

## Basic Study

## Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma

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Author contributions: All authors contributed to the manuscript.

Supported by University Grant Commission, New Delhi, No. 2060930310.

**Institutional review board statement:** The study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh (India) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals (IAEC/284-295 at Sr. No. 48).

**Institutional animal care and use committee statement:** All procedures involving animals were reviewed and approved by the Institutional Animal Care and Use Committee of the Panjab University, Chandigarh, India [IACUC protocol number: (IAEC/284-295 at Sr. No. 48)].

**Conflict-of-interest statement:** The authors declare that there are no conflicts of interest.

**Data sharing statement:** No additional data are available.

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**Manuscript source:** Invited manuscript

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Telephone: +91-172-2534119

Received: May 4, 2016

Peer-review started: May 5, 2016

First decision: June 6, 2016

Revised: June 20, 2016

Accepted: July 20, 2016

Article in press: July 22, 2016

Published online: October 18, 2016

### Abstract

#### AIM

To investigate the effect of lycopene extracted from tomatoes (LycT) on ultrastructure, glycolytic enzymes, cell proliferation markers and hypoxia during N-Nitrosodiethylamine (NDEA)-induced hepatocarcinogenesis.

#### METHODS

Female BALB/c mice were randomly divided into four groups: The Control, NDEA (200 mg NDEA/kg b.w. given i.p.), LycT (5 mg/kg b.w. given orally on alternate days) and LycT + NDEA group. The mRNA and protein expression of various cell proliferation markers (PCNA, Cyclin D1, and p21) were assessed by reverse transcription-polymerase chain reaction and enzyme linked immunosorbent assay, respectively. The ultrastructure of hepatic tissue was analyzed using scanning and transmission electron microscopy. The enzymatic activity of glycolytic enzymes was estimated using standardized protocols, while glucose-6-phosphate dehydrogenase activity level was estimated using a kit obtained from Reckon Diagnostic P. Ltd. (India).

#### RESULTS

Uncontrolled proliferation in the liver of NDEA ( $P \leq 0.001$ ) mice was evident from the high expression of cell-proliferation associated genes (PCNA, Cyclin D1, and p21) when compared to control and LycT mice. In addition, enhanced activities of hexokinase, phosphoglucoisomerase, aldolase, glucose-6-phosphate

dehydrogenase and hypoxia-inducible factor-1 $\alpha$  were observed in NDEA mice as compared to control ( $P \leq 0.001$ ) and LycT ( $P \leq 0.001$ ) mice. The alterations in hepatic ultrastructure observed in the NDEA group correlated with the changes in the above parameters. LycT pre-treatment in NDEA-challenged mice ameliorated the investigated pathways disrupted by NDEA treatment. Moreover, hepatic electron micrographs from the LycT + NDEA group showed increased macrophages, apoptotic bodies and well-differentiated hepatocellular carcinoma (HCC) in comparison to undifferentiated HCC as observed in the NDEA treated group.

### CONCLUSION

This study demonstrates that dietary supplementation with LycT has a multidimensional role in preventing HCC development.

**Key words:** Hepatocellular carcinoma; Ultrastructure; Hypoxia; Cell proliferation; Lycopene; Glycolysis

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**Core tip:** The present study was designed to evaluate the chemopreventive role of lycopene extracted from tomatoes (LycT) against N-Nitrosodiethylamine-induced hepatocellular carcinoma (HCC). The findings suggested the mechanism underlying LycT-mediated chemoprevention of HCC.

Gupta P, Bhatia N, Bansal MP, Koul A. Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma. *World J Hepatol* 2016; 8(29): 1222-1233 Available from: URL: <http://www.wjgnet.com/1948-5182/full/v8/i29/1222.htm> DOI: <http://dx.doi.org/10.4254/wjh.v8.i29.1222>

## INTRODUCTION

The continuous rising trend in cancer worldwide necessitates potential action against this deadly disease. Cancer is a complex disease and requires attention in multiple directions to prevent its development. Nearly two-thirds of all cancer cases are linked to inadequate components in the diet, environmental exposure to pollutants and occupational exposure to toxic materials<sup>[1,2]</sup>. Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and is the second most common cause of cancer-related death<sup>[3]</sup>. N-Nitrosodiethylamine (NDEA) is a known potent environmental hepatic carcinogen and has been used as an initiator in several hepatic cancer models<sup>[4,5]</sup>. Besides direct exogenous exposure, humans are also exposed to endogenously produced nitrosamines<sup>[6]</sup>. NDEA is metabolized in the liver to its active ethyl radical metabolite and various other reactive metabolites, which are highly reactive towards DNA, proteins and lipids, thus exhibiting sequential cellular and molecular alterations leading to its hepatocarcinogenic effect<sup>[4,7-9]</sup>.

Natural and experimental chemical hepatocarcino-

genesis is accompanied by altered cellular redox status, altered cytochemical pathways, altered molecular phenomena, chromosomal instability and altered physiological environment in cells. Of the various carcinogenic insults, uncontrolled proliferation, dysregulated carbohydrate metabolism and hypoxia play very crucial roles in HCC development. Aerobic glycolysis and diversion to a biosynthetic pathway, *i.e.*, the pentose phosphate pathway are the metabolic hallmarks of carcinogenesis<sup>[10]</sup>. In recent years, interest in these pathways has been renewed in the tumor microenvironment which has a profound effect on core tumor metabolism. Hypoxia inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) is involved in many compensatory pathways such as angiogenesis, glucose metabolism, survival and tumor development<sup>[11]</sup>. Although there have been advances in therapeutic approaches a complete cure is still unavailable. Dietary multi-targeted agents have attracted the attention of cancer biologists as these agents may provide a solution to this complex problem by targeting multiple targets simultaneously<sup>[12]</sup>. A large body of evidence has revealed an association between phytochemicals and a reduced risk of developing chronic diseases<sup>[13,14]</sup>.

Lycopene, a polyunsaturated hydrocarbon imparting red colour to various fruits is a nutritionally important carotenoid exhibiting beneficial health effects by virtue of its antioxidant activity with minimal side effects<sup>[15]</sup>. A large number of studies have shown an association between lycopene and a reduced risk of developing chronic diseases such as cancer, diabetes, cardiovascular disorders and degenerative diseases<sup>[15-18]</sup>. In addition to its antioxidant property, lycopene is known to modulate other non-oxidative pathways such as regulation of gap junction communication, the hormonal system, the immune system and the metabolic pathways of xenobiotics<sup>[16,19]</sup>. Phytochemicals also tend to be more effective for long-standing health problems that do not respond well to synthetic medicines<sup>[20]</sup>.

In our previous studies we developed a standardized detailed protocol for lycopene extracted from tomatoes (LycT), its characterization and its beneficial effect in inhibiting NDEA-induced HCC development in terms of histopathological observations, tumor statistics, apoptosis, antioxidative capacity and toxicity<sup>[21-23]</sup>. However, further studies are warranted to determine the modulating effect of lycopene on dysregulated glucose metabolism and hypoxia, as these processes play critical roles in cancer. Thus, the present study was designed to explore the influence of LycT on various glycolytic and non-glycolytic enzymes, the expression of *HIF-1 $\alpha$*  and potent cell proliferation-associated genes, while preventing NDEA-induced HCC. Moreover, an attempt was made to demonstrate the impact of an imbalance between energy production and metabolic demands on the gross morphology and ultrastructure of hepatocytes in HCC.

## MATERIALS AND METHODS

### Chemicals

Azino-bis(ethylbenzthiazoline sulfonic acid) (ABTS),

diaminobenzidine, ethidium bromide, TRI-reagent and NDEA were obtained from Sigma Chemicals (St. Louis, MO, United States). Primary and secondary antibodies were obtained from Santa Cruz Biotechnology, CA, United States. Invitrogen superscript (III) one step reverse transcription-polymerase chain reaction (RT-PCR) was purchased and used for RT-PCR analysis. Other chemicals were purchased from local reputable companies including Sisco Research Laboratory (P) Ltd. Detailed information regarding extraction and characterization of LycT has been reported previously (Gupta *et al.*<sup>[21]</sup>).

#### **Animal model and experimental conditions**

The animal protocol was designed to minimize pain or discomfort to the animals. All animals were acclimatized to laboratory conditions, *i.e.*, temperature of 21 °C ± 1 °C and humidity of 50%-60% for one week prior to the various treatments. All the mice were provided with drinking water and a standard animal pellet diet *ad libitum*. Female BALB/c mice (25-30 g) were randomly divided into four groups ( $n = 7$  per group). Animals in Group I (Control) received 0.1 mL olive oil (vehicle) orally throughout the experiment. Group II (NDEA) animals received a cumulative dose of 200 mg NDEA/kg body weight (b.w.) given intraperitoneally in 8 wk as described previously<sup>[5]</sup>. Group III (LycT) mice received LycT orally at a dose of 5 mg/kg b.w. thrice a week for 24 wk. Group IV (LycT + NDEA) animals received NDEA in the same manner as Group III and were also given LycT at a dose of 5 mg/kg b.w. thrice a week for 24 wk. LycT administration was commenced two weeks prior to NDEA treatment. Animals were sacrificed after the 24<sup>th</sup> week to evaluate the modulatory effect of LycT in NDEA-induced hepatocarcinogenesis. The experimental study was reviewed and approved by the Institutional Animal Ethics Committee (IAEC) of Panjab University, Chandigarh (India) and conducted according to the Indian National Science Academy guidelines for the use and care of experimental animals (IAEC/284-295 at Sr. No. 48).

#### **Scanning and transmission electron microscopy**

After 24 wk, liver tissues from animals in the different groups were immediately fixed in 2% para-formaldehyde and 2.5% glutaraldehyde prepared in 100 mmol/L phosphate buffer (pH 7.4) for 6 h at 4 °C. Critical point drying, trimming of the tissue and gold coating were carried out and the tissues were viewed under a LEO 435 VP scanning electron microscope. Secondary fixation, treatment with a mixture of propylene oxide and epoxy resin (1:1), embedding in freshly prepared epoxy resin and ultrathin sections mounted on colloid on-carbon coated grids were carried out and examined with a Philips CM-10 transmission electron microscope.

#### **Estimation of the activities of glycolytic enzymes, glucose-6-phosphate dehydrogenase and glycogen content**

The specific activity of hexokinase, phosphoglucosomerase (PGI) and aldolase was estimated according

to the reported standard protocols<sup>[24-26]</sup>. Glucose-6-phosphate dehydrogenase (G6PD) activity level was estimated in a tissue homogenate using an ENZOPAK G6PD kit obtained from Reckon Diagnostic P. Ltd. (India). The activity of G6PD in the samples was further calculated using 6.22 mmol/L per centimeter as the extinction coefficient of NADPH at 340 nm. The glycogen content in liver was estimated using the protocol described by Seifter *et al.*<sup>[27]</sup>. The amount of glycogen in the aliquot was determined using a glucose standard.

#### **mRNA expression analysis**

Total RNA isolation from liver tissue was carried out using TRI-reagent. For RT-PCR analysis, primers for *HIF-1 $\alpha$* , *PCNA*, *p21* and *Cyclin D1* were searched from the database "Gene Runner" and were synthesized by Sigma-Aldrich (United States). The lengths of the primers chosen were approximately 20bp (Table 1). RT-PCR was performed according to the described protocol of the Superscript (III) one step RT-PCR kit. The DNA bands were visualized in agarose gel using an ultraviolet transilluminator and photographed on Gel Doc. Densitometric analysis of the bands was performed using Image J software (National Institute of Health, United States).

#### **Quantitation of protein expression**

**Sample preparation:** The animals were fasted overnight before liver dissection. Mice were euthanized by cervical dislocation under light ether anesthesia. Liver perfusion was carried out with 0.9% NaCl and the liver was carefully removed and placed in a Petri plate containing ice-cold saline. The tissue was homogenized in ice-cold 100 mmol/L potassium phosphate buffer (pH 7.4) containing 150 mmol/L KCl in an ice-chamber to obtain 25% homogenate (w/v) using a mechanically driven Teflon fitted Potter Elvehjem homogenizer. The homogenate (25%) was then subjected to centrifugation at 10000 rpm for 30 min at 4 °C for preparation of the post-mitochondrial fraction.

**ELISA:** Post mitochondrial fractions obtained from the hepatic tissue of different groups were quantitated for protein concentration by the method of Lowry *et al.*<sup>[28]</sup>. Two point five microgram protein was loaded onto an ELISA strip containing carbonate buffer. Further, protein expression of *HIF-1 $\alpha$* , *PCNA*, *p21* and *Cyclin D1* were analyzed according to the standard protocol of ELISA using specific primary antibodies and enzyme conjugated secondary antibodies. ABTS in citrate buffer was added along with hydrogen peroxide for color generation. The color thus obtained was quantified at 405 nm.

#### **Statistical analysis**

The statistical methods used in this study were reviewed by Dr. Neha Arora Chugh, Department of Biophysics, Panjab University, Chandigarh. Data were expressed as mean ± SD. The results were subjected to analysis of variance (one-way ANOVA) followed by the post

**Table 1** List of primer pairs used

Gene	Strand	Primer
<i>HIF-1<math>\alpha</math></i>	Sense	5'-GGT/CAG/ATG/ATC/AGA/GTC/C-3'
	Antisense	5'-TGC/TTG/GTG/CTG/ATT/TGTG/A-3'
<i>PCNA</i>	Sense	5'-GAT/GTG/GAG/CAA/CTT/GGA/AT-3'
	Antisense	5'-AGC/TCT/CCA/ACT/TGC/AGA/AAA-3'
<i>p21</i>	Sense	5'-CCG/TGG/ACA/GTG/AGC/AGT/TG -3'
	Antisense	5'-TGG/GCA/CTT/CAG/GGT/TTT/CT-3'
<i>Cyclin D1</i>	Sense	5'-CAC/AAC/GCA/CTT/TCT/TTC/CA-3'
	Antisense	5'-GAC/CAG/CCT/CTT/CCT/CCA/C-3'
$\beta$ -actin	Sense	5'-ATC/CGT/AAA/GAC/CTC/TAT/GC-3'
	Antisense	5'-AAC/GCA/GCT/CAG/TAA/CAG/TC-3'

hoc test for statistical significance using SPSS (version 14.0) software.  $P \leq 0.05$  was considered statistically significant.

## RESULTS

Scanning electron microscopy (SEM) of the control and LycT groups revealed normal hepatic surface morphology with polyhedral hepatocytes radially arranged around central veins in cords separated by sinusoids (Figure 1A-C). Bile canaliculi were observed on the apical surface of hepatocytes. A few red blood cells were also visible in the sinusoids. However, serious and irreversible alterations in liver architecture were observed in the NDEA and LycT + NDEA groups. Smoothing or rounding of the hepatocytes with hyperplastic tumor along with clumps of hepatocytes with intercellular surfaces covered with numerous microvillus projections were visible (Figure 1D-E). The discernible nodules were of irregular shape and size. Necrotic tumor nodules and uncontrolled cell density revealed the presence of undifferentiated HCC in the NDEA group. In contrast, the surface morphology of liver tissue from the LycT + NDEA group revealed well differentiated HCC characterized by stromal invasion and a trabecular pattern of two to three cells thick plates of hepatocytes (Figure 1H). High cell density with pleomorphism of tumor cells was also evident (Figure 1F). Apoptotic bodies were observed indicating a high rate of apoptosis (Figure 1G). Thus, LycT pre-treatment of NDEA-challenged mice significantly reduced the severity caused by NDEA. Table 2 shows the results of a quantitative comparison of hepatic tissues from the NDEA and LycT + NDEA groups using SEM.

Transmission electron microscopy (TEM) of the control and LycT groups revealed normal hepatic ultrastructural architecture (Figure 2A-C). At low magnification, hexagonal hepatocytes radially arranged around blood vessels with an intact cell membrane, clear and granulated cytoplasm comprising oval-shaped mitochondria, rough endoplasmic reticulum (RER) and smooth endoplasmic reticulum were observed. A smooth, rounded and prominent nucleus with intact double layered nuclear membrane, a darkly stained single and prominent nucleolus along with uniformly distributed chromatin in the nucleoplasm

**Table 2** Comparative analysis of the N-Nitrosodiethylamine and lycopene extracted from tomatoes + N-Nitrosodiethylamine group by scanning electron microscopy

Groups/parameters	NDEA	LycT + NDEA
Cell density	+++	+
Rounding of hepatocytes	+++	+
Trabecular structures	-	+++
Necrotic tumor nodules	+++	+
Apoptotic bodies	-	+++
Type of HCC	Undifferentiated	Well-differentiated

Where "+++" indicates that more than 70%-90% of mice in a group showed this feature; "+" indicates that < 70% of mice showed this feature; "-" indicates that the < 20% of mice showed this feature. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; HCC: Hepatocellular carcinoma.

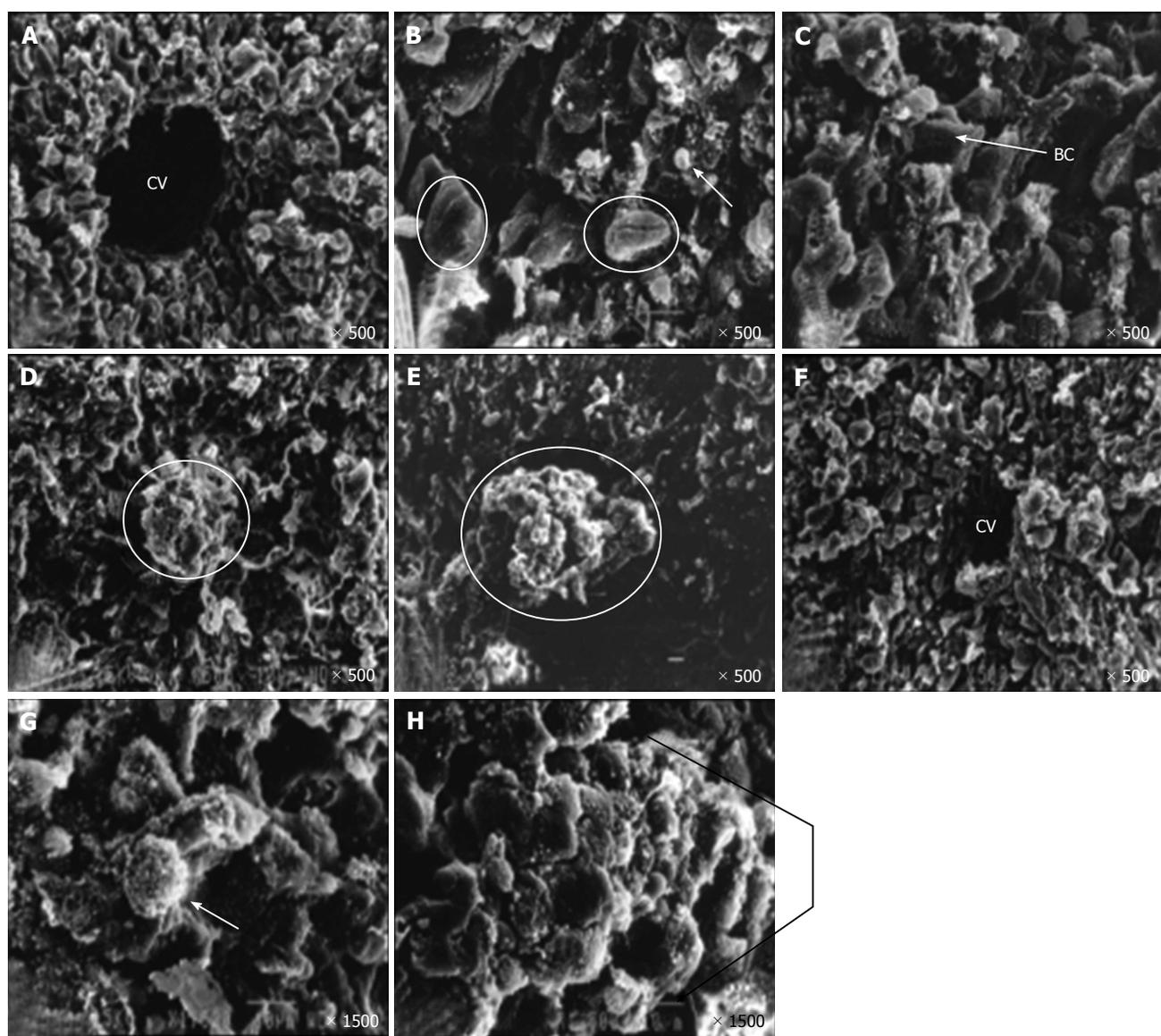
were also visible. Irregular non-membrane bound, faintly stained granules of hepatocellular glycogen were also observed. The presence of lipid granules as darkly stained spots and a few bi-nucleated hepatocytes were found in the LycT group in addition to the above features. Several irreversible alterations in the nucleus of liver cells from the NDEA group were observed (Figure 2D-E). A prominent large and irregular nucleus with interrupted nuclear membrane, multiple prominent nuclei and pseudo-inclusions were observed in liver sections from the NDEA group with an increased nuclear/cytoplasmic ratio. Karyotin (reticular material) deposition along the nuclear membrane was the striking difference when compared with normal nuclei. Loss of organization of cytoplasmic components such as pleomorphic mitochondria varying in shape and size, dilated cisternae of RER associated with mitochondria and decreased lysosomes were observed in tumor cells from the NDEA group. Variable light and dark granules of fat and glycogen deposits were also observed. The liver cells from the LycT + NDEA group showed mild damage to the nuclear membrane with fewer karyotin deposits (Figure 2F). Hepatocytes were evident with prominent nucleoli, pleomorphic mitochondria and a higher number of lysosomes. Moreover, liver sections showed many macrophages in sinusoids and apoptotic cells (Figure 2G-H). Table 3 shows the results of a quantitative comparison of hepatic tissues from the NDEA and LycT + NDEA groups using TEM.

A significant increase in the activities of liver hexokinase, PGI and aldolase in the NDEA and LycT + NDEA groups was evident when compared to the control and LycT groups. However, LycT pre-treatment significantly lowered the activity of hexokinase and PGI in the LycT + NDEA group when compared to the NDEA group. No significant change in aldolase level was observed in the LycT + NDEA group when compared to the NDEA group (Figure 3A-C). Moreover, NDEA treatment caused a significant increase in liver G6PD activity in the NDEA group when compared to the control and LycT groups. Moreover, a significant increase was also observed in the levels of liver G6PD in the LycT + NDEA group when compared to the control group. However, G6PD level

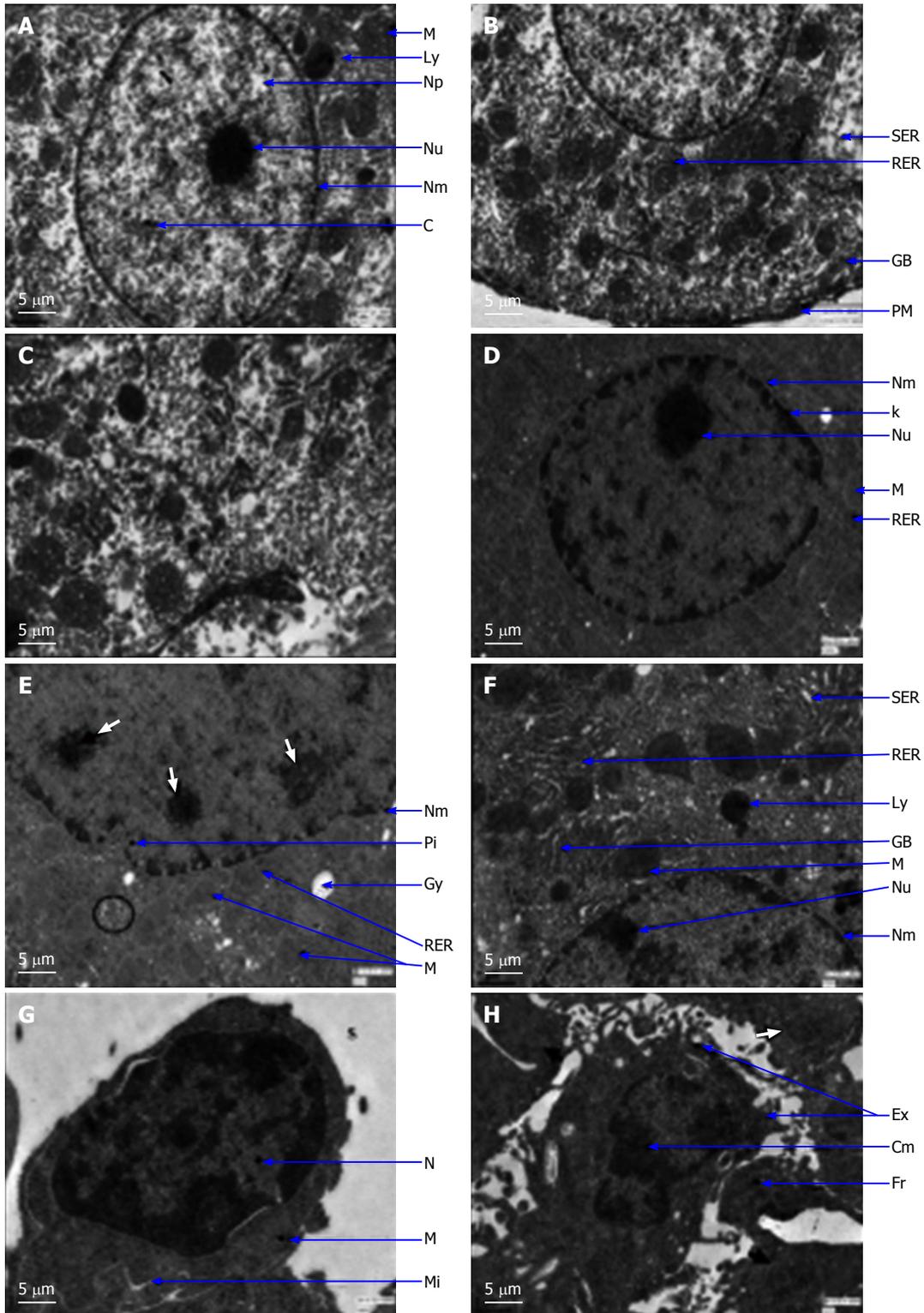
**Table 3** Comparative analysis of the N-Nitrosodiethylamine and lycopene extracted from tomatoes + N-Nitrosodiethylamine group by transmission electron microscopy

Groups/parameters	NDEA	LycT + NDEA
Hepatocytes	Rounded, smaller in size	Polygonal but rounded edges
Nucleus	Large and irregular shape	Oval shaped
Nuclear membrane	Not uniform and pseudoinclusions	Not uniform
Nucleoli	Large, irregular, multiple	One-two
Nuclear/cytoplasmic ratio	High	Low
Karyotin (reticular material)	Deposition along nuclear membrane	Fewer karyotin deposits
Cytoplasm	Loss of organization, dense	Organized
Mitochondria	Pleomorphic with increased density	Pleomorphic but number less than that in the NDEA group
Lysosomes	Few in number	High in number
Fat and glycogen globules	Variable	High
Macrophages	Very few	Many
Apoptotic bodies	No	Clearly visible in the section

LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.



**Figure 1** Scanning electron micrographs of liver tissue at  $\times 500$  and  $\times 1500$ . A and B: Control group, illustrating the central vein (CV), hexagonal hepatocytes (encircled) and biconcave RBCs in sinusoids (arrowed); C: LycT group illustrating hexagonal hepatocytes with bile canaliculi (BC) on their surface (arrowed); D and E: NDEA group illustrating tumor nodules (encircled) with abnormal cell proliferation and disturbed ultrastructure; F-H: LycT + NDEA group respectively illustrating high cell density, CV, apoptotic bodies (arrowed) along with various alterations and two-three cell plate thickening indicating well-differentiated hepatocellular carcinoma, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; RBCs: Red blood cells.



**Figure 2** Transmission electron micrographs ( $\times 2550$ ) of liver tissue. A and B: Control group illustrating round nucleus (N), nuclear membrane (Nm), nucleolus (Nu), and chromatin (C) and nucleoplasm (Np). Clear cytoplasm with mitochondria (M), dark bodies as lysosomes (Ly), smooth endoplasmic reticulum (SER), rough endoplasmic reticulum (RER), and Golgi bodies (GB) surrounded by plasma membrane (PM) were observed; C: LycT group illustrating similar characteristics to those in the control liver micrograph; D and E: NDEA group illustrating deformed N with disrupted and convoluted Nm, darkly masses in the nucleolus (white arrows), deposition of karyotin (k) and pseudo-inclusions (Pi). Cytoplasm was also found to be compactly packed with multiple pleomorphic M and RER. Percentage of other organelles was found to be lower, and variable light and dark granules of fat (encircled) and glycogen (Gy) deposits were also observed; F-H: LycT + NDEA group illustrating perforated nuclear membrane. Cytoplasm contained was occupied with pleomorphic mitochondria, with a however higher percentage of organelles such as GB, Ly, RER and higher glycogen deposits. Macrophages in the sinusoid (S) were characterized by a large nucleus to cytoplasmic ratio, abundant mitochondria and microvilli (Mi). Apoptotic body characterized by cell shrinkage and condensation of nuclear chromatin into delineated masses (Cm), forming extensions (Ex), and crowded with closely packed cellular organelles (white arrow) and fragments of nucleus (Fr). LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

**Table 4** Effect of N-Nitrosodiethylamine and/or lycopene extracted from tomatoes on protein expression in mice hepatic tissue using ELISA

Protein (absorbance at 405 nm)	Control	NDEA	LycT	LycT + NDEA
PCNA	0.31 ± 0.01	0.47 ± 0.01 <sup>a</sup>	0.31 ± 0.01 <sup>b</sup>	0.39 ± 0.01 <sup>a,b,c</sup>
Cyclin D1	0.25 ± 0.02	0.38 ± 0.02 <sup>a</sup>	0.24 ± 0.02 <sup>b</sup>	0.36 ± 0.01 <sup>a,c</sup>
p21	0.40 ± 0.02	0.33 ± 0.02 <sup>e</sup>	0.39 ± 0.02 <sup>f</sup>	0.37 ± 0.01 <sup>g</sup>
HIF-1 $\alpha$	0.22 ± 0.03	0.41 ± 0.01 <sup>a</sup>	0.23 ± 0.03 <sup>b</sup>	0.36 ± 0.04 <sup>a,c</sup>

<sup>a</sup> $P \leq 0.05$ , compared to the NDEA group; <sup>b,c,e</sup> $P \leq 0.001$ , compared to the control group, NDEA group and LycT group, respectively; <sup>f,g</sup> $P \leq 0.01$ , compared to the control group and NDEA group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; PCNA: Proliferating cell nuclear antigen; p21: Cyclin-dependent kinase inhibitor 1A; HIF-1 $\alpha$ : Hypoxia inducible factor-1 $\alpha$ .

following LycT pre-treatment in NDEA-challenged mice was observed to be significantly lower than that in the NDEA group. No significant change was observed in the level of G6PD in the LycT group when compared to the control group (Figure 3D). A significant decrease in liver glycogen level was observed in the NDEA group when compared to the control and LycT groups. LycT pre-treatment in NDEA-challenged mice caused a significant increase in the levels of tissue glycogen when compared to the NDEA group. However, a significant decrease in tissue glycogen level was observed when compared to the control and LycT groups (Figure 3E). No significant change was observed in the activities of these enzymes and the level of glycogen in the LycT group when compared to the control group.

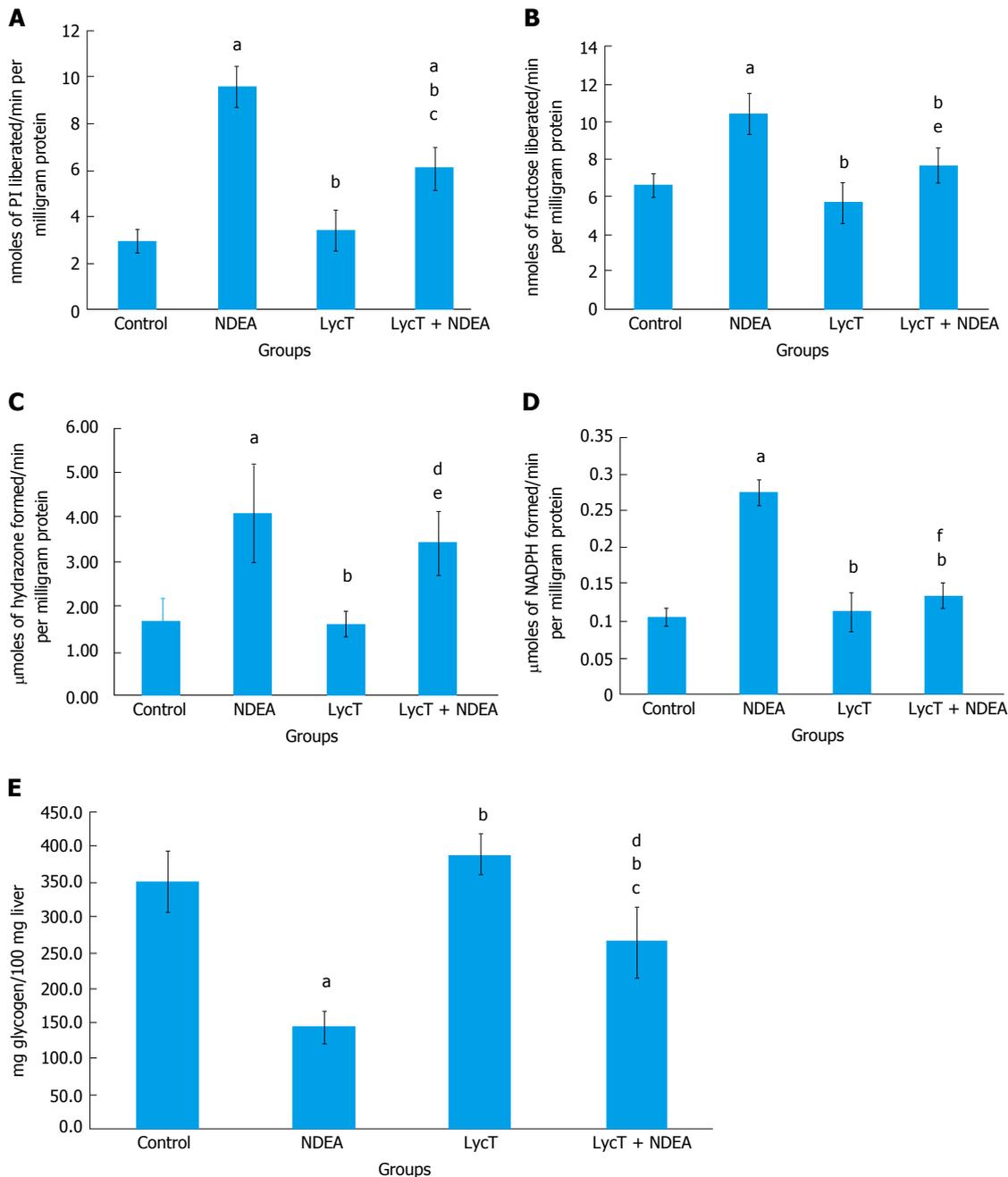
Figure 4 shows the mRNA expression of various genes involved in proliferation during HCC in the different treatment groups. Densitometric analysis of *HIF-1 $\alpha$*  expression revealed a significant increase in the NDEA and LycT + NDEA groups when compared to the control and LycT groups (Figure 4). A significant increase in the expression of *PCNA* and *Cyclin D1* was observed in the NDEA group when compared to the control and LycT groups. The LycT + NDEA group showed a significant decrease in mRNA expression of *PCNA* and *Cyclin D1* when compared to the NDEA group. A significant increase in the expression of *Cyclin D1* was observed in the LycT + NDEA group when compared to the control and LycT groups. Densitometric analysis of *p21* expression revealed a significant decrease in the NDEA group when compared to the control and LycT groups. A significant increase in the expression of *p21* was observed in the LycT + NDEA group when compared to the control, NDEA and LycT groups. No change in the expression of these genes was observed when the LycT group was compared to the control group.

Table 4 shows the protein expression of various genes related to proliferation during HCC in the different treatment groups. The expression of *HIF-1 $\alpha$*  was significantly increased in the NDEA and LycT + NDEA groups when compared to the control and LycT groups. Significantly enhanced expression of *PCNA* and *Cyclin D1* was attributed to significantly higher absorbance at 405 nm in the NDEA group when compared to the control and LycT groups (Table 4). A significantly lower absorbance at 405 nm was observed for *PCNA* expression following

LycT administration in NDEA-challenged mice when compared to the NDEA group. However, significantly enhanced expression of *PCNA* and *Cyclin D1* was observed in the LycT + NDEA group when compared to the control and LycT groups. Protein expression of *p21* was analyzed using ELISA in all treatment groups (Table 4). A significantly lower absorbance at 405 nm was found in the NDEA group when compared to the control and LycT groups. Significantly increased expression of *p21* was observed following LycT pre-treatment in NDEA-challenged mice when compared to the NDEA group. No significant change in the expression of *HIF-1 $\alpha$* , *PCNA*, *Cyclin D1*, and *p21* was observed between the LycT and control groups.

## DISCUSSION

Previously, we observed that LycT yielded lycopene phyto-complex (LycT) which delayed and reduced the severity of NDEA-induced HCC as indicated by histopathology, tumor statistics and antioxidant defence system analysis<sup>[21]</sup>. The presence of a myriad number of compounds in the extract has been reported to enhance the medicinal properties of active components through synergistic effects<sup>[29,30]</sup>. The therapeutic activity of a medicinal plant is not due to a single component or a few components. However, one substance is so dependent on the presence of another substance that the plant or part of the plant when used in its entirety often yields better results than any single component if used in isolation. Lycopene extraction following basic solvent separation has been proved to be a better agent as there is substantial evidence to show that synergism further enhances its activity and efficacy<sup>[31]</sup>. Lycopene phyto-complex has also shown high efficacy in triggering apoptosis in addition to its anti-oxidative property<sup>[31]</sup>. However, the study would be incomplete if the effects of LycT on other hallmark pathways of HCC development were not demonstrated. One of the essential and necessary alterations for the development of almost all cancers is the induction of aerobic glycolysis (Warburg effect). Recently, scientists have linked sustained aerobic glycolysis to oncogenic mutations leading to abnormal cell proliferation and apoptosis<sup>[32]</sup>. Limited literature is available regarding the irreversible alterations in hepatic architecture during HCC development and their

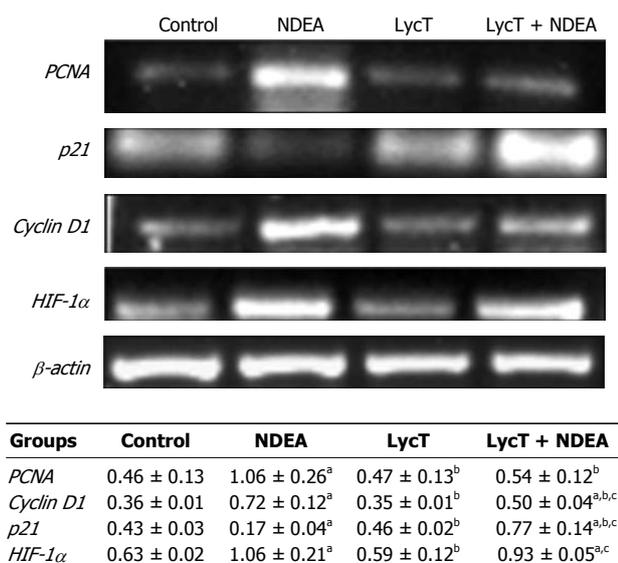


**Figure 3** Effect of lycopene extracted from tomatoes and/or N-Nitrosodiethylamine. A: Hexokinase activity (nmol of pseudo-inclusions liberated/min per milligram protein); B: Phosphoglucosomerase (nmol of fructose liberated/min per milligram protein); C: Aldolase (μmol of hydrazone formed/min per milligram protein); D: G6PD (μmol of NADPH formed/min per milligram protein); E: Glycogen (mg glucose/100 mg liver). <sup>a</sup>*P* ≤ 0.05, compared to the control group; <sup>b,c,d</sup>*P* ≤ 0.001, compared to the control group, NDEA group and LycT group, respectively; <sup>e,f</sup>*P* ≤ 0.01, compared to the control group and LycT group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

association with other dysregulated carcinogenic insults. With this in mind, the present study was designed to provide an insight into the alterations in ultrastructure, cell proliferation and aerobic glycolysis in NDEA-induced HCC and the effects of LycT on NDEA-induced HCC.

Several irreversible distortions using SEM and TEM were clearly observed and indicated the transformation of well-differentiated HCC to undifferentiated HCC in the NDEA group. Rapidly dividing tumor cells attaining a round contour during crowding of the cells has been reported in the literature<sup>[33]</sup>. Gross changes in nuclear mor-

phology, epigenetic regulation, chromatin packing and overall nuclear architecture can be related to alterations in the molecular machinery<sup>[34]</sup>. However, LycT pre-treatment in NDEA-challenged mice resulted in reduced severity as depicted in micrographs. Increased lysosomal bodies and the presence of apoptotic bodies in hepatic tissue from the LycT + NDEA treated group revealed a high apoptotic rate and thus confirms the observations and strengthened our reported data. Aerobic glycolysis arises as a compensatory mechanism due to altered respiration in tumor cells to fulfil the ATP requirements during



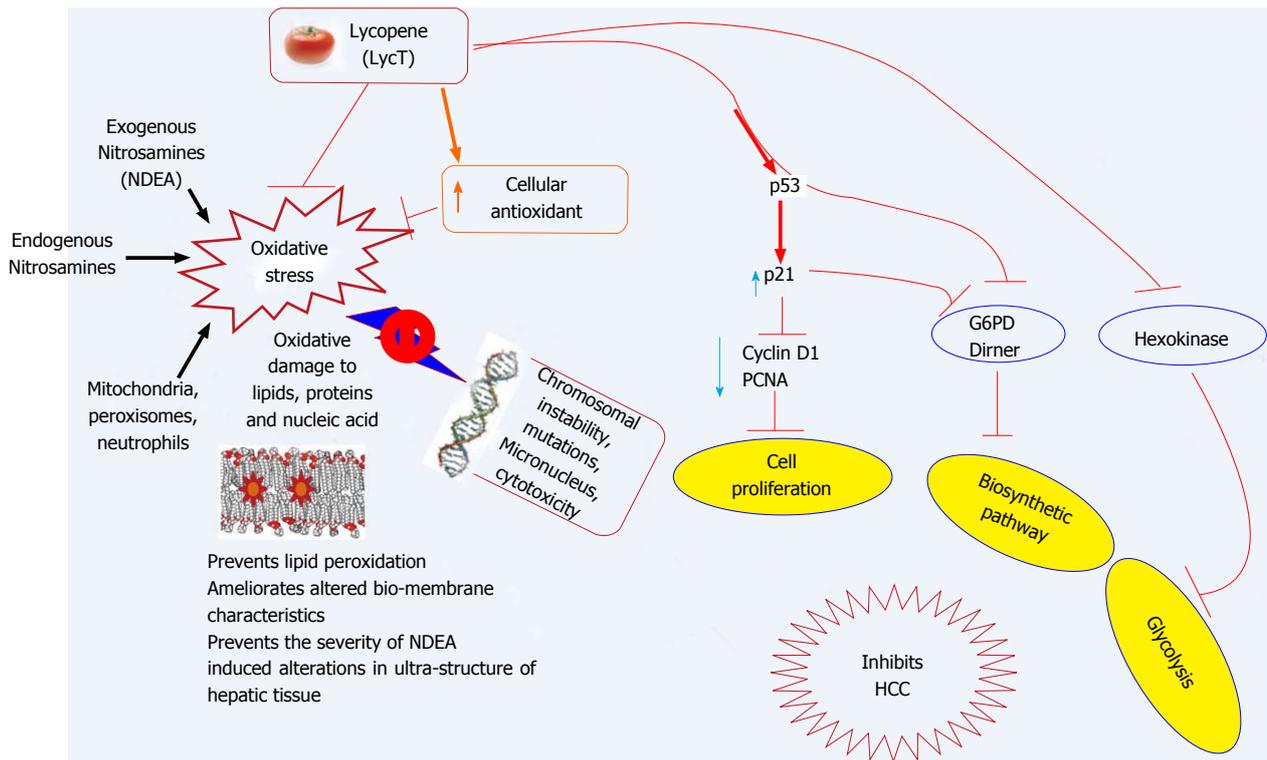
**Figure 4** Effect of lycopene extracted from tomatoes on mRNA expression of *PCNA*, *Cyclin D1*, *p21* and *HIF-1α* during N-Nitrosodiethylamine - induced hepatocarcinogenesis in mice. <sup>a,b,c</sup>*P* ≤ 0.001, compared to the N-Nitrosodiethylamine group, LycT group and control group, respectively. LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine.

abnormal cell proliferation<sup>[35]</sup>. Similarly, enhanced hepatic hexokinase, PGI and aldolase activities in the NDEA group can be attributed to the high cell density observed. Such observations are in accordance with the available literature where enhanced glycolytic enzymes have been linked with chemically induced HCC<sup>[36]</sup>. Elevated levels of PGI have recently emerged as an excellent response to cancer and PGI level is used as a marker of metastatic growth in patients<sup>[37]</sup>. Moreover, significant reductions in the activities of glycolytic enzymes following LycT pre-treatment in NDEA-challenged mice were inversely related to HCC development. Histopathological and ultrastructural observations revealed well-differentiated HCC in the LycT + NDEA group, whereas poorly to undifferentiated HCC was observed in the NDEA group<sup>[21]</sup>. These structural observations could be correlated with the observed modulation of the glycolytic pathway. Various studies based on <sup>18</sup>F-FDG uptake on PET scans in different HCCs revealed that well differentiated HCC showed lower <sup>18</sup>F-FDG uptake in comparison with poorly differentiated HCC<sup>[38]</sup>.

Ectopic expression of G6PD promotes the survival of tumor cells by maintaining both extracellular pH and redox potential<sup>[39]</sup>. Moreover, loss of p53 or mutated p53 has been linked with enhanced glucose consumption *via* increased activity of G6PD<sup>[40]</sup>. In the current study, LycT pre-treatment in NDEA-challenged mice resulted in significantly low expression of G6PD indicating the inhibitory role of lycopene in HCC by affecting metabolic pathways. Although there is limited research on the role of lycopene in regulating G6PD, some researchers have demonstrated the inhibitory effect of phytochemicals by regulating the activity of G6PD<sup>[41]</sup>. Decreased expression of Bcl-2 and enhanced p53 expression in

the LycT + NDEA group may be responsible for reduced G6PD activity. In the current study, liver glycogen content in HCC was found to be decreased in the NDEA group. However, the reasons for the lack of glycogen accumulation were not fully explored. The transformation of liver cells to tumor cells causes a loss of glucose production *via* gluconeogenesis. According to the literature, overproduction of the molecule, microRNA-23a, is responsible for inhibiting gluconeogenesis<sup>[42]</sup>. Glycogen metabolism then acts as an alternate energy source, enabling growth of the cell under metabolic stress. A significant increase in the level of glycogen content was observed in the LycT + NDEA group when compared to the NDEA group. The current observations indicate that lycopene may interfere with glycogen conversion to glucose or might be due to less severe early HCC followed by lower ATP requirement. Such observations are in accordance with the previously reported effect of lycopene in CCL<sub>4</sub>-challenged mice<sup>[43]</sup>.

These observations in the NDEA group clearly point to a high rate of cell proliferation, which was further evident from increased *PCNA* and *Cyclin D1* expression. Enhanced proliferation attributed to increased expression of *PCNA* and *Cyclin D1* has been reported in the literature<sup>[44]</sup>. Decreased expression of *p21* in the NDEA group could be correlated with enhanced expression of *Cyclin D1* as *p21* is known to inhibit the activity of cyclin-CDKs complexes<sup>[45]</sup>. *p21* is an important downstream mediator of p53 and its anti-proliferative property plays an important role in preventing tumor development. In our previous study it was observed that reduced p53 expression in the NDEA group was correlated with evasion of apoptosis<sup>[22]</sup>. Moreover, the LycT + NDEA group showed significantly enhanced expression of *p21* and reduced expression of *PCNA* when compared to the NDEA group indicating the anti-proliferative activity of LycT. Although it is difficult to comment on how lycopene or its metabolites inhibit HCC, the literature shows that treatment with lycopene or its metabolite increased *p21* expression and hence aided in preventing NDEA-induced cancer<sup>[46]</sup>. This observation is also supported by the current observation of increased cell density as shown by SEM and the hepatic glycolysis rate. Moreover, enhanced mRNA and protein expression of *HIF-1α* clearly indicated the existence of hypoxic conditions in NDEA-treated liver tissue. Various research groups have observed that the expression of *HIF-1* modulates apoptosis in HCC<sup>[11]</sup>. Reports have demonstrated that hypoxia enhances VEGF expression and decreases the ratio of Bax/Bcl-2, thus blocking apoptosis<sup>[47]</sup>. Pre-treatment with LycT in NDEA-challenged mice resulted in a significant reduction in the expression of *HIF-1α* at week 24 when compared to the NDEA group. Many reports have demonstrated similar observations where lycopene had an inhibitory response on HIF-1 in both *in vivo* and *in vitro* studies. Upadhyay *et al.*<sup>[48]</sup> performed a comparative study of different antioxidants in order to assess their cancer preventive activity through the inhibition of HIF-1 activity. According to the results, HIF-1α operated in the presence of free radicals and antioxidants with maximum scavenging



**Figure 5 Overall mechanism of lycopene extracted from tomatoes mediated chemoprevention.** LycT: Lycopene extracted from tomatoes; NDEA: N-Nitrosodiethylamine; HCC: Hepatocellular carcinoma; G6PD: Glucose-6-phosphate dehydrogenase.

efficiency for ROS cause inhibition of HIF-1 $\alpha$ <sup>[49]</sup>. The literature also supports the consumption of tomatoes and lycopene mostly inhibited the expression of *HIF-1 $\alpha$*  during prostate carcinogenesis<sup>[50]</sup>. Such reports strengthen our current observations, that the delay in HCC development may be attributed to the anti-proliferative effect of lycopene. In summary, our report demonstrates the potential of lycopene as a multi-targeted approach against chemically induced HCC.

Data from the present study and previously published studies show that LycT has beneficial effects against NDEA-induced HCC. Electron micrographs (SEM and TEM) of liver biopsies from the different treatment groups provided a picture of 3-D *in vivo* tissue modulations and thus served as an efficient and accurate tool for demonstrating carcinogenesis and the efficacy of chemopreventive agents along with histopathological observations. Moreover, aerobic glycolysis is also a potential target for determining the chemopreventive efficacy of lycopene and other phytochemicals in preventing carcinogenesis. As shown in the present study, LycT pre-treatment ameliorated disturbed metabolism, however, further studies are warranted to understand the in-depth pharmacokinetics and pharmacodynamics related to lycopene in experimental models of cancer. Finally, an attempt was made to represent diagrammatically the anti-carcinogenic effect of lycopene based on the current study and previous publications (Figure 5).

## ACKNOWLEDGMENTS

Financial assistance provided by the University Grant

Commission, New Delhi, (India) to carry out the present research is gratefully acknowledged.

## COMMENTS

### Background

Cancer is a complex disease and requires attention in multiple directions to prevent its development. Among the various carcinogenic insults, uncontrolled proliferation, dysregulated carbohydrate metabolism and hypoxia play very crucial roles in the development of hepatocellular carcinoma (HCC). Although there have been advances in therapeutic approaches a complete cure is still unavailable. Dietary multi-targeted agents have attracted the attention of cancer biologists as these agents may provide a solution to this complex problem by targeting multiple targets simultaneously. Lycopene, a polyunsaturated hydrocarbon imparting red colour to various fruits, is a nutritionally important carotenoid exhibiting beneficial health effects due to its antioxidant activity with minimal side effects. In addition to its antioxidant property, lycopene is known to modulate other non-oxidative pathways such as regulation of gap junction communication, the hormonal system, immune system and metabolic pathways of xenobiotics. Previously, we demonstrated that lycopene from tomatoes is a potent agent for inhibiting HCC development in terms of histopathological observations, tumor statistics, apoptosis, antioxidative capacity and toxicity. However, studies are warranted to determine the modulating effect of lycopene on dysregulated glucose metabolism and hypoxia, as these processes play critical roles in cancer. Thus, in the current study, the influence of lycopene extracted from tomatoes on various glycolytic and non-glycolytic enzymes, the expression of hypoxia inducible factor-1 $\alpha$  (*HIF-1 $\alpha$* ) and potent cell proliferation-associated genes while preventing N-Nitrosodiethylamine (NDEA)-induced HCC was investigated. Moreover, an attempt was made to demonstrate the impact of an imbalance between energy production and metabolic demands on the gross morphology and ultrastructure of hepatocytes in HCC.

### Research frontiers

Important areas related to the current study include: (1) carcinogenesis: Incidence rate, statistics, prognosis, consequences, mortality, molecular and biochemical markers, altered cellular pathways and therapeutic limitations;

and (2) chemoprevention: Natural agents, multifaceted approach, lycopene a colored pigment and its antioxidative and anti-carcinogenic potential.

### Innovations and breakthroughs

Despite significant research efforts, cancer is considered an incurable disease due to its high incidence rate, poor prognosis, high mortality rate, multifactorial causative agents, and side effects associated with chemotherapeutics. As natural agents are safer and have a multifaceted approach they are considered an alternative therapy. Lycopene is known to be a potent antioxidant and phytoagent with a protective effect against chronic diseases such as cancer. However, the detailed mechanism underlying its anti-carcinogenic effects is unclear. The basic requirement in the present investigation was to design a study that could demonstrate the action of lycopene at various stages to cover the maximum number of carcinogenic bioprocesses. The present study is part of this research design, in which hepatic tissue from different groups, *i.e.*, control, LycT, NDEA and LycT + NDEA groups was studied at different levels including structural markers, electro-physical markers, morphological markers, biochemical markers, and molecular markers that are known to be involved in the development of cancer. This type of study provides innovation and achievement for futuristic analyses of natural agents in disease models.

### Applications

The outcomes of the current study provide deeper insight into the mechanistic targets of lycopene which shows ameliorating effects. Lycopene administration directly or indirectly stimulated various molecular targets such as p53, which have been correlated with decreased G6PD activity or biosynthetic pathways that play important roles during tumor proliferation. The protective effect of lycopene can be studied at the ultrastructural level by scanning and transmission electron microscopy (SEM and TEM). A detailed explanation regarding hepatic SEM and TEM during carcinogenesis is also information which is ambiguous in the literature.

### Peer-review

This manuscript entitled "Lycopene modulates cellular proliferation, glycolysis and hepatic ultrastructure during hepatocellular carcinoma" investigated the effect of lycopene on ultra-structure, glycolytic enzymes, cell proliferation markers and hypoxia during NDEA induced hepatocarcinogenesis.

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**P- Reviewer:** Camacho J, Xu MY **S- Editor:** Qi Y  
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