Investigations on in vitro anti-carcinogenic potential of L-carnosine in liver cancer cells

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Abstract This study was carried out to investigate the anti-carcinogenic effect of L-carnosine in human carcinoma cells (SNU-423). The SNU-423 cancer cells were cultured at a density of 2×10^4 cells/well in Dulbecco modified Eagle medium. After 24 h of adherence, the cells were treated with L-carnosine (0.2 and 1 mg/mL) for 48 h. Then, cell viability was assessed by sulforhodamine assay, while mitochondrial dysfunction was measured by fluorescence microscopy using chromatin-specific dye Hoechst 33258. Intracellular levels of ROS were assayed by fluorescence spectroscopy with 2',7'-dichlorofluorescein diacetate (DCFDA). L-Carnosine significantly inhibited the growth of the SNU-423 cells ($p < 0.05$).

The inhibitory effect of L-carnosine was confirmed by results from mitochondrial fragmentation assay. The relative fluorescent unit was increased in a dose-dependent manner by L-carnosine, with values of 79.43, 186.87 and 400.89 for 0.6, 0.8 and 1 mg/mL of L-carnosine, respectively ($p < 0.05$). These results demonstrate that L-carnosine exerts anti-carcinogenic effects in human liver cancer cells.

Keywords L-Carnosine · Liver cancer · ROS · Inhibition · Natural substance

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Introduction

Apoptosis is a highly regulated process of programmed cell death (Muthuraman et al. 2016a). During the apoptotic process, cancer cells undergo a well-regulated program which plays a significant role in the cancer prevention and treatment (Reed 2007). Darzynkiewicz et al. (1997) have reported that cell shrinkage and nuclear condensation are characteristic features of apoptosis. Un-regulated cell growth is a key event in tumor development (Muthuraman et al. 2014, 2016b).

Natural compounds have been recognized as important factors in disease prevention and treatment. L-carnosine, a dipeptide composed of beta-alanine and histidine, is present at high levels in animal brain and skeletal muscle (Muthuraman et al. 2016c). The primary sources of L-carnosine are chicken, lean beef,

and fish. It is recommended that humans take in 300–600 mg of L-carnosine daily. Studies have shown that L-carnitine possesses antioxidant, anti-glycating, anti-inflammatory, and metal-chelating properties. The anti-proliferative effect of L-carnitine has been reported in mammalian cells (Iovine et al. 2012), and in NIH3T3-HER2/neu mouse model (Renner et al. 2012).

The present study was aimed at investigating the anticancer effect of carnosine in human liver cancer cells.

Materials and methods

Materials

Dulbecco modified Eagle medium (DMEM), fetal bovine serum (FBS), penicillin–streptomycin, and trypsin–EDTA were obtained from Sigma-Aldrich (St. Louis, MO, USA). L-carnosine, dimethyl sulphoxide (DMSO), sulforhodamine B (SRB), Mito Tracker Red and Hoechst 33258 were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit polyclonal anti-caspase-3 antibodies were purchased from Santa Cruz Biotechnology. Donkey Anti-Rabbit IgG H&L (FITC) conjugated secondary antibody was purchased from Abcam (Cambridge Science Park, Cambridge, UK).

Cell culture

Liver carcinoma cells (SNU-423) were purchased from ATCC (CRL-2238TM) and were cultured in DMEM supplemented with 10% FBS and 1% penicillin–streptomycin. The SNU-423 cells were maintained in a 5% CO₂ incubator at 37 °C.

Sulforhodamine B (SRB) assay

SNU-423 cells were cultured (2×10^4 cells/well) and grown in 96-well plates and allowed to adhere for 24 h at 37 °C. The cells were then incubated with L-carnosine at different concentrations (0.2–1 mg/mL) for 48 h. At the end of 48 h, they were fixed with acetone and air-dried. Thereafter, 100% SRB solution was added to each well, and the wells were incubated for 3 h at room temperature. The micro plates were then washed with 1% acetic acid dried in a drying oven and photographed using an inverted light microscope. Finally, 10 mM Tris base was added, and the plates

were left overnight for complete dissolution of SRB. The absorbance of the solution was measured at 540 nm (Muthuraman et al. 2015).

Assay of mitochondrial dysfunction

Nuclear changes indicative of apoptosis were determined using chromatin-specific dye Hoechst 33258. The SNU-423 cancer cells were cultured at a density of 2×10^4 cells/well in DMEM supplemented with FBS and penicillin–streptomycin. After 24 h of adherence, the cells were treated with L-carnosine (0.6, 0.8, and 1 mg/mL) for 48 h. At the end of treatment period, cells were washed twice with PBS and exposed to 40 nM Mito Tracker Red for 30 min at room temperature and washed twice with PBS. Then, cells were exposed to 0.5 µg/mL of Hoechst 33258 for 15 min at room temperature (Bonner et al. 2010). Thereafter, the cells were washed thrice with PBS and examined under a fluorescent microscope (Axiovert 2000, Carl Zeiss, Germany).

Determination of intracellular ROS levels

SNU-423 cancer cells were cultured at a density of 2×10^4 cells/well in DMEM supplemented with FBS and penicillin–streptomycin. After 24 h of adherence, the cells were treated with L-carnosine (0.6, 0.8, and 1 mg/mL) for 48 h. Thereafter, the growth medium was removed, and the cells were washed thrice with PBS and incubated with 10 µM of 2',7'-dichlorofluorescein diacetate (DCFDA) for 30 min. Then, fluorescence was determined in a fluorescent plate reader (Axiovert 2000, Carl Zeiss, Oberkochen, Germany) (Muthuraman et al. 2015), and the fluorescence intensity was expressed as relative fluorescence unit (RFU).

Statistical analysis

Data are expressed as mean $\pm$ SEM. Statistical analysis was performed using one-way analysis of variance ANOVA (SPSS 17, Chicago, IL, USA) and Student “t” test was carried out. $p < 0.05$ was considered statistically significant.

Results and discussion

L-Carnosine significantly inhibited the growth of SNU-423 cancer cells when compared to the control

($p < 0.05$), with up to 25% growth inhibition at the highest concentration used (Figs. 1, 2). Normal mitochondrial morphology was found in the untreated control cells. However, L-carnosine treatment (0.8 and 1 mg/mL) significantly altered the mitochondrial morphology of the SNU-423 cancer cells. In the L-carnosine treated liver cancer cells, mitochondria appeared as a condensed and clumpy (Fig. 3).

Intracellular ROS levels were significantly increased in a dose-dependent manner. The fluorescence intensity was measured and expressed as relative fluorescence unit ($p < 0.05$). The RFU was increased in a dose-dependent manner by L-carnosine, with values of 79.43, 186.87 and 400.89 for 0.6, 0.8 and 1 mg/mL of L-carnosine, respectively ($p < 0.05$, Fig. 4).

Our present study investigated anticancer effect of natural dipeptide substance L-carnosine against human liver cancer cells. Our experimental result indicates that L-carnosine could be potential therapeutic agent to inhibit the liver cancer cells. In this

* $P < 0.05$, ** $P < 0.01$ & *** $P < 0.001$

Fig. 2 Effect of L-carnosine on SNU-423 liver cancer cell viability. Cell growth inhibition is expressed as a percentage of control. * $p < 0.05$. Values are mean $\pm$ SEM

study, L-carnosine inhibited cancer cell growth even at the lowest physiological concentration.

Renner et al. (2008) have reported that L-carnosine incubation significantly inhibited the

Fig. 1 Effect of L-carnosine on SNU-423 liver cancer cell viability

Fig. 3 Effect of L-carnosine on mitochondria. SNU-423 liver cancer cells were exposed to the different concentration of carnosine. Cells were stained with Mito Tracker® Red and Hoechst 33258 to visualize mitochondria and nuclei

growth of primary glioblastoma cells. The potential of L-carnosine as a treatment strategy for autistic spectrum disorder has been reported (Chez et al. 2012). Several studies have shown that L-carnosine is a dipeptide model for studying the uptake of materials in the brain cells (Schulz et al. 1987). The removal of cellular ROS is necessary for the maintenance of cellular homeostatic. Increased cellular levels of ROS inhibit cancer cell growth. L-Carnosine has been reported to have several

functional roles in the cellular system such as metal chelation, neurotransmitter, pH-buffering and anti-cell death properties (Dickinson and Chang 2012; Ramsey and Sharpless 2006). Kohen et al. (1988) have reported the ROS scavenging potential of L-carnosine, which is believed to be an essential property of an anticancer agent. Moreover, L-carnosine has been reported to reduce lipid peroxidation and senescence (Gallant et al. 2000; Fontana et al. 2002; Hipkiss et al. 1998).

Fig. 4 Effect of L-carnosine on intracellular ROS level. Values mean $\pm$ SEM

Conclusion

The results obtained in this study demonstrate that L-carnosine significantly inhibited the growth of SNU-423 liver cancer cells in a dose-dependent manner. Thus, L-carnosine has promising potential as a dipeptide for the effective arrest of the proliferation of human liver cancer cells.

Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

Human participants and animals participants Research involving Human Participants and/or Animals: This article does not contain any studies with human participants or animals performed by any of the authors.

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