Protective Effect of Curcumin Against Chlormadinine Acetate Induced Genotoxic Damage In Cultured Human Peripheral Blood Lymphocytes

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Summary

Chlormadinone acetate (CMA) is a synthetic progestin. The prolonged use has been reported to be carcinogenic in various experimental animal models. In the present study the effect of CMA was studied along with curcumin at various doses in cultured human lymphocytes using cell viability, lipid peroxidation and DNA damage assay as a parameters. The treatment of curcumin results in a significant reduction of the toxic effects induced by CMA alone. The results suggest that the curcumin has potential to reduce the genotoxic effects of CMA in cultured human lymphocytes.

Keywords: Chlormadinone acetate, curcumin, DNA damage, cell viability, lipid peroxidation assay.

Introduction

Chlormadinone acetate (CMA) is a synthetic progestin used in the formulations of oral contraceptives in combination with estrogen (1). It is the derivative of 17α-hydroxyprogesterone (Pregnanes) (2). Prolonged uses of oral contraceptives have been reported to cause various types of cancer (3). It has been reported to develop mammary tumors in dogs and also increase the mammary gland hyperplasia and mammary nodules (4). It was reported to form DNA adducts in rat liver *in vitro* (5), human lymphocytes *in vitro* (6) and micronucleus in rat liver cells in *vivo* (7). Our earlier studies showed that CMA is potent to induce chromosomal aberrations and sister chromatid exchanges in cultured human peripheral blood lymphocytes (8) and mice bone marrow cells (9). Increase in the genotoxicity is associated with an increased overall risk of cancer (10-11). Natural plant products are reported to reduce the genotoxic effects of certain synthetic progestins (12-16), anticancerous drugs (17-18) and steroids (19). The reduction in the genotoxic effects reduces the possible risk of cancer formation (20). Curcumin (diferuloylmethane), the main yellow bioactive component of turmeric has been shown to have a wide spectrum of biological actions useful for humans (21). In the present study the effect of curcumin was studied on the cell viability, lipid peroxidation and DNA damage induced by chlormadinone acetate in cultured human blood lymphocytes.

Materials and methods

Chemicals: RPMI-1640, Fetal Calf Serum, Phytohaemagglutinin and antibiotic-antimycotic mixture were procured from Invitrogen, USA. Chlormadinone acetate (CAS No. 302-22-7), curcumin and 1-methyl-2-phenylindole were procured from Sigma. Acetonitrile, Methanol, HCl, Tris-Cl, EDTA, Triton X-100, Trichloroacetic acid, Diphenyl amine were procured from SRL, India. Trypan Blue was procured from Loba, India.

Human lymphocyte culture: Duplicate peripheral blood cultures of two healthy female donors were treated according to Carballo et al. (22). Briefly, heparinized blood sample (0.5 ml), was obtained from a healthy donor and was placed in a sterile culture tube containing 7 ml of RPMI 1640 medium, supplemented with fetal calf serum (1.0 ml), antibiotic antimycotic mixture (1.0 ml) and phytohaemagglutinin (0.1 ml). The culture tubes were placed in an incubator at 37^{o}C for 24 h.

Cell viability assay by Trypan blue exclusion: Cell viability assay was performed according to the method of Cook and Mitchell (23). After 24 h of the initiation of culture 30 and 40 μM of CMA treatments were given separately. About, 30 μM of CMA was also given along with 5, 10 and 15 μM of curcumin respectively. Similar treatment was given along with 40 μM of CMA. After 48 h the cells were centrifuged at 800 rpm for 10 min. The supernatant was removed and the cell suspension was used for assay. The cell suspension from each of the treatments was made to about containing ~5x10^{4} cells. The equal amount of 0.2% trypan blue (in phosphate buffer saline) was mix and allowed to incubate 1-2 min at room temperature. The samples were load on to the hemacytometer (Neubauer type- Paul-Marienseld, Germany) and the total number of cells and the number of unstained cells in five major section of the hemacytometer were counted. Percent viability was calculated by the following formula:

$$\% \text{ viability} = \frac{\text{number of unstained cells}}{\text{total number of cells}}$$

Lipid peroxidation assay: The present method of lipid peroxidation assay is based on the reaction of malonaldehyde with two molecules of 1-methyl-2-phenylindole at 45^{o}C at 586 nm (24). After 24 h of the initiation of culture the treatments of 30 and 40 μM of CMA were given separately as well as in combination with 5, 10 and 15 μM of curcumin, respectively. After 48 h of incubation, the treated blood cultures were centrifuged at 3000 g for 20 min and the supernatant was collected and the estimation was performed according to as described by Siddique et al. (25). Briefly, to a fresh tube 1.3 ml of 10mM 1-methyl-2-Phenyl lindole, dissolved in acetonitrile was taken. About 1 ml of supernatant was diluted 10 times with Tris buffer (20 mM; pH 7.4) and about 200μl of diluted supernatant was taken from each of the treated culture along with 200μl of distilled water separately and vortexed. To the tubes about 300μl of 37% of HCl was added and vortexed. The tubes were incubated at 45^{o}C for 40 min. The tubes were cooled on ice and centrifuged at 15000g for 10 min at 4^{o}C. The reding were noted at 586nm.

Quantitative assay for DNA fragmentation: The quantification of DNA fragmentation was performed according to the protocol of Burton (26). After 24 h of the incubation the treatments were given similarly as described earlier in the text. After the incubation of 48 h, the cell suspension containing 1-10x10^{6} cells in a 1 ml volume was prepared for each of the treated culture. About 0.8 ml of cell suspension was transformed to micro centrifuge and 0.7 ml of ice cold lysis buffer (5 mM Tris Cl, pH 8.2; 20 mM EDTA / 0.5% v/v Triton X-100)

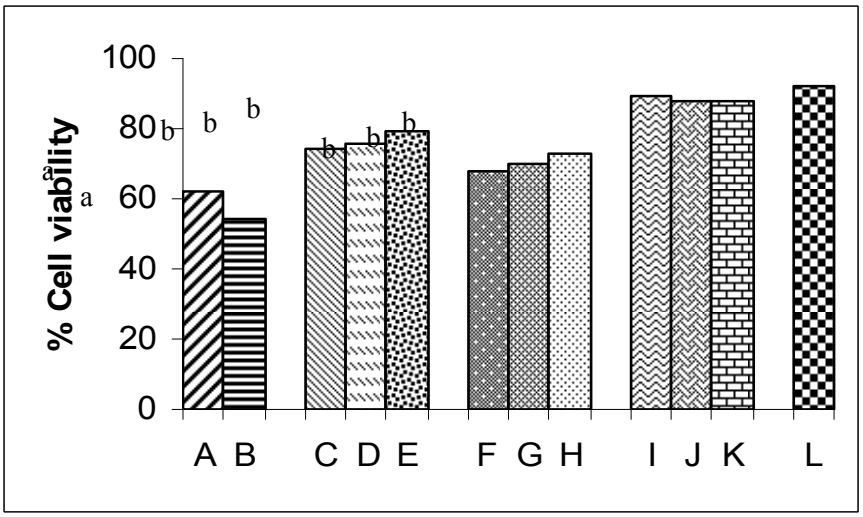
was added. The tubes were vortexed and allowed to lysed for 30 min at 4°C. The tubes were centrifuged for 15 min at 15000 g (4°C) and the supernatant was transferred to labelled conical glass tube. About 0.65 ml of 5% Trichloroacetic acid (TCA) was added to the pellet in microcentrifuge tube and 1.5 ml of 10% TCA to the sample in labelled glass tube. The samples were precipitated overnight at 4°C. The tubes were centrifuged for 10 min at 2500 g at room temperature and the supernatant was removed and about 0.65 ml of 5% TCA to the pellet. The tubes were boiled for 15 min in 100°C water both. After cooling down to room temperature, the samples were centrifuged at 2500 g for 5 min. About, 0.5 ml of each supernatant (from both glass and micro centrifuge tubes) was transferred to the labelled glass tubes. About, 1 ml of diphenyl amine reagent was added to each tube and was incubated for 4h at 37°C. Finally the absorbances were noted at 600 nm in spectrophotometer and the results were expressed as the percentage of DNA fragmented:

$$\% \text{ fragmented DNA} = \frac{\text{absorbance of the supernatant}}{\text{absorbance of supernatant + pellet}} \text{ X } 100$$

Statistical analysis: Statistical analysis was performed by using statistical soft Inc., USA (Chi square).

Results

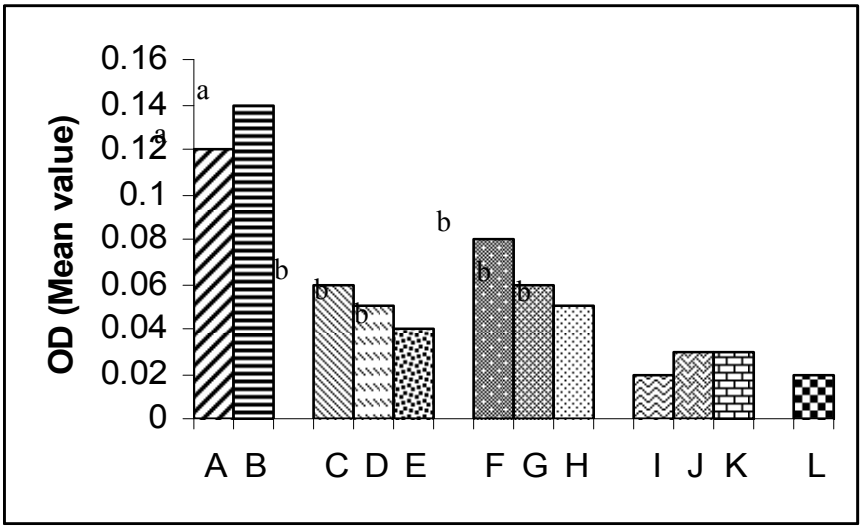
Cell viability assay: The cell viability assay showed a significant decrease at 30 μM (62%) and 40 μM of CMA, as compared to untreated (92%). The treatment of 30 μM of CMA along with 5, 10 and 15 μM of curcumin results in a dose dependent significant increase in cell viability i.e. 74% and 79% respectively (Fig. 1). Similar results were obtained when the treatment of 40 μM of CMA was given along with 5, 10 and 15 μM of curcumin. (Fig. 1).



A = CMA (30 μM); B = CMA (40 μM); C = CMA (30 μM) + Curcumin (5 μM);
D = CMA (30 μM) + Curcumin (10 μM); E = CMA (30 μM) + Curcumin (15 μM);
F = CMA (40 μM) + Curcumin (5 μM); G = CMA (40 μM) + Curcumin (10 μM);
H = CMA (40 μM) + Curcumin (15 μM); I = Curcumin (5 μM); J = Curcumin (10 μM); K = Curcumin (15 μM); L = Untreated

Fig. 1 Effect on cell viability after the treatment of chlormadinone acetate (CMA) and Curcumin. ([a]P<0.05 vs. untreated; [b]P<0.05 vs. CMA treatment)

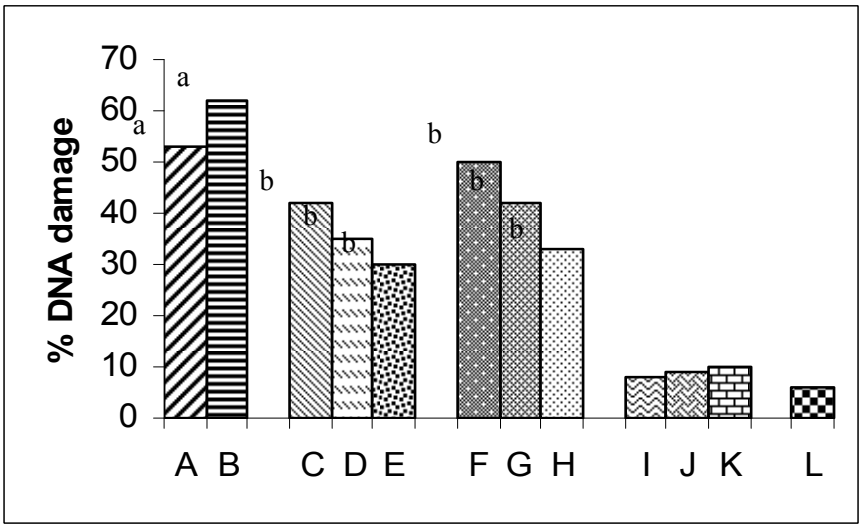
Lipid peroxidation assay: The treatments of 30 and 40 μM of CMA were associated with a mean absorbance value of 0.12 and 0.14 respectively (Fig. 2). The treatment of 30 μM of CMA along with 5, 10 and 15 μM of curcumin was associated with mean absorbance value of 0.06, 0.05 and 0.04, respectively (Fig. 2). Similar, results were obtained when the treatment of 40 μM of CMA was given along with 5, 10 and 15 μM of curcumin. (Fig. 2).



A = CMA (30 μM); B = CMA (40 μM); C = CMA (30 μM) + Curcumin (5 μM);
D = CMA (30 μM) + Curcumin (10 μM); E = CMA (30 μM) + Curcumin (15 μM);
F = CMA (40 μM) + Curcumin (5 μM); G = CMA (40 μM) + Curcumin (10 μM);
H = CMA (40 μM) + Curcumin (15 μM); I = Curcumin (5 μM); J = Curcumin (10 μM); K = Curcumin (15 μM); L = Untreated

Fig. 2 Effect on the absorbance after treatment of chlormadinone acetate (CMA) and Curcumin. ([a]P<0.05 vs. untreated; [b]P<0.05 vs. CMA treatment).

DNA Damage assay: The treatments of 30 and 40 μM of CMA were associated with 53% and 62% of DNA damage respectively (Fig. 3). The treatment of 30 μM of CMA along with 5, 10 and 15 μM of curcumin results in a significant decrease in DNA damage i.e. 42%, 35% and 30% respectively (Fig. 3). Similar results were obtained when the treatment of 40 μM of CMA, was given along with 5, 10 and 15 μM of curcumin (Fig. 3).



A = CMA (30 μM); B = CMA (40 μM); C = CMA (30 μM) + Curcumin (5 μM);
D = CMA (30 μM) + Curcumin (10 μM); E = CMA (30 μM) + Curcumin (15 μM);
F = CMA (40 μM) + Curcumin (5 μM); G = CMA (40 μM) + Curcumin (10 μM);
H = CMA (40 μM) + Curcumin (15 μM); I = Curcumin (5 μM); J = Curcumin (10 μM); K = Curcumin (15 μM); L = Untreated

Fig. 3 DNA damage after the treatment of chlormadinone acetate (CMA) and Curcumin. ($^{a}P<0.05$ vs. untreated; $^{b}P<0.05$ vs. CMA treatment)

Discussion

The results of the present study reveal that curcumin is potent in reducing the damage induced by chlormadinone acetate (CMA) in cultured human lymphocytes. In earlier study with CMA, it was found to be genotoxic by generating reactive oxygen species (ROS) (8). These ROS can induce lipid peroxidation and peroxidative fatty acid fragments and further these radicals can vice-verse lead to the formation of ROS (24). Curcumin is a well known antioxidant and its mechanism is attributed to its unique conjugated structure that includes two methoxylated phenols (28). Curcumin inhibits the generation of ROS that are responsible for the DNA and membrane damage (29). ROS i.e. superoxide, hydrogen peroxide, hydroxyl radicals are responsible for the oxidative damage of the DNA (30). The International Agency of Research on Cancer (IARC) mainly on the basis of epidemiological studies classifies steroidal estrogens and estrogen-progestin combination among agents carcinogenic to humans (Group 1) (31). At higher doses estrogen-progestins are reported to induce liver cancer in humans (31). Natural plant products are reported to protect against xenobiotics either by inducing detoxifying enzymes or by inhibiting oxidative enzymes. The treatment of curcumin along with the chlormadinone acetate reduced the toxic effects of CMA, and this is due to the possible scavenging of free radicals in the system.

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