The influence of piperine on radioprotective effect of curcumin in irradiated human lymphocytes

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ABSTRACT

Objective: Ionizing radiation (IR) induces DNA damage on normal cell leads to genotoxicity. The radioprotective effects of co-treatment curcumin and piperine were investigated against genotoxicity induced by ionizing radiation in human normal lymphocytes.

Material and methods: Human blood samples were pretreated with curcumin at different concentrations (5, 10 and 25 µg/ml) and/or piperine (2.5 µg/ml) and then were exposed to IR at dose 1.5 Gy. The radioprotective effects of curcumin and piperine were assessed by micronucleus (MN) assay.

Results: It was found that curcumin and piperine reduced the percentage of MN induced by IR in lymphocytes. Piperine alone significantly reduced genotoxicity induced by IR as compared to curcumin alone at all concentrations. An additive radioprotective effect was observed in combination of piperine and curcumin at low concentration 5 µg/ml while this synergistic effects were not observed with curcumin at higher concentrations 10 and 25 µg/ml.

Conclusion: This result provides that piperine acts as a potent radioprotective effect at low concentration as compare to curcumin. However, an additive radioprotective effect was observed with co-treatment piperine and curcumin at low concentration, while piperine increased the percentage of MN in normal lymphocytes when was co-treated with curcumin at higher concentration.

Keywords: Curcumin; Piperine; Radioprotective; Genotoxicity; Ionizing radiation

Running title: Radioprotective effect of curcumin and piperine

Introduction
Ionizing radiation (IR) is widely used for cancer treatment in patients. In this strategy, IR produces free radicals and reactive oxygen species (ROS) when passing through cell. These toxic substances react with critical macromolecules such as DNA results in genotoxicity and cell death. However, IR is focusing on cancerous cells, the unwanted exposure to normal cells results to normal tissue damage. The side effects induced by IR are limited to use of radiotherapy in patients. Radioprotective agents are enable to protect normal cells against genotoxicity and deaths induced by IR. Several protection mechanisms are proposed for radioprotective agents such as free radical scavenging and increasing endogenous cellular antioxidants enzymes. Curcumin is a natural component which is prepared from *Curcuma longa* and widely used as an additive flavoring in foods. This compound has several benefit biological properties as antioxidant, anti-inflammatory and anti-cancer. Curcumin enable to protect cell against genotoxicity and death induced by IR. Poor bioavailability in oral consumption is the main disadvantage of curcumin for clinical application. It is interesting that some of natural compounds to act as an enhancer of curcumin through oral absorption. Piperine is a natural product which is prepared from black pepper (*Piper nigrum* Linn). This natural product is consumed with *Curcuma longa* as spices in food. Piperine is used as anticancer as well as natural bioenhancer for curcumin. Piperine enhances the protective effects of curcumin against oxidative stress related diseases in animal model. Also, synergistic effects of curcumin and piperine were observed is suppression of tumor proliferation in animals. With respect to beneficial effects of piperine and curcumin on oxidative stress and prevention of cancer, the aim of this study was to investigate the influence of piperine on radioprotective effect of curcumin against genotoxicity induced by IR on normal human lymphocytes.

**Material and Methods**
Materials

Curcumin was prepared from Sami Labs (India) and piperine was from Qingado BNP Co. (China). Phytohemagglutinin M (PHA-M), Roswell Park Memorial Institute (RPMI-1640) medium, fetal bovine serum (FBS), penicillin and streptomycin, L-glutamine were purchased from Gibco-Brl, (USA). Cytochalasin-B was purchased from Sigma Chemicals Co. (St. Louis, USA). Giemsa stain, methanol, and acetic acid were obtained from Merck, (Germany).

Blood treatment

After obtaining permission from research and ethical committees of the Mazandaran University of Medical Sciences, this study was performed. Four healthy, non-smoking male volunteers, aged from 22 and to 28 years were enrolled in this study. Twelve milliliter whole blood were collected in heparinized tubes and divided in centrifuge tubes at 0.9 mL each. Blood samples were pretreated with 100 μL solution of curcumin at the concentrations of 5, 10, or 25 μg/ml and/or piperine (2.5 μg/ml.) These samples were incubated for three hours at 37 °C. Curcumin (CUR) and piperine (P) were dissolved in DMSO and diluted in RPMI cultural medium. The twelve samples groups are following: control, ionizing radiation (IR), 5 μg/ml CUR+IR, 10 μg/ml CUR+IR, 25 μg/ml CUR+IR, 5 μg/ml CUR+2.5 μg/ml(P)+IR, 10 μg/ml CUR+2.5 μg/ml(P)+IR, 25 μg/ml CUR+2.5 μg/ml(P)+IR, 2.5 μg/ml(P)+IR, 25 μg/ml CUR, 2.5 μg/ml(P), 5 μg/ml CUR+2.5 μg/ml(P). The curcumin concentrations were selected based on previous studies. Piperine concentration was selected based on previous studies that P has an IC₅₀ 61 μg/ml on HeLa cell line and it did not exhibited any genotoxicity and cellular toxicity up to 60 μM (17 μg/ml). Control samples were treated with diluted DMSO in RPMI at same concentration to other curcumin and/or piperine samples.
Ionizing radiation and micronucleus test

Whole blood samples in micro tubes were kept on the plastic box containing water as a phantom and then were irradiated with 6 MV X-ray beam produced by a Linear accelerator (Siemens, Primus, Germany) at a dose of 1.5 Gy with a dose rate of 1.9 Gy/min. Samples from four volunteers were allocated to the control (non-irradiated samples). After irradiation, subsequently, 0.5 mL of each sample (control and irradiated samples in duplicate) was added to 4.4 mL of RPMI 1640 culture medium, which contained a mixture of 10% FBS, 100 µL PHA. All cultures were incubated at 37 °C. Cytochalasin B (100 µL at final concentration: 6 µl/ml) was added after 44 h of culture. Following 72 h of incubation, the cells were collected by centrifugation, re-suspended in cold 0.75 M potassium chloride. Cells were immediately fixed in a fixative solution as made of methanol: acetic acid (6:1 V:V) two times. The fixed cells were dropped onto clean microscopic slides, air-dried and stained with 10% Giemsa solution. All slides were evaluated at 1000 × magnification in order to determine the frequency of micronuclei in the cytokinesis-blocked binucleated cells with a well-preserved cytoplasm 16. For each treated group from each volunteer, a total of 1,000 binucleate cells (in the duplicate cultures) were examined to record the frequency of micronuclei-containing cells. All slides were evaluated by an expert person using a light microscope. A total of 4000 binucleated lymphocytes were blindly counted in each treated group from three volunteers, and totally 48,000 binucleated lymphocytes were counted for twelve treated groups in this study. The criteria for scoring micronuclei were a diameter between 1/16th and 1/3rd of the main nuclei, non-refractile, not linked to the main nuclei and not overlapping the main nuclei 16.

Statistical analysis

The data values are presented as mean ± standard division (SD). Statistical analysis was performed using one-way analysis of variance (ANOVA), as well as post hoc Tukey multiple
comparison tests. \( P \) value < 0.05 was considered as significant and highly significant (Prism 7 Software, 2016, USA).

**Results**

A typical binucleated lymphocyte with micronucleus is shown in figure 1. The mean percentage of micronuclei in irradiated samples was 8.57 ± 0.09, while that was 0.71 ± 0.06 in non-irradiated control samples. It showed a statistically significant increase (12-fold rise) in the frequency of micronuclei in irradiated samples at dose of 1.5 Gy (Table 1, Figure 2) \( (P < 0.001) \). In irradiated samples with CUR pre-treatment, the frequency of micronuclei at the concentrations of 5, 10, or 25 \( \mu \text{g/ml} \) were 6.10% ± 0.14, 4.47% ± 0.09 and 4.35% ± 0.19 (Table 1). The data demonstrate that pre-treated samples with CUR at concentrations of 5, 10, or 25 \( \mu \text{g/ml} \) exhibited a significant decrease in the frequency of micronuclei as compared to irradiated samples without CUR addition \( (P < 0.001) \). Total micronuclei frequencies were reduced by 1.40, 1.92 and 1.97 fold in irradiated samples with CUR treatment at concentrations of 5, 10 or 25 \( \mu \text{g/ml} \), respectively, as compared to alone irradiated samples (Table 1). The maximum protection of lymphocytes was observed with CUR treatment at concentration of 25 \( \mu \text{g/ml} \). It was observed a dose-manner protective effect with CUR at concentrations 5, 10 and 25 \( \mu \text{g/ml} \) \( (P < 0.01) \). However, non-irradiated sample with CUR treatment at concentration 25 \( \mu \text{g/ml} \) did not showed any increased genotoxicity as compared to control group.

In irradiated samples with CUR + P pre-treatment, the frequency of micronuclei at the concentrations of 5 \( \mu \text{g/ml} \) CUR + 2.5 \( \mu \text{g/ml} \) (P) + IR, 10 \( \mu \text{g/ml} \) CUR + 2.5 \( \mu \text{g/ml} \)(P) + IR and 25 \( \mu \text{g/ml} \) CUR + 2.5 \( \mu \text{g/ml} \) (P) + IR were 3.07% ± 0.24, 4.87% ± 0.26 and 5.25% ± 0.29, respectively (Table 1). The data demonstrate that pre-treated samples with CUR (5 \( \mu \text{g/ml} \)) and P (2.5 \( \mu \text{g/ml} \)) exhibited significant decrease in the frequency of micronuclei as compared to irradiated samples with CUR alone samples at all concentrations 5, 10 and 25 \( \mu \text{g/ml} \). It is
interesting to see an increased frequencies of micronuclei in human lymphocytes treated with CUR + P + IR as compared to CUR (10 µg/ml) + IR or CUR (25 µg/ml) + IR (P < 0.05). Piperine significantly reduced the frequency of micronuclei in irradiated lymphocytes as compare to irradiation alone. The frequency of micronuclei lymphocytes with P at concentration of 2.5 µg/ml was insignificant as compared to control sample, while the combination of CUR (25 µg/ml) and P (2.5 µg/ml) increased significantly the frequency of micronuclei in binucleated lymphocytes as compared to control sample (Table 1 and Figure 2).

**Discussion**

Curcumin, as a natural product, is widely used in food and drug composition and has several biological and pharmacological properties. Curcumin exhibits as an anti-cancer, anti-inflammatory agent and antioxidant 3-4. Curcumin enable to scavenge free radicals and ROS that are generated by toxic substances such as IR. The anti-inflammatory effect was reported for curcumin through diminishing cytokines and interleukins that are involved in inflammation process 18. Oxidative stress and inflammation are two suggested main mechanisms are involved in cellular toxicity induced by IR, then curcumin acts as a radioprotective agent through two mentioned mechanisms. Recently we showed that curcumin had protective effect against genotoxicity induced by iodine radioactive in human lymphocyte 5. Curcumin could sensitize selectively thyroid cancer cell to death induced by iodine radioactive without any toxicity on non-malignant fibroblast cells 13. In the present study, we showed that curcumin significantly protected human healthy lymphocyte against genotoxicity induced by external IR. These results showed curcumin has radioprotective effect on normal cell and radiosensitizing effect on cancer cell, that is promised using this natural agent in cancer therapy.
The highest radioprotection of lymphocytes with curcumin alone treatment was observed at concentration 25 µg/ml. However, this maximum protection is interesting, this concentration should be reached in vivo by oral administration of curcumin. Curcumin could not achieve its expected therapeutic outcome in vivo due to its low solubility and poor bioavailability. Poor oral bioavailability of curcumin is due to limited intestinal uptake and rapid metabolism and it is the most limitation of this natural product for human usage. Several strategies have been applied for enhancement of oral bioavailability of curcumin such as improvement of its formulation and bioavailability enhancer. Piperine, as a major plant alkaloid, is widely used as condiments and flavoring agent for many types of dishes. Piperine acts as an enhancer of bioavailability and pharmacological activity of curcumin. There are two suggested mechanisms for piperine as a bioenhancer are including promoting rapid absorption of drugs and nutrients, and inhibiting enzymes involving in biotransformation of drugs. Piperine is a potent inhibitor of P-gp efflux transporter present in gastrointestinal wall. However, the most enhancer effect of piperine has been studied in vivo for improvement of oral bioavailability, there are limited in vitro studies on the co-treatment of curcumin with piperine for cytoprotective effect or cytotoxicity. The uptake of curcumin was evaluated with curcumin-piperine mixture emulsion in Caco-2 cell cultures as a model for intestinal uptake. The extent of curcumin uptake was improved markedly by piperine addition. The combination effect of curcumin and piperine was studied on human osteogenic sarcoma cells. Curcumin combined with piperine suppressed the osteoclastogenesis in vitro without causing any cytotoxic effects in periodontal ligament cells. Our study showed that piperine alone significantly reduced genotoxicity induced by IR in lymphocytes at concentration 2.5 µg/ml (8.7 µM) that was higher potent than CUR at concentration 25 µg/ml (68 µM). Piperine exhibited 1.3-fold decrease in the frequency of micronuclei as compared to CUR while molar concentration of piperine was 8-fold-lower than CUR. In this study, piperine was used at concentration 2.5 µg/ml which was
lower than other reported protective effects of pipeine in vitro \textsuperscript{26-27}. At first time, this study showed that piperine exhibited radioprotective effect in vitro on normal cells and more potent than CUR. Recently the comparative efficacy of piperine and curcumin in deltamethrin (DLM; a potent immunotoxicant) induced splenic apoptosis and altered immune functions was evaluated. This study strongly demonstrated that piperine displayed the more anti-oxidative, anti-apoptotic and chemo-protective properties in the DLM induced splenic apoptosis as compared to curcumin \textsuperscript{28}. Other studies have been shown the protective effect of piperine against cellular toxicity induced by oxidative stress in cellular and animal model. The mechanisms of protective effect of piperine are antioxidant, reduction the intracellular ROS level, reduction the levels of pro-inflammatory mediators and anti-apoptotic \textsuperscript{29-30}. Piperine has synergistic effect with CUR in reduction of micronuclei frequency in lymphocytes at low concentration of CUR (5 µg/ml). In this study, co-treatment CUR (5 µg/ml) with piperine (2.5 µg/ml) showed highest radioprotective effect against genotoxicity induced by IR on human lymphocytes, while it was not observed any additive protective effects with CUR at concentrations 10 and 25 µg/ml with piperine. It is interesting that addition of piperine to CUR at concentrations 10 and 25 µg/ml resulted in reducing in protective efficacy as compared to CUR alone at these concentrations. In other side, CUR alone at concentrations 10 and 25 µg/ml is more potent than addition of piperine to CUR (10 and 25 µg/ml) for its radioprotection. It is clear that the synergistic effect of CUR and piperine is concentration dependent and diminishing radioprotective effect was observed with increasing concentration of CUR with piperine. It was observed an increased genotoxicity in co-treatment of CUR and piperine at concentration 25 µg/ml and 2.5 µg/ml, respectively, on human normal lymphocytes. The exact mechanism of the cellular toxicity of piperine and CUR at high concentrations is unclear and future studies are needed for finding the exact mechanism.
Conclusion

In this study, piperine exhibited potential radioprotective effect at low concentration 2.5 µg/ml that was more potent than curcumin at concentration up to 25 µg/ml. The addition of piperine to curcumin at low concentration 5 µg/ml exhibited synergistic effect as compare to curcumin alone in radioprotective effect while additional protection was not observed at higher concentrations of curcumin with piperine.

Acknowledgments

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Conflict of Interest

No potential conflict of interest relevant to this article was reported.

References


**Table 1.** The frequency of micronuclei induced *in vitro* by 1.5 Gy X-ray radiation (IR) in cultured blood lymphocytes at different concentrations of curcumin (CUR) and/or piperine (P) 
(N = 4)"
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<th>II</th>
<th>III</th>
<th>IV</th>
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<td>CUR25+IR</td>
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</table>

1000 binucleated lymphocyte were examined in each sample, and 4000 binucleated lymphocyte from four volunteers in each group.

* $P < 0.001$ compared to control, **$P < 0.001$ compared to IR, ##$p<0.01$ compared to control group, † $P < 0.01$ compared to CUR5+IR, CUR10+IR, CUR25+IR, ‡ Non significant compared to control, § $P < 0.05$ compared to control.
C, control; IR, ionizing radiation; CUR5, curcumin 5 µg/ml; CUR10, curcumin 10 µg/ml; CUR25, curcumin 25 µg/ml; P, piperine 2.5 µg/ml.
Figure 1: A typical binucleated lymphocyte with micronucleus in our study
Figure 2: The effect of curcumin (CUR) and piperine (P) on frequency of micronuclei induced by 1.5 Gy X-ray radiation (IR) in cultured blood lymphocytes (N = 4)