The Influence of Piperine on the Radioprotective Effect of Curcumin in Irradiated Human Lymphocytes

Piperinin Işınlanmış İnsan Lenfositlerinde Kurkuminin Radyoprotektif Etkilerine Etkisi

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ABSTRACT

Objectives: Ionizing radiation (IR) induces DNA damage in normal cells, leading to genotoxicity. The radioprotective effects of co-treatment with curcumin and piperine were investigated against genotoxicity induced by IR in human normal lymphocytes.

Materials 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 a dose 1.5 Gy. The radioprotective effects of curcumin and piperine were assessed by micronucleus (MN) assay.

Results: 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 with combination of piperine and curcumin at the low concentration of 5 μg/mL, while this synergistic effect was not observed with curcumin at the higher concentrations of 10 and 25 μg/mL.

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

Key words: Curcumin, piperine, radioprotective genotoxicity, ionizing radiation

ÖZ

Amaç: İyonize radyasyon (IR) normal hücrelerde DNA hasarına neden olarak genotoksisiteye neden olur. Curcumin ve piperin ile ortak tedavinin insan normal lenfositlerinde iyonlaştırıcı radyasyonun neden olduğu genotoksisiteye karşı koruyucu etkileri araştırıldı.

Gereç ve Yöntemler: İnsan kan numuneleri, farklı konsantrasyonlarda (5, 10 ve 25 μg/mL) kurkumin ve/veya (2.5 μg/mL) piperin içerisinde ile ön muamele edildi ve daha sonra 1.5 Gy'lik bir dozda IR'ye maruz bırakıldı. Curcumin ve piperinin radyo-koruyucu etkileri mikronükleus (MN) testi ile değerlendirildi.

Bulgular: Kurkumin ve piperin, lenfositlerde IR tarafından indüklenen MN yüzdesini azaltmıştır. Sadece piperin, tüm konsantrasyonlarda tek başına uygulanan kurkumin ile karşılaştırıldığında, IR tarafından indüklenen genotoksisiteyi önemli ölçüde azaltmıştır. Düşük konsantrasyonda 5 μg/mL'de piperin ve kurkumin kombinasyonu ile ilave bir radyo-koruyucu etki gözlenirken, bu sinerjistik etki 10 ila 25 μg/mL'lik yüksek konsantrasyonlarda kurkuminle gözlenmedi.

Sonuç: Piperin, kurkuminle karşılaştırıldığında düşük konsantrasyonda güçlü bir radyo-koruyucu etkiye sahiptir. Bununla birlikte, düşük konsantrasyonda piperin ve kurkumin ile birlikte yapılan muamele ile ilave bir radyo-koruyucu etki gözlenirken, piperin yüksek konsantrasyonda kurkumin ile birlikte muamele edildiğinde normal lenfositlerde MN oranını artırmıştır.

Anahtar kelimeler: Kurkumin, piperin, radyo-koruyucu genotoksisite, iyonize radyasyon

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 cells. These toxic substances react with critical macromolecules such as DNA, resulting in genotoxicity and cell death. While IR is focused on cancerous cells, unwanted exposure to normal cells results in damage to normal tissue. The side effects induced by IR limit the use of radiotherapy in patients. Radioprotective agents protect normal cells against genotoxicity and death induced by IR.[1,2] Several protection mechanisms are proposed for radioprotective agents such as free radical scavenging and increasing endogenous cellular antioxidants enzymes.[2] Curcumin is a natural component that is prepared from *Curcuma longa* and widely used as an additive for flavoring in foods. This compound has several beneficial biological properties such as antioxidant, anti-inflammatory, and anticancer.[3,4] Curcumin protects cells from genotoxicity and death induced by IR.[5,6] Poor bioavailability in oral consumption is the main disadvantage of curcumin for clinical application.[7] It is interesting that some natural compounds act as an enhancer of curcumin through oral absorption. Piperine is a natural product prepared from black pepper (*Piper nigrum* L.). This natural product is consumed with *C. longa* as a spice in food. Piperine is used as an anticancer agent as well as a natural bioenhancer for curcumin.[8] Piperine enhances the protective effects of curcumin against oxidative stress-related diseases in animal models.[9,10] Moreover, synergistic effects of curcumin and piperine were observed in the suppression of tumor proliferation in animals.[11,12] With respect to the beneficial effects of piperine and curcumin on oxidative stress and prevention of cancer, the aim of the present study was to investigate the influence of piperine on the radioprotective effect of curcumin against genotoxicity induced by IR on normal human lymphocytes.

MATERIALS 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 (USA). Cytochalasin-B was purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Giemsa stain, methanol, and acetic acid were obtained from Merck (Germany).

Blood treatment

After obtaining permission from the research and ethical committees of Mazandaran University of Medical Sciences, this study was performed. Four healthy, nonsmoking male volunteers, aged from 22 to 28 years were enrolled. Twelve milliliters of whole blood was collected in heparinized tubes and divided among centrifuge tubes with 0.9 mL in each. Blood samples were pretreated with 100 μL of solution of curcumin at a concentration of 5, 10, or 25 μg/mL and/or piperine (2.5 μg/mL). These samples were incubated for 3 h at 37°C. Curcumin

(CUR) and piperine (P) were dissolved in DMSO and diluted in RPMI cultural medium. The 12 samples groups were as follows: 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.[5,13] Piperine concentration was selected based on previous studies that showed P has a IC_{50} of 61 μg/mL on the HeLa cell line[14] and it did not exhibit any genotoxicity or cellular toxicity up to 60 μM (17 μg/mL).[15] Control samples were treated with diluted DMSO in RPMI at the same concentration as the other curcumin and/or piperine samples.

Ionizing radiation and micronucleus test

Whole blood samples in microtubes were kept on a plastic box containing water as a phantom and then were irradiated with a 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 as controls (nonirradiated 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 and 100 μL of 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 and resuspended in cold 0.75 M potassium chloride. The cells were immediately fixed in a fixative solution 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 1000 binucleate cells (in the duplicate cultures) were examined to record the frequency of micronuclei-containing cells. All slides were evaluated by an expert 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 the 12 treated groups in this study. The criteria for scoring micronuclei were a diameter between 1/16 and 1/3 of the main nuclei, nonrefractile, not linked to the main nuclei, and not overlapping the main nuclei.[16]

Statistical analysis

The data values are presented as mean ± standard deviation. The statistical analysis was performed using one-way ANOVA, as well as *post hoc* Tukey multiple comparison tests. A p value <0.05 was considered significant and highly significant (Prism 7 Software, 2016, USA).

RESULTS

A typical binucleated lymphocyte with a micronucleus is shown in Figure 1. The mean percentage of micronuclei in the irradiated samples was 8.57±0.09, while it was 0.71±0.06 in

the nonirradiated control samples. It showed a statistically significant increase (12-fold rise) in the frequency of micronuclei in irradiated samples at a dose of 1.5 Gy (Table 1, Figure 2) (p<0.001). In irradiated samples with CUR pretreatment, the frequency of micronuclei at the concentrations of 5, 10, or 25 µg/mL was 6.10±0.14%, 4.47±0.09%, and 4.35±0.19% (Table 1). The data demonstrate that samples pretreated with CUR at concentrations of 5, 10, or 25 µg/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 µg/mL, respectively, as compared to just irradiated samples (Table 1). The maximum protection of lymphocytes was observed with CUR treatment at a concentration of 25 µg/mL. A dose-manner protective effect was observed with CUR at concentrations of 5, 10, and 25 µg/mL (p<0.01). However, the nonirradiated sample with CUR treatment at a concentration of 25 µg/mL did not show any increased genotoxicity as compared to the control group.

In irradiated samples with CUR+P pretreatment, the frequency of micronuclei at the concentrations of 5 µg/mL CUR+2.5 µg/mL (P)+IR, 10 µg/mL CUR+2.5 µg/mL (P)+IR, and 25 µg/mL

Figure 1. A typical binucleated lymphocyte with a 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)

CUR+2.5 µg/mL (P)+IR was 3.07±0.24%, 4.87±0.26%, and 5.25±0.29%, respectively (Table 1). The data demonstrate that pretreated samples with CUR (5 µg/mL) and P (2.5 µg/mL) exhibited significant decreases in the frequency of micronuclei as compared to irradiated samples with CUR alone at all concentrations (5, 10 and 25 µg/mL). It is interesting to see 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 compared to irradiation alone. The frequency of micronucleus lymphocytes with P at a concentration of 2.5 µg/mL was insignificant as compared to control samples, 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 samples (Table 1, Figure 2).

DISCUSSION

Curcumin, a natural product, is widely used in food and drug compositions and has several biological and pharmacological properties. Curcumin exhibits anticancer, anti-inflammatory, and antioxidant effects.[3,4] Curcumin scavenges free radicals and ROS[17] generated by toxic substances such as IR. The anti-inflammatory effect was reported for curcumin through diminishing cytokines and interleukins involved in the inflammation process.[18] Oxidative stress and inflammation are two suggested main mechanisms involved in cellular toxicity induced by IR. Curcumin acts as a radioprotective agent through
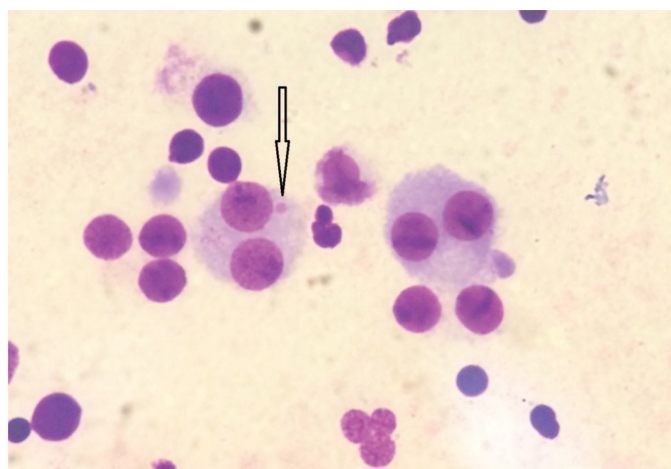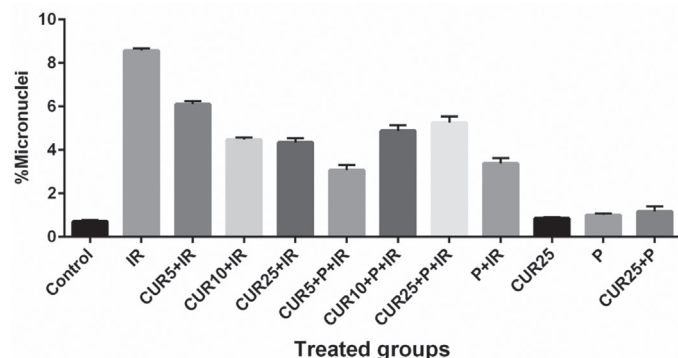
Table 1. The frequency of micronuclei induced *in vitro* by 1.5 Gy X-ray radiation (IR) in cultured blood lymphocytes at different concentrations of curcumin and/or piperine (P) (n=4)[a]

Volunteer treated group	% Micronuclei in binucleated lymphocytes				
	I	II	III	V	Mean ± standard deviation
Control	0.8	0.66	0.7	0.7	0.71±0.06
IR	8.6	8.5	8.7	8.5	8.57±0.09[b]
CUR5+IR	6	6.1	6.3	6	6.10±0.14[c]
CUR10+IR	4.6	4.5	4.4	4.4	4.47±0.09[c]
CUR25+IR	4.6	4.4	4.2	4.2	4.35±0.19[c]
CUR5+P+IR	2.9	3.1	2.9	3.4	3.07±0.24[c,d]
CUR10+P+IR	5	5.1	4.5	4.9	4.87±0.26[c]
CUR25+P+IR	5	5.5	5.5	5	5.25±0.29[c]
P+IR	3.5	3.5	3	3.5	3.37±0.25[c]
CUR25	0.8	0.9	0.8	0.9	0.85±0.06[e]
P	1	1.1	0.9	1	1.00±0.08[e]
CUR25+P	1.00	1.5	1.2	1	1.17±0.24[f]

[a]1000 binucleated lymphocytes were examined in each sample, and 4000 binucleated lymphocytes from four volunteers in each group, [b]p<0.001 compared to control, [c]p<0.001 compared to IR, ##p<0.01 compared to control group, [d]p<0.01 compared to CUR5+IR, CUR10+IR, CUR25+IR, [e]Nonsignificant compared to control, [f]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

the two mentioned mechanisms. Recently we showed that curcumin had a protective effect against genotoxicity induced by radioactive iodine in human lymphocytes.[5] Curcumin could selectively sensitize thyroid cancer cells to death induced by radioactive iodine without any toxicity on nonmalignant fibroblast cells.[13] In the present study, we showed that curcumin significantly protected human healthy lymphocytes from genotoxicity induced by external IR. These results showed curcumin has a radioprotective effect on normal cells and a radiosensitizing effect on cancer cells, and so is promising for use as a natural agent in cancer therapy.

The highest radioprotection of lymphocytes with curcumin alone treatment was observed at a concentration of 25 µg/mL. Although this maximum protection is interesting, this concentration should be achieved *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. The poor oral bioavailability of curcumin is due to its limited intestinal uptake and rapid metabolism and this is the biggest limitation of this natural product for human usage.[7] Several strategies have been applied for enhancement of the oral bioavailability of curcumin such as improvement of its formulation[19,20] and bioavailability enhancement.[21] Piperine, as a major plant alkaloid, is widely used as a condiment and flavoring agent for many types of dishes. Piperine acts as an enhancer of the bioavailability and pharmacological activity of curcumin.[11,22] There are two suggested mechanisms for piperine as a bioenhancer: promoting rapid absorption of drugs and nutrients and inhibiting enzymes involved in the biotransformation of drugs. Piperine is a potent inhibitor of the P-gp efflux transporter present in the gastrointestinal wall.[21,23] Although the enhancing effect of piperine has been extensively 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.[24] The combined effect of curcumin and piperine was studied on human osteogenic sarcoma cells. Curcumin combined with piperine suppressed osteoclastogenesis *in vitro* without causing any cytotoxic effects in periodontal ligament cells.[25] Our study showed that piperine alone significantly reduced genotoxicity induced by IR in lymphocytes at a concentration of 2.5 µg/mL (8.7 µM) and was more potent than CUR at a concentration of 25 µg/mL (68 µM). Piperine exhibited a 1.3-fold decrease in the frequency of micronuclei as compared to CUR, while the molar concentration of piperine was 8-fold lower than that of CUR. In the present study, piperine was used at a concentration of 2.5 µg/mL which was lower than that in other reports of the protective effects of piperine *in vitro*.[26,27] For the first time, the present study showed that piperine exhibited a radioprotective effect *in vitro* on normal cells that was more potent than the effect of CUR. Recently the comparative efficacy of piperine and curcumin in deltamethrin (DLM) (DLM; a potent immunotoxicant)-induced

splenic apoptosis and altered immune functions was evaluated. That study strongly demonstrated that piperine displayed more anti-oxidative, anti-apoptotic, and chemoprotective properties in the DLM-induced splenic apoptosis as compared to curcumin.[28] Other studies have shown the protective effect of piperine against cellular toxicity induced by oxidative stress in cellular and animal models. The mechanisms of the protective effect of piperine are antioxidant, reduction of intracellular ROS level, reduction of levels of pro-inflammatory mediators, and anti-apoptotic.[29,30] Piperine has a synergistic effect with CUR in reduction of micronucleus frequency in lymphocytes at a low concentration of CUR (5 µg/mL). In the present study, co-treatment with CUR (5 µg/mL) and piperine (2.5 µg/mL) showed the highest radioprotective effect against genotoxicity induced by IR on human lymphocytes, while no additive protective effects were observed with CUR at concentrations of 10 and 25 µg/mL with piperine. It is interesting that addition of piperine to CUR at concentrations of 10 and 25 µg/mL resulted in a reduction in protective efficacy as compared to CUR alone at these concentrations. On the other hand, CUR alone at concentrations of 10 and 25 µg/mL is more potent than addition of piperine to CUR (10 and 25 µg/mL) for radioprotection. It is clear that the synergistic effect of CUR and piperine is concentration dependent and a diminishing radioprotective effect was observed with increasing concentration of CUR with piperine. Increased genotoxicity was observed in co-treatment with CUR and piperine at concentrations of 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.

CONCLUSIONS

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

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