female Balb/c mice were randomly divided into four groups i.e. Control, NDEA, LycT and LycT + NDEA. Hepatic tissue from NDEA treated mice exhibited enhanced expression of anti-apoptotic gene bcl-2 and decreased expression of pro-apoptotic genes caspase 3, 9 and p53 when compared to control group. LycT intervention to NDEA challenged mice exhibited enhanced expression of caspase 3, 9 and p53 and decreased expression of bcl-2 when compared with NDEA treated animals. Enhanced DNA damage was revealed in NDEA and LycT + NDEA groups as revealed by comet assay. However, TUNEL assay indicated enhanced apoptosis in LycT + NDEA group when compared to NDEA group. Hepatic tissue of NDEA treated mice showed persistently high lipid peroxidation levels and glutathione redox ratio during the process of hepatocarcinogenesis. The observed enhanced apoptosis in LycT + NDEA group may be attributed to its differential effects on apoptosis associated genes and its ability to act as a pro-oxidant. These findings provide a rational mechanistic insight into the growth-inhibitory effects of lycopene against hepatic cancer.

1. Introduction

Exposure to chemical carcinogens leads to various biochemical and genetic alterations in the cell. N-nitrosodiethylamine (NDEA), a known environmental hepatic carcinogen, has been used as an initiator in several hepatic cancer models [1]. Cytochrome P450 (CYP) mediated NDEA metabolism generates reactive oxygen species (ROS) and other free radicals, which may be responsible for its hepatocarcinogenic effects [2]. Hepatocellular carcinoma (HCC) is ranked among the most common cancer in the world, and is one of the leading causes of cancer associated deaths [3]. In fact, lack of curative therapies and their associated adverse effects demands new strategies to address the issue of liver cancer with lesser or no side effects. One such strategy might include incorporating large amounts of natural compounds thought to have chemopreventive effects against cancer in diet or to ingest a dietary supplement containing an efficacious quantity of such a compound. Therefore, research towards preparation of phytochemical formulations and use of natural dietary supplements for combating carcinogenesis has gained momentum [1,4,5].

Lycopene, a polyunsaturated hydrocarbon, is the most abundant carotenoid, present in tomatoes (*Lycopersicum* esculentum) with concentrations ranging from 0.9 to 4.2 mg/100 g [6]. It is a potent antioxidant with highest singlet oxygen quenching efficiency and may modulate mutagenesis and carcinogenesis [7,8]. For these reasons several epidemiological and small scale studies have given lycopene, a privileged status in natural product research and have associated it with decreased risk of cancer and cardiovascular diseases [9,10]. Therefore, unraveling the pathways intervened by lycopene during tumorigenesis could provide a rational approach for using it as a chemopreventive agent. Apoptosis is arguably one of the most potent defenses against cancer because evasion of apoptosis is one of the hallmarks for the promotion and progression of cancer as well as treatment resistance [11,12]. Therefore, considering the importance of apoptosis in cancer, the present study was designed to determine the involvement of lycopene in influencing the apoptotic pathway while counteracting NDEA induced hepatocarcinogenesis.

2. Material and methods

2.1. Materials

Lycopene (LycT) was extracted from tomatoes as described previously [13]. Oligonucleotides were synthesized by Sigma–Aldrich (USA). Antibodies against bcl-2, caspase 3, caspase 9, p53, β-actin and peroxidise-conjugated anti-rabbit secondary antibody were purchased from Santa Cruz Biotechnology, Santa Cruz, CA (USA).

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2.2. Animal model and experiment conditions

Female Balb/c mice, 25–30 g each were randomly divided into four groups. Group I (Control) animals received 0.1 ml olive oil (vehicle) orally thrice a week for 24 weeks. Group II (NDEA) animals received a cumulative dose of 200 mg NDEA/kg body weight intraperitoneally in 8 weeks as previously described [1]. Group III (LycT) animals were administered LycT orally at a dosage of 5 mg/kg body weight in olive oil thrice a week throughout the experiment (24 weeks). The first dose of NDEA was commenced after 2 weeks of pre- treatment with LycT in group IV (LycT + NDEA). The experimental animal studies were approved by the ethical committee, Panjab University, Chandigarh and conducted adhering to the Indian National Science Academy Guidelines (New Delhi, India) for the use and care of experimental animals. The treatment schedule carried out in the study is shown in Fig. 1.

2.3. Apoptosis analysis

2.3.1. TUNEL assay

Apoptosis was demonstrated after 24 weeks of treatment in different groups by the terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) method using a standard procedure included in TACS.XL-DAB detection kit from TREVIGEN (Gaithersburg). Incorporation of brominated nucleotides mixture (B-dNTP) by terminal deoxynucleotidyl transferase enzyme (TdT) at the site of DNA fragments are detected using highly specific and sensitive biotinylated anti-BrdU antibody. It generates dark brown staining in the apoptotic cells that is easily visualized against green counterstain. Apoptotic index was determined by counting the number of TUNEL (+ve) cells over the total number of cells in the selected liver tissue section (300 cells), and apoptotic index was expressed based on this percentage.

2.3.2. Comet assay

After 24 weeks single cell gel electrophoresis (SCGE) was performed as described by Tice et al. [14]. Briefly, hepatocytes were prepared by collagenase perfusion as previously described [15]. Comet assay is based upon the ability of denatured, cleaved DNA fragments to migrate out of the nucleoid under the influence of an electric field, whereas undamaged DNA migrates slower and remains within nucleoid. Approximately 200 cells were examined per slide for comet images and scored blindly by two observers.

2.3.3. mRNA expression analysis by RT-PCR

Total RNA was isolated from the hepatic tissue using Tri-Reagent (Molecular Research Centre, Inc., Cincinnati, Ohio) after 24 weeks of treatment. For the RT-PCR analysis primers for the following genes: bcl-2, caspase 3, caspase 9, p53 and β-actin were designed from the sequence data with the help of software 'Gene Runner'. Primers designed for different genes are mentioned in Table 1. mRNA expression was demonstrated by RT-PCR method using the standard procedure described in Superscript III one step RT-PCR kit from Invitrogen (California). The final PCR products formed were analyzed on 1.5% agarose gel electrophoresis and densitometric analysis of the bands was done by Image J software (NH, USA).

2.3.4. Quantitation of protein expression by ELISA

10% (w/v) liver homogenates were prepared in 50 mM Tris–HCl (pH 7.4) under ice-cold conditions and treated with triton-X 100 and phenylmethylsulfonyl fluoride, a protease inhibitor. Homogenates were then centrifuged at 11,800 g for 30 min. PMF thus

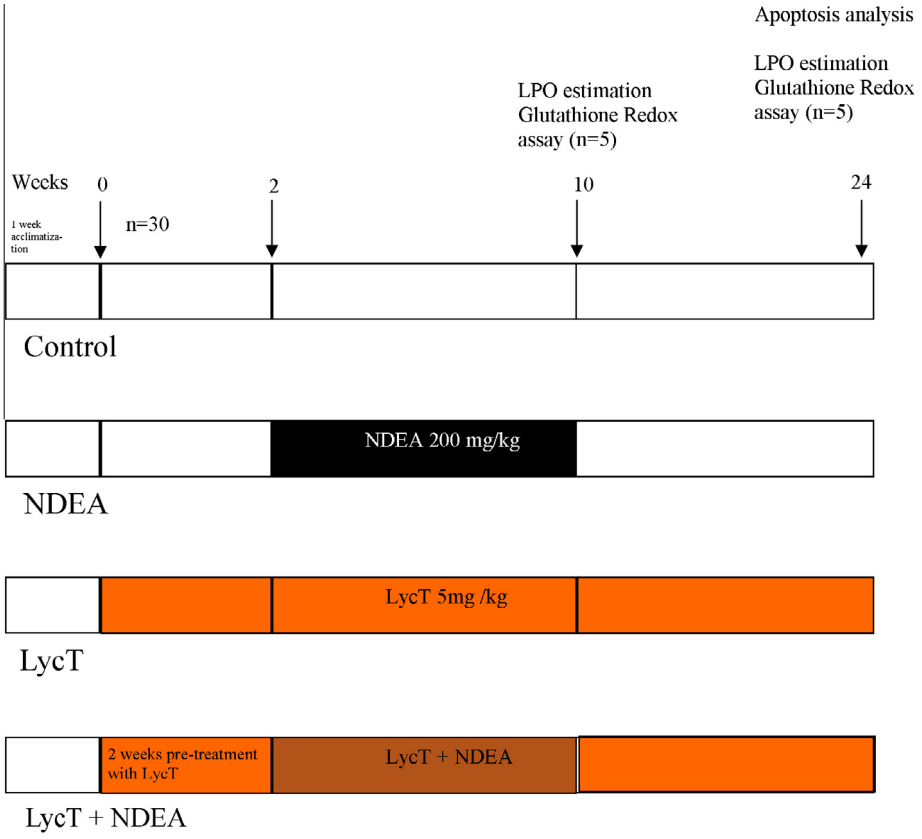


Fig. 1. Treatment protocol in different groups of animals.

Table 1
List of primer pairs used.

Gene	Primer	Refs.
Caspase 3	Sense 5′-AGT CAG TGG ACT CTG GGA TC-3′ Antisense 5′-GTA CAG TTC TTT CGT GAG CA-3′	[36]
Caspase 9	Sense 5′-TGC ACT TCC TCT CAA GGC AGG ACC-3′ Antisense 5′-TCC AAG GTC TCC ATG TAC CAG GAG-3′	[37]
Bcl-2	Sense 5′-AGA GGG GCT ACG AGT GGG AT-3′ Antisense 5′-CTC AGT CAT CCA CAG GGC GA-3’	[38]
p53	Sense 5′-GGG ACA GCC AAG TCT GTT ATG-3′ Antisense 5’ –GGA GTC TTC CAG TGT GAT GAT-3’	[39]
β-Actin	Sense 5’-ATC CGT AAA GAC CTC TAT GC-3′ Antisense 5′-AAC GCA GCT CAG TAA CAG TC-3′	[40]

obtained was used for ELISA. Quantitation of apoptosis related protein expression was proceeded as described previously [16].

2.3.5. Lipid-peroxidation (LPO) and glutathione redox ratio

LPO levels were estimated by the method described by Wills, after 10 and 24 weeks of treatment to observe the status of oxidative stress [17]. The estimation of total glutathione levels were done by the method of Zahler and Cleland, [18]. The method was based on reduction of cellular glutathione with dithioerythritol and determination of resulting total reduced glutathione at 412 nm and expressed as nanomole of GSH/mg of protein [19].

Oxidized glutathione (GSSG) levels were determined by subtracting the value of GSH from total glutathione. Redox ratio was calculated as GSSG/GSH.

2.3.6. Statistical analysis

Data was expressed as mean ± SD. The results were subjected to analysis of variance (one-way ANOVA) followed by post hoc test

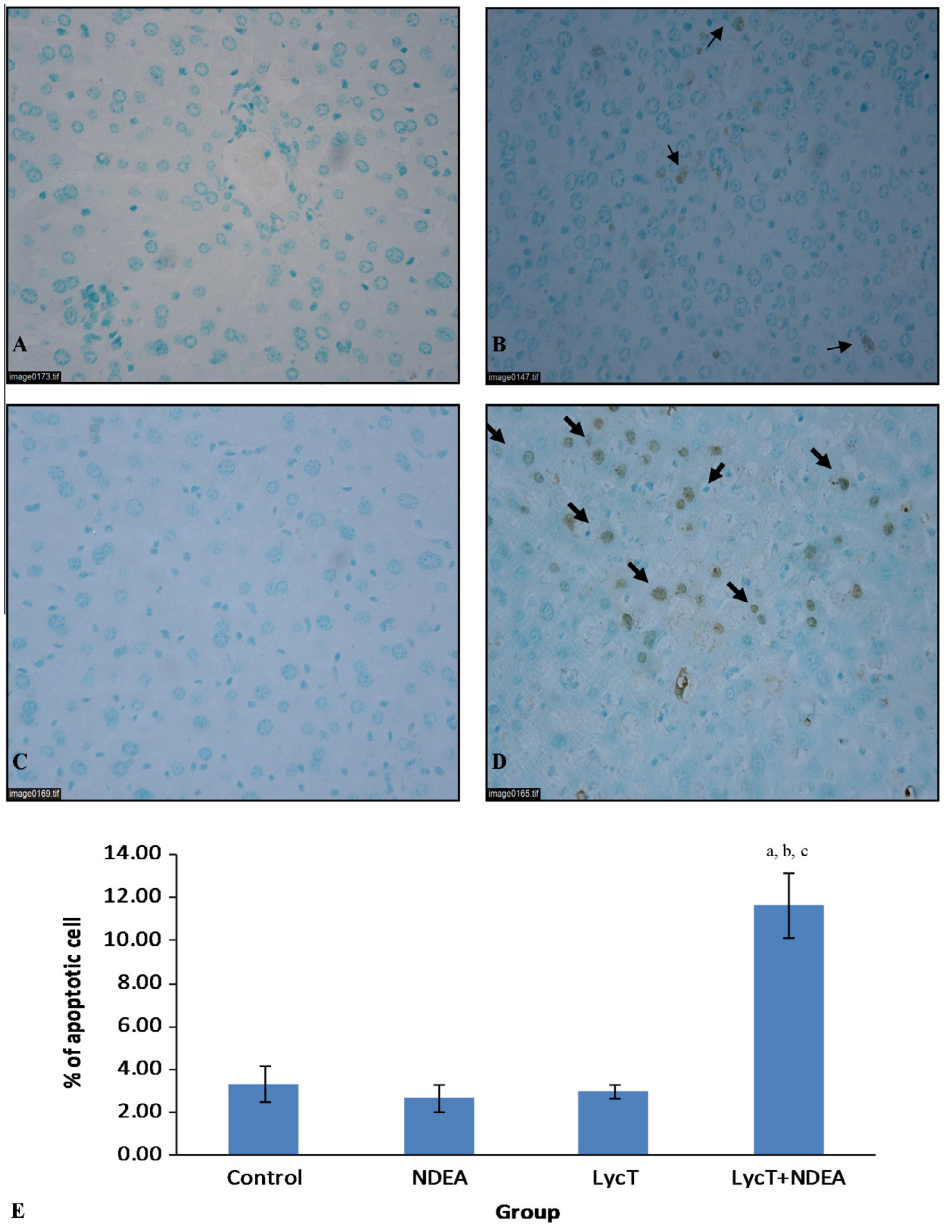


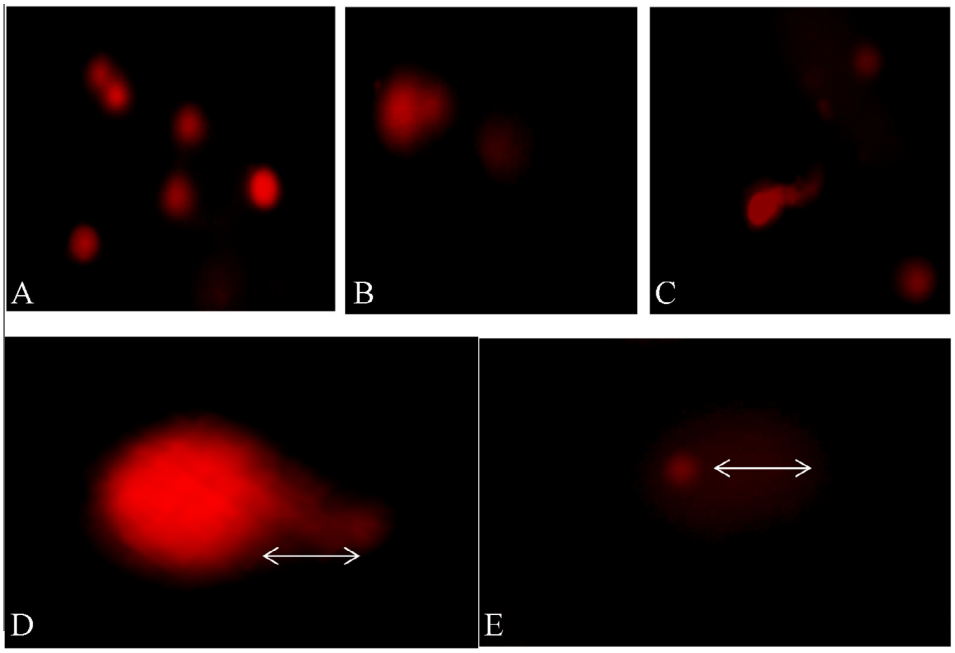
Fig. 2. Effects of NDEA and/or LycT on apoptotic index through TUNEL assay in hepatic tissues. (A) Round blue-green stained cells represent normal hepatocytes at 400X; (B) NDEA exposed liver section showing cell proliferation, very few brown stained apoptotic hepatocytes were visible at 400X; (C) LycT treated liver section showing normal blue stained hepatocytes at 400X; (D) LycT + NDEA treated liver tissue showing large number of brown stained hepatocytes (arrowed) indicating high apoptosis rate (400X); (E) represent apoptotic index in various groups. Values are expressed as mean ± SD ($n = 5$); statistical significance and symbols ((a) $p \leqslant 0.001$ wrt control group; (b) $p \leqslant 0.001$ wrt NDEA group; (c) $p \leqslant 0.001$ wrt LycT group). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

for statistical significance using SPSS (version 14.0) software. $p \leqslant 0.05$ was considered as statistically significant.

3. Results

After 24 weeks of treatment quantitative analysis of TUNEL-positive cells (brown stained cells) against normal (blue-green stained cells) in random microscopic fields of tissue sections from different groups revealed a significant increase in the hepatic apoptotic index in LycT + NDEA group when compared with NDEA group ($p \leqslant 0.05$). However, a non-significant decrease was observed in NDEA group when compared with control group. No significant change in apoptosis was found between control and LycT group (Fig. 2). Significant increase in the comet formation was observed in NDEA and LycT + NDEA group when compared with control group ($p \leqslant 0.05$; $p \leqslant 0.05$). However, the increase observed in LycT + NDEA group when compared with NDEA group was not statistically significant. No significant change in comet index was found between control and LycT group (Fig. 3).

Fig. 4 and Table 2 depict the alterations in the mRNA and protein expression of bcl-2, caspase 3, caspase 9 and p53 in the various groups studied. A significant reduction in the expression of caspase 3 ($p \leqslant 0.05$) and caspase 9 ($p \leqslant 0.05$), respectively were observed in NDEA group when compared to control group. However, LycT intervention to NDEA administered mice caused a significantly increased mRNA and protein expression of caspase 3 and caspase 9 respectively when compared to NDEA group ($p \leqslant 0.05$; $p \leqslant 0.05$). In contrast, NDEA administration showed a significant increase in the expression of Bcl-2 when compared to control group ($p \leqslant 0.05$). LycT intervention to NDEA challenged mice lowered the expression of Bcl-2 when compared to NDEA group. There



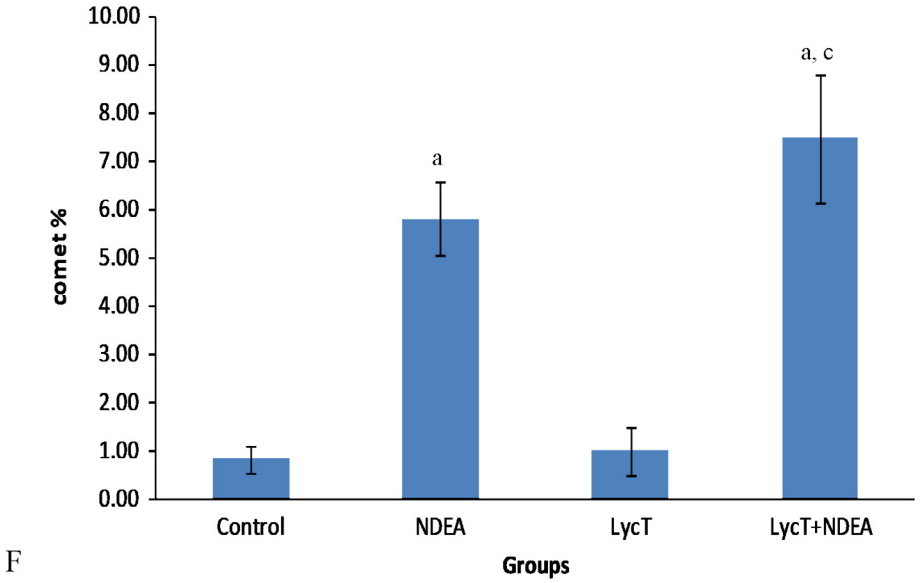


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Fig. 3. A–E shows different shapes of nuclei in COMET assay ((A) represent normal nuclei with no DNA damage; (B) represent nuclei damage showing one macronuclei and other micronuclei; (C–E) represent nuclei with DNA damage; double headed arrow indicate tail portion of comet); (F) represents comet percentage in various groups. Values are expressed as the mean ± SD ($n = 5$); statistical significance and symbols ((a) $p \leqslant 0.001$ wrt control group; (c) $p \leqslant 0.001$ wrt LycT group).

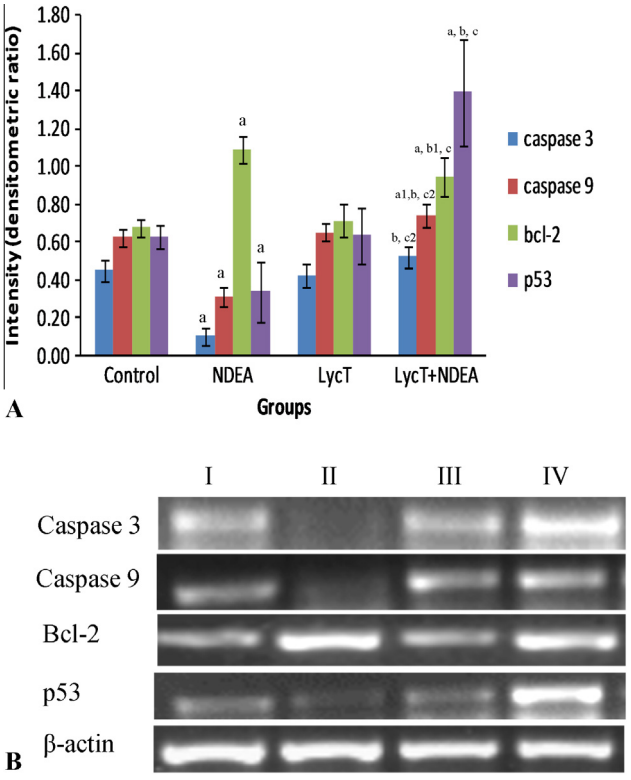

Fig. 4. mRNA expression of anti-apoptotic and pro-apoptotic genes in various treatment groups by RT-PCR. (A) represents intensity (densitometric ratio) of mRNA expression. Values are expressed as mean ± SD ($n = 5$); statistical significance and symbols ((a) $p \leqslant 0.001$ wrt control group; (a1) $p \leqslant 0.01$ wrt control group; (b) $p \leqslant 0.001$ wrt NDEA group; (b1) $p \leqslant 0.01$ wrt NDEA group; (c) $p \leqslant 0.001$ wrt LycT group; (c2) $p \leqslant 0.05$ wrt LycT group). (B) represent RT-PCR gel showing mRNA expression. Lane I = control; Lane II = NDEA; Lane III = LycT; and Lane IV = LycT + NDEA.

Table 2
Effects of LycT and/or NDEA on protein expression of apoptotic related genes.

$A_{405\ nm}$	Control	NDEA	LycT	LycT + NDEA
Caspase 3	0.31 ± 0.00	0.19 ± 0.01 a	0.34 ± 0.06	0.39 ± 0.01 a_2 b
Caspase 9	0.26 ± 0.00	0.14 ± 0.01 a	0.25 ± 0.02	0.31 ± 0.01 a b c
Bcl-2	0.22 ± 0.02	0.49 ± 0.01 a	0.24 ± 0.01	0.26 ± 0.01 a_1 b
p53	0.32 ± 0.01	0.14 ± 0.02 a	0.32 ± 0.00	0.44 ± 0.02 a b c

Data are expressed as mean ± SD and is analyzed by one-way ANOVA followed by post hoc test.
(a) $p \leqslant 0.001$; (a_1) $p \leqslant 0.01$; (a_2) $p \leqslant 0.05$ significant wrt control; (b) $p \leqslant 0.001$ significant wrt NDEA; (c) $p \leqslant 0.001$ significant wrt LycT.

was no alteration in mRNA and protein expression of caspases and bcl-2 was found between control and LycT group. Moreover, p53 expression in NDEA exposed mice was lower than control liver ($p \leqslant 0.05$). LycT + NDEA group showed a significant elevated expression of p53 when compared to NDEA group ($p \leqslant 0.05$). No significant alteration in mRNA and protein expression of p53 was observed between control and LycT group.

Hepatic MDA level after 10 weeks of treatment in NDEA group was significantly higher than the control group. However, when LycT pre-treated mice were challenged with NDEA, a significant decline in hepatic MDA level was observed as compared to NDEA group ($p \leqslant 0.05$). No alteration in LPO levels was observed when LycT group was compared to control group.

Interestingly, the scenario of hepatic MDA level was found to be quite different after 24 weeks of treatment. The hepatic MDA level in NDEA group was significantly higher than control group. However, hepatic MDA level of LycT + NDEA was also observed to be significantly higher than normal groups ($p \leqslant 0.05$) (Table 3).

The redox ratio supports the above levels of lipid peroxidation. Significantly high redox ratio was observed in NDEA treated mice when compared with control group after 10 and 24 weeks of treatment. However, when LycT pre-treated mice were challenged with NDEA, glutathione redox ratio was found to be significantly decreased when compared to NDEA group after 10 weeks of treatment. After 24 week redox ratio was found to be significantly high in LycT + NDEA group when compared to control group ($p \leqslant 0.05$) (Table 3).

4. Discussion

Production of free radicals during NDEA metabolism in liver may be responsible for its hepatocarcinogenic effects [20]. The use of potent antioxidants like lycopene seems to be a rational approach to counteract oxidative stress induced carcinogenesis. Studies point out that lycopene can modulate cellular processes such as cell proliferation, cell differentiation, cell signaling, apoptosis, etc., in several model of tumorigenesis [5,7,8]. *In-vitro* studies have shown higher efficacy of lycopene phyto-complex in triggering apoptosis in phytodynamic therapy [21]. We further extended this work to gain insight into the demonstration of apoptosis in NDEA induced hepatocarcinogenesis and its intervention using LycT.

TUNEL assay was performed to provide quantitative analysis of apoptotic index. NDEA treatment resulted in the formation of hepatic tumors and caused no significant change in apoptotic index when compared to control group. Studies have reported decreased rate of apoptosis with NDEA administration [22]. However, significant increase in the number of TUNEL positive apoptotic cells were observed on LycT treatment to NDEA challenged mice when compared to NDEA treated mice. The present observation is in agreement with the previously reported induction of apoptosis on administration of various identified chemopreventive agents in a variety of premalignant or malignant cell types *in vitro* and in a few animal models *in vivo* and in clinical trials [23]. However, the present study demonstrated an increase in number of comet shaped cells in NDEA treated mice compared to the healthy control mice. The present observations are in corroboration with the previous studies reporting NDEA induced DNA damage as indicated by comet shaped cells. The increment in comets formation may be due to the production of pro-mutagenic DNA lesions of NDEA bio-transformed compounds, playing an important role in inducing hepatocarcinogenesis [24]. LycT administration to NDEA challenged mice in the present study showed a further increase in comet formation when compared to NDEA group, however statistically non-significant. Induction of apoptosis in cancer cells by lycopene has been observed in certain *in-vivo* and *in-vitro* studies. Lycopene has been shown to induce mitochondrial apoptosis in LNaCP cells and HuCC cells [25].

In the present study, NDEA induced hepatocarcinogenesis was associated with alterations in the apoptosis-related proteins such as bcl-2, caspase 3, caspase 9 and p53. An enhanced expression of bcl-2, protein responsible for inhibiting apoptosis by preventing the release of mitochondrial release of cytochrome c; diminished expression of caspase 3 and caspase 9, proteins responsible for the deliberate disassembly of the cell into apoptotic bodies and reduced expression of p53, tumor suppressor protein observed in the NDEA group may have facilitated evasion of apoptosis and development of carcinomas. Similar observations have been reported showing alterations in the expression of apoptosis associated genes on NDEA exposure [26]. Moreover, LycT administration to NDEA challenged mice significantly down-regulated the expression of anti-apoptotic gene bcl-2 and enhanced the expression of

Table 3
Effects of NDEA and/or LycT on levels of lipid peroxidation (LPO) and glutathione redox ratio in hepatic tissues during hepatocarcinogenesis.

	Control	NDEA	LycT	LycT + NDEA
LPO (10th week)	0.11 ± 0.01	0.18 ± 0.01 a	0.10 ± 0.03 b	0.12 ± 0.02 b
LPO (24th week)	0.11 ± 0.01	0.16 ± 0.02 a1	0.12 ± 0.01	0.18 ± 0.02 a c
Glutathione redox ratio (10th week)	0.53 ± 0.15	2.01 ± 0.37 a	0.56 ± 0.13 b	0.58 ± 0.18 b
Glutathione redox ratio (24th week)	0.48 ± 0.09	1.38 ± 0.39 a	0.43 ± 0.09 b	1.61 ± 0.22 a c

Values are expressed as the mean ± SD ($n = 5$); units of LPO: nmoles MDA formed mg^{-1} protein; statistical significance and symbols (a) $p \leqslant 0.001$ wrt control group; (a1) $p \leqslant 0.01$ wrt control group; (b) $p \leqslant 0.001$wrt NDEA group; (c) $p \leqslant 0.001$ wrt LycT group).

caspase 3, caspase 9 and p53 when compared to the NDEA group. These observations demonstrate the induction of apoptosis in LycT intervened group. Some studies inferred that lycopene's metabolite product, rather than the intact molecule, may act at the gene level to modulate the expression of relevant genes and induce apoptosis in cancer cells. It has been suggested that it may depend on the intracellular formation of auto-oxidant products of lycopene rather than to lycopene itself [27]. Studies have reported apoptotic effect of lycopene in gastric cancer elucidating its effect on p53-dependent apoptosis. Lycopene metabolite reduced cell viability by inducing apoptosis in HL-60 cells through the activation of caspases and reduction of bcl-2 gene [28].

In the present study persistently elevated LPO levels and redox ratio (GSSG/GSH) observed in NDEA group demonstrate the alteration in cellular redox environment during hepatocarcinogenesis. LPO is correlated with increased production of ROS and plays an important role in carcinogenesis induced by NDEA [29]. LPO leads to the formation of several toxic products, such as malondialdehyde (MDA) and 4-hydroxy-2-nonenal which can attack cellular targets including DNA, thereby modulating signal pathways associated with proliferation and apoptosis [30]. The current observation is in concordance with the previous report that NDEA injection caused depletion of GSH, which may be responsible for the increased LPO [31,32].

Lycopene administration to NDEA challenged mice showed differential effects on the levels of LPO and redox ratio during hepatocarcinogenesis. A significantly decreased LPO level and GSSG/GSH ratio after 10 weeks of treatment was presumably due to the ability of LycT to scavenge hydroxyl and peroxyl NDEA radicals. Studies have indicated a decrease in the initiation of liver preneoplastic foci by NDEA with dietary intake of lycopene [33]. Interestingly, marked increase in LPO and redox ratio after 24 weeks was observed in LycT + NDEA group. This present observation may be inferred to be associated with the consequences of the enhanced apoptosis with LycT administration. Similar observations have been previously reported that lycopene administration has been linked to oxidative stress induced apoptosis [27].

Apoptosis has also been associated with the changes in cellular redox environment. Bcl-2 protects cells from oxidative stress induced cell death via different unraveled mechanisms including a reduction in the formation of ROS, the prevention of oxygen radical-mediated LPO, the inhibition of cytochrome c release from mitochondria or the alteration of the cellular glutathione pool [34]. Bcl-2 inhibits the release of GSH through cystic fibrosis transmembrane conductance regulator (CFTR), which further enhances GSH accumulation. Combined depletion of bcl-2 and GSH has been reported and has been linked to the increase anti-tumor efficacy. Recently, direct binding of glutathione with Bcl-2 has also been described, although bcl-2 does not appear to substitute for GSH [35]. Down-regulation of bcl-2 gene expression in cancerous cells may contribute a protective effect leading to apoptosis. Such reports strengthen our present observations and provide explanation behind lycopene induced programmed cell death during NDEA induced hepatocarcinogenesis.

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