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Protective effects of lupeol against mancozeb-induced genotoxicity in cultured human lymphocytes

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ABSTRACT

Background: Lup-20(29)-en-3H-ol (Lupeol), a dietary pentacyclic triterpenoid has been shown to possess multiple medicinal activities including anti-inflammatory, anti-oxidant and anti-carcinogenic effects. Mancozeb is a widely used broad-spectrum fungicide with well-known carcinogenic hazards in rodents.
Purpose: The present study has been designed to investigate the protective effects of lupeol against mancozeb-induced genotoxicity and apoptosis in cultured human lymphocytes (CHLs).
Methods: The genotoxic effect of mancozeb was evaluated by chromosomal aberration and micronucleus assays. The cell cycle kinetics and intracellular reactive oxygen species (ROS) generation was measured by flow cytometry. The levels of anti-oxidant enzymes and lipid peroxidation (LPO) were estimated by enzymatic assays. The localization of p65NF-κB was measured by immunocytochemical analysis. The differential expression of genes associated with genotoxicity was measured by qRT-PCR.
Results: Mancozeb exposure (5 μg/ml) for 24 h caused significant induction of chromosomal aberrations (CAs) and micronuclei (MN) formation in CHLs. Pre-and post-treatment (25 and 50 μg/ml) of lupeol for 24 h significantly ($p < 0.05$) reduced the frequency of CAs and MN induction, in a dose-dependent manner in mancozeb treated CHLs. Concomitantly, lupeol pre-treatment for 24 h significantly increased the levels of anti-oxidant enzymes, superoxide dismutase (SOD) and catalase and decreased ROS generation and LPO. Additionally, lupeol pre-treatment significantly reduced mancozeb-induced apoptosis as shown by Sub-G1 peak analysis and annexin V-PI assay, in a dose dependent manner. Moreover, pre-treatment with lupeol attenuated mancozeb-induced NF-κB activation in CHLs. Furthermore, the results of qRT-PCR showed that lupeol pre-treatment significantly ($p < 0.05$) decreased mancozeb-induced expression of DNA damage (*p53, MDM2, COX-2, GADD45α* and *p21)* and increased expression of DNA repair responsive genes (*hOGG1* and *XRCC1*) in CHLs.
Conclusion: Taken together, our findings suggest that lupeol could attenuate mancozeb-induced oxidative stress, which in turn could inhibit NF-κB activation and thus provide protection against mancozeb-induced genotoxicity and apoptosis. So, lupeol could be used as a potent anti-oxidant regimen against pesticide induced genotoxicity in agricultural farm workers.

Introduction

In recent years, the use of plant based dietary agents has gained the substantial attention as chemopreventive agents, because these compounds can prevent the progression of carcinogenesis (Shukla and Gupta. 2005). Lupeol, is one of the plant based dietary agents present in fruits such as mango, olive, strawberry, grape, fig and in several medicinal plants, have shown strong chemopreventive and therapeutic potential in diverse experimental models (Chaturvedi et al. 2008). It is also used for the treatment of various skin diseases in North America, Japan, China, Latin America, and Caribbean islands (Chaturvedi et al. 2008). Lupeol

Abbreviations: CA, chromosomal aberration; MN, micronuclei; chls, cultured human lymphocytes; ROS, reactive oxygen species; DDR, DNA damage response; DMSO, dimethyl sulfoxide; PBS, phosphate buffered saline.

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possesses strong anti-oxidant, anti-inflammatory, anti-mutagenic, and anti-malarial activity *in-vitro* and *in-vivo* systems. It also acts as a strong inhibitor of protein kinases and serine proteases and inhibits the activity of DNA topoisomerase II, a well-known target for cancer therapy (Lima et al. 2007; Prasad et al. 2008a). It has also been shown that lupeol induces differentiation of mouse melanoma cells (Hata et al. 2003). Previous works from our laboratory have shown that lupeol exhibits significant inhibition of prostate cancer cells proliferation by induction of apoptosis and also shows promising anti-tumor-promoting activity in two-stage model of mouse skin carcinogenesis (Nigam et al. 2007; Prasad et al. 2008c). Reports published from many laboratories show that lupeol also has strong anti-cancer effects. Palanimuthu et al. (2012) evaluated the anti-tumorigenic potential of lupeol against the 7,12-dimethylbenz[a]anthracene (DMBA)-induced oral carcinogensis. Saleem et al. (2004) demonstrated that lupeol administration could inhibit tumor promotion in two-stage mouse skin carcinogenesis through modulating NF-κB and PI3-kinase (PI3K)/Akt pathways. Additionally, Zhang et al. (2009) studied the lupeol induced apoptosis in hepatocellular carcinoma. Experimental evidence has also shown that lupeol acts through the alterations of phase I and II drug metabolizing enzymes, and could altered the rates of DNA repair activity and scavenging of free radical species (Preetha et al. 2006).

NF-κB is present as a dimer of protein components (p65/p50) in a latent/inactive form, bound to inhibitory protein IκB in the cytoplasm (Shalini and Bansal. 2007). Activation of NF-κB by a variety of extracellular signals leads to degradation of IκB. The activated NF-κB then rapidly translocate into the nucleus, where it regulates transcription of target genes by binding to the consensus κB sites as present in the promoters of the target genes (Wang et al. 2002). Recent studies show that the activation of redox-sensitive NF-κB plays a pivotal role in modulating the cellular signaling mechanism for oxidative stress-induced inflammation during aging (Tilstra et al. 2011). Normally, NF-κB exists in an inactive cytoplasmic form, but enters into the nucleus in response to various stimuli including infection, inflammation, and oxidative stress (Stancovski and Baltimore. 1997). On activation, NF-κB regulates the expression of over 400 different genes, which include enzymes (*e.g.*, COX-2 and iNOS), cytokines (such as TNF, IL-1, IL- 6, IL-8, and chemokines), adhesion molecules, cell cycle regulatory molecules, and angiogenic factors (Ahn and Aggarwal. 2005).

So far, we have evaluated the anti-cancerous potential of lupeol against skin cancer (Nigam et al. 2009) prostate cancer (Prasad et al. 2008c) and liver cancer (Prasad et al. 2007). In addition, we have studied the protective effects of lupeol against DMBA and B(a)P-induced DNA damage (Nigam et al. 2007; Prasad et al. 2008b). It would be of great interest to examine whether lupeol exerts its protective effects against pesticides induced genotoxicity and apoptosis. Extending the previous studies, we examined the protective effects of lupeol against mancozeb-induced genotoxicity and apoptosis in CHLs. Additionally, it was also investigated whether treatment with lupeol could impede the mancozeb-induced aberrant gene expression of DNA damage (p53, MDM2, GADD45α- growth arrest and DNA-damage-inducible α, p21, COX-2- Cyclooxygenase 2) and repair (hOGG1- human 8-oxoguanine DNA glycosylase 1, XRCC1- X-ray cross-complementing group 1, XRCC3- X-ray cross-complementing group 3, ERCC1- excision repair cross-complementing) responsive genes.

Materials and methods

Chemicals

Mancozeb, Lupeol, Histopaque-1077, RPMI-1640, 2',7'-dichlorodihydrofluorescein diacetate dye (DCFH-DA), Propidium iodide (PI), 3[4-dimethylthiazol-2-yl]-2-5-diphenyl tetrazolium bromide (MTT), N-Acetyl-cysteine (NAC) and Giemsa, were purchased from Sigma Chemical Company (St Louis, CA, USA). Annexin V/PI kit was procured from Becton-Dickinson, (San Jose, CA, USA). NF-κB antibody was procured from Cell Signaling Technology (Beverly, USA). The rest of the chemicals were of analytical grade of purity and were procured locally.

Lymphocyte isolation and culture

Lymphocytes were isolated from peripheral blood of healthy non-smoking donors. The lymphocyte's isolation was performed by centrifugation in a density gradient of Histopaque-1077(Srivastava et al. 2012). Then, isolated lymphocytes were cultured in RPMI-1640 supplemented with 20% FBS. PHA (0.1 ml) and streptomycin (100 μg/ml) were added in CHLs and incubated at 37°C for a period of 72 h depending upon the experimental conditions.

Cell proliferation assay

The effect of lupeol on the growth and proliferation of CHLs was determined using MTT assay as described earlier (Srivastava et al. 2012) with slight modifications. Briefly, cells were plated at 1×10^4 cells per well in 100 μl of complete culture medium and treated with 0–80 μg/ml concentrations of lupeol. Lupeol stock solution (10 mg/ml) was mixed with fresh medium to achieve the desired final concentrations. After incubation for 24 h at 37°C, the effect of lupeol on growth inhibition was assessed as % activity, where vehicle (only DMSO in complete medium) treated cells were taken as 100% active.

Pre and post-treatment of CHLs with lupeol

Mancozeb solutions were prepared immediately before use in DMSO. The final DMSO concentration never exceeded 0.5% (v/v). The protective effects of lupeol against mancozeb-induced genotoxicity in human lymphocytes were assessed using two protocols: pre-treatment and post-treatment. The pre-treatment protocol started at 24 h of culture and ended at 48 h. After incubation for 24 h, the cells were treated with lupeol and incubated again. At 48 h, these cells were washed with RPMI-1640, treated with a genotoxic compound mancozeb, and incubated up to 72 h. The post-treatment process with lupeol started at 48 h of culture and ended at 72 h. After incubation for 24 h, cells were treated with mancozeb and incubated again for 48 h. After completion of 48 h, the lymphocytes were then washed with RPMI-1640, treated with the lupeol, and incubated for 72 h. For other assays, like ROS measurement, estimation of anti-oxidant enzymes (SOD and Catalase), LPO, cell cycle analysis, annexin V/PI staining, NF-κB activation and quantitative RT-PCR analysis, only pre-treatment protocol was followed. Cells used as control were incubated with the vehicle (DMSO) alone.

In-vitro chromosomal aberration assay

A general technique described by Preston et al. (1987) for chromosome aberration assay was followed in this study. Cells (1×10^6) was added to 3.5 ml of RPMI-1640 supplemented with 20% FBS. PHA (0.1 ml) and streptomycin (100 μg/ml) were added and CHLs incubated at 37°C for a period of 72 h. Both, pre and post treatment protocol was followed as described above. The clastogen B(a)P (5 μg/ml) and anti-oxidant NAC (50 μM) were used as a positive control. The cells were treated with colchicine (8 μg/ml) for 2 h before harvesting. The cultures were processed and slides were prepared. All the slides, including mancozeb treated, negative and positive controls were independently coded prior to microscopic