Repressive effect of curcumin against 2-amino-3-methylimidazo [4, 5-f] quinoline induced hepato- and immunotoxicity in mice

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The mutagenic heterocyclic amine, 2-amino-3-methylimidazo [4,5-f] quinoline (IQ), produced during cooking of protein rich foods is known to affect liver. Curcumin, a component of turmeric Curcuma longa L., is a safe agent characterized with powerful therapeutic potential. In this study, we explored the possible protective role of curcumin against IQ induced liver damages on immunological, serological, inflammatory and apoptotic levels. Mice were administered IQ alone or IQ and curcumin together for 1, 15 and 30 days. Our results revealed that IQ exerts time dependent down regulation of p53 and upregulation of bcl2 in mice liver, no significant changes were observed in bax and cleaved caspase 3 levels. Curcumin co-administration increased the levels of p53, Bax and cleaved caspase 3 and reduced the level of Bcl2 compared with their levels in IQ treated mice. In addition, it was found that curcumin co-administration protects against IQ-induced modulations of some selected serum proteins, leucocytes percentage and cytokines level. Also, the curcumin protected liver cells from IQ-induced inflammation as detected by mRNA level of the inflammatory marker Ccl5. We conclude that the dietary curcumin is a powerful repressive natural agent against IQ-induced liver injury.

Keywords: Apoptosis, Curcuma longa, Cytokines, Heterocyclic aromatic amines, Inflammation, IQ mutagenicity, Liver injury, p53, Spice, Tumor suppressor protein, Turmeric

Harmful chemicals, such as heterocyclic aromatic amines (HAA) are known to be formed during food preparations, particularly cooking foods containing high creatine, free amino acid and sugars¹. The most harmful component of HAA found in the human diet is 2-amino-3- methylimidazo [4,5-f] quinoline (IQ)². IQ is known to induce high mutagenicity and tumours in liver, colon, and lungs of rats³ and hepatocellular carcinomas in cynomolgus monkeys⁴. Pathological conditions induced by IQ are due to its ability to generate abnormally high intracellular ROS levels⁵,⁶. Furthermore, it has been reported that IQ mutagenicity is according to formation of high levels of DNA adducts⁷,⁸. The tumour suppressor protein p53 is a 53-kDa nuclear protein mediates cell cycle arrest or apoptosis depends on the cellular context and specific stress stimuli⁹. Inactivation of the p53 pathway is observed in most human cancers, which induced mutations in p53¹⁰. IQ association with p53 gene mutations suggests that these mutations might be induced by the formation of DNA adducts of IQ in the p53 gene¹¹. Also, IQ promotes hepatocarcinogenesis through activation of transforming growth factor and wnt/b catenin signaling pathway¹².

Curcumin, the yellow active ingredient of turmeric is a potent antioxidant and anti-inflammatory agent with hepatoprotective, anticarcinogenic, antiapoptotic, antimicrobial, nematicidal, antiepileptogenic and neuroprotective properties¹³-¹⁶. Curcumin enhanced apoptotic cell death in different cancer cell lines including multi drug resistant cells without cytotoxic effects on healthy cells¹⁷. Further, it exhibits broad spectrum modulatory actions on cells including interaction with blood proteins¹⁸, modulation of the activity of leucocytes¹⁹ and altering the function of different apoptotic proteins²⁰. The action of curcumin against IQ induced genotoxicity and ROS generation has been addressed earlier²¹,²². Here, we investigated whether the dietary curcumin is able to antagonize the damaging effect of IQ on the immunological, serological, inflammatory and apoptotic levels.

Materials and Methods

Materials
IQ (J & K Scientific LTD., China), tween-80, tween 20, EDTA (ethylene diamine tetra-acetic acid), EGTA (ethylene glycol tetra-acetic acid), sodium
dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) chemicals, nitrocellulose membrane, protease inhibitor cocktail, mouse anti-p53 IgG, mouse anti bcl2 IgG and mouse anti bax IgG were purchased from Sigma (USA). SuperSignal West Pico chemiluminescent substrate was from Pierce Biotechnology (USA). Mouse anticaspase 3 IgG, goat antimouse IgG-HRP, goat anti-β actin IgG and mouse anti-goat IgG-HRP are from Santa Cruz Biotechnology (USA). RNeasy Mini Kit was from Invitrogen (USA). Protein assay kit was from Bio-Rad (Australia). All other chemicals of good quality, if not mentioned, were obtained from local commercial sources.

Animals and experimental design

Sixty five adult male Swiss albino mice Mus musculus, weighing 25-30 g used in this experiment were maintained in Assiut University Joint Animal Breeding Unit, College of medicine, Assiut, Egypt. All experimental procedures were conducted in strict compliance with ethics prepared by INSA, (WHO/UNESCO), NIH and PHS. The temperature was maintained at 23±2°C and Ambient light was maintained on a 12-h light: dark schedule. Animals were allowed to adapt to the laboratory housing conditions for at least 1 wk before the experiment started and continued for 30 days. Animals were randomly divided into nine groups of 5-10 animals each. Group I (control 1) was kept untreated, group II (control 2), treated with corn oil by oral gavage for 30 days, group III (control 3) with 0.5% tween-80 for 30 days. Groups IV–VI were treated orally with IQ (50 mg/kg body wt., dissolved in corn oil) for 1, 15 and 30 days, respectively whereas Groups VII–IX were had IQ (50 mg/kg body wt.) and curcumin (0.5 mg/kg body wt., dissolved in 0.5% tween 80) for 1, 15 and 30 days, respectively. Curcumin was given 30 min after the administration of IQ. Turmeric rhizome powder freshly prepared was dissolved in 100 mL absolute ethanol and filtered; the filtrate was boiled indirectly in water bath until evaporation. Ten grams of curcumin extract were dissolved in 20 mL of 0.5 % tween 80, filtered and used as stock solution for mice administration.

Western blot analysis

For western blotting, liver were removed and homogenized in RIPA Lysis buffer (Nonidet-P40 1%, TritonX-100 1%, Na dioxycholate 0.5%, NaCl 150 mM, EDTA 5 mM, EGTA 10 mM, Tris-HCl 50 mM., PMSF 1 mM, Leupeptin/pepstatin mix 1% and protease inhibitor cocktail. Tissue samples were lysed on ice by grinding and briefly sonication and then protein concentration was estimated. Twenty micrograms of total protein were resolved by 10% SDS-PAGE and transferred onto nitrocellulose membrane. Membranes were then blocked with 5 % skim milk in TBS with 0.05% Tween 20. Incubation with primary antibodies (overnight, 4°C) and HRP-conjugated secondary antibodies (1 h, 24°C) in blocking solution was carried out according to manufacturer's recommendations. Immunoreactive bands were visualized by chemiluminescent substrate kit. Anti-β actin goat polyclonal antibody was used for equal loading confirmation. The optical density of bands was estimated using Image J software referenced to the corresponding β actin band.

Estimation of serum protein fractions

Blood was collected from jugular vein during mice sacrifice in non-heparinized sterile tubes. Serum samples were obtained from clotted blood after centrifugation at 5000 rpm for 5 min. Total serum protein concentration was estimated. Twenty micrograms of serum proteins were subjected to 10% SDS-PAGE. The gel was stain with coomassie blue for 1/2 h and distaining with 40 % methanol and 10 % acetic acid. To confirm the identity of each serum protein, the mobility of each protein was calculated and blotted against molecular weight standards expressed on a semi-logarithmic scale. The optical density of each band was estimated using Image J software.

Differential leucocytes count and cytokines quantification

Blood serum was collected as previously mentioned and differential white blood cells were done as described23. Determination of tumor necrosis factors- alpha and interleukin-1 levels was carried out by Enzyme- Linked Immuno Sorbent Assay (ELISA) using quantikine (USA) mouse TNF-α & IL-1 immunoassays kits according to the manufacturer manual.

Quantitative Real time RT-PCR

Total RNA was extracted from liver tissue samples using RNeasy Mini Kit (Invitrogen) according to the manufacturer protocol. Reverse transcription was done using (SMART_PCR cDNA synthesis kit, Clontech Inc., Palo Alto, CA). Quantitative RT-PCR was performed in duplicate in 25 μL mixture
containing 1 μL template cDNA, SYBR green PCR Master mix (Applied Biosystems), 10 pmol of Primers for Ccl5 forward, TGCCCACGTCAA-GGATATTT and reverse, TTTCTGGTGGTG-CAACACAT and for GAPDH forward, AACTTTGGCATTGTGGAAGG and reverse, GTCTTCTGGTG- GCCAGTGAT. Reactions were run in I Cycler iQ (Bio-Rad). The results were normalized to GAPDH mRNA level.

**Statistical analysis**

All data were presented as mean ± SD. Statistical analyses were performed by using an ANOVA with treatment and time of study included as factors. *P* value <0.05 was considered significant.

**Results**

**Curcumin upregulated p53 in IQ treated mice**

Treatment with IQ for 1, 15 and 30 days decreased the level of p53 vs. those of control (Fig. 1A) as calculated from densitometric measurement (Fig. 1B). One day of IQ treatment was enough for reducing p53 to almost half the level of control. IQ and curcumin coadministration upregulated the level of p53 (Fig. 1A and B). Though the effect of curcumin for one day was not clear, 15 and 30 days exhibited almost 1.3 and 2.2 fold increase in p53 level, respectively compared to the control.

**Curcumin induced apoptosis in the liver of IQ treated mice**

The antiapoptotic protein; bcl2 was found to be upregulated by IQ treatment whereas, no important change in the level of the pro-apoptotic protein; bax except after 30 days of IQ treatment (Fig. 2A). Bcl2 level increased almost two folds at one and 15 days of IQ treatment and almost 7 folds at 30 days compared to the control (Fig. 2B, upper panel). Curcumin treatment induced sharp decrease in bcl2 level at all-time points (Fig. 2B, upper panel). In contrast, curcumin increased the level of bax by 10, 15 and 19 folds compared with control (Fig. 2B, middle panel).

Further, we determined the bcl2 /bax ratio in IQ treatment and in IQ and curcumin co-treatment. As the antiapoptotic character of IQ is enforced by its upregulation of bcl2/bax ratio, here, the pro-apoptotic effect of curcumin was clearly visible from its downregulation of this ratio (Fig. 2C). For confirmation of the role of curcumin as potent apoptotic agent, the activation of caspase 3 was studied. IQ treatment had no effect on caspase 3 cleavage state compared with control (Fig. 2A). But curcumin co-treatment resulted in high cleaved caspase 3 level almost 2.5 folds higher than control at 15 and 30 days (Fig. 2B, lower panel).

**The protective role of curcumin against IQ-modulation of serum proteins levels**

The effect of IQ administration and IQ and curcumin co-administration on the levels of some selected serum proteins namely; albumin, transferrin, anti-trypsin and immunoglobulin G heavy chain (IgG HC) was demonstrated in Fig. 3. The IQ treated group of mice for 1, 15 and 30 days showed decreased albumin level by 29.3, 50.7 and 80 %, respectively. Co-administration of curcumin prevented the modulation effect of IQ on the level of both proteins. However, long IQ and curcumin co-treatment (30 days) resulted in almost 25 % reduction of transferrin level lower than control. The levels of both anti-trypsin and IgG were found to be downregulated by IQ treatment. The same protective role of curcumin on restoring the level of serum protein is also detectable in the case of anti-trypsin and IgG proteins.
The protective role of curcumin against IQ modulation of leucocytes percentage and cytokines levels.

The percentage of blood lymphocytes gradually decreased under the effect of IQ treatment. The average percentage of control untreated mice lymphocytes was 78% of the total leucocytes. This percentage decreased to 66% after one day of IQ administration and reached 30 and 15% at 15 and 30 days of treatment, respectively (Fig. 4A, upper panel). Curcumin antagonized the IQ effect, however, the percentage of lymphocytes did not restore completely to the control value by curcumin co-treatment (Fig. 4A, upper panel). IQ had the same effect on monocytes as lymphocytes. The percentage of monocytes decreased by time after IQ administration, reaching almost 1% after 30 days out of 7% of total leucocytes count in control untreated mice (Fig. 4A, lower panel). In contrast, IQ had an upregulatory effect on neutrophils percentage. Neutrophils reached 31% of total leucocytes count after 30 days of IQ administration, whereas it was almost 11% in control untreated mice (Fig. 4A,
middle panel). In all leucocytes subtypes currently studied, curcumin had an antagonistic effect against IQ modulation on leucocytes percentage. In addition, IQ has been shown to gradually decrease both IL-1 and TNF-α level over time. The reduction reached 74 and 82% for IL-1 level and 75 and 85% for TNF-α level at 15 and 30 days of IQ treatment, respectively (Fig. 4B). The antagonistic effect of curcumin against IQ induced reduction of TNF-α was clearly stronger than that of IL-1 (Fig. 4B).

The inhibitory role of curcumin against IQ induced hepatic inflammation

The mRNA of inflammatory marker C-C motif Ligand 5 (Ccl5) was quantified in liver tissues by qRT-PCR in control and in different treatments. IQ administration for different time points resulted in gradual elevation of Ccl5 mRNA compared to control untreated mice (Fig. 5). This increase reached almost 3 and 5 folds higher than the control untreated mice at 15 and 30 days of IQ administration, respectively. Inversely, curcumin co-administration resulted in significant attenuation of Ccl5 mRNA level compared to the corresponding treatment of IQ alone. This observation confirms the inhibitory characters of curcumin against IQ-induced inflammation.

Discussion

The quantum of studies carried out from time to time to understand the biological and therapeutical characters of curcumin and the development of its pharmacological derivatives reflects the importance of curcumin as a natural and safe agent for human health. IQ (2-amino-3-methylimidazo [4,5-f] quinoline) is an HAA compound, produced during high-temperature cooking of protein-rich foods. Different reports have
recorded its injury and carcinogenicity targeting of multiple organs including liver, lung and intestine in rodent. It has been reported that IQ is associated with p53 gene mutations in hepatocellular carcinomas which might be induced by the formation of DNA adducts of IQ in the p53 gene. In the present study, curcumin administration has stimulatory effect on the level of p53 in liver of IQ treated mice. Also, IQ administration significantly stimulated the levels of bcl2 and increases bcl-2/bax ratio in the liver, whereas curcumin downregulated bcl2 and decreased bcl2/bax ration. Our results have shown that curcumin administration stimulated the level of cleaved caspase-3 in liver tissue vs. those of IQ-treated mice, which is in agreement with reports that suggested the involvement of caspases in curcumin-induced apoptosis.

Determination of plasma proteins concentrations has immense value in evaluation of severity and progression of the disease. The amount of albumin synthesis in the liver is highly dependent on the nutritional state particularly the amount of protein ingested. In the current study, IQ administration significantly inhibited the level of serum albumin that ensures the use of serum albumin as a sensitive biomarker of exposure to heterocyclic aromatic amines. In the present study, we found that IQ administration significantly stimulated the level of serum transferrin. High serum level of transferrin is an indicator of the presence of chronic inflammation. Accordingly, IQ-induced liver inflammation was investigated in our study by measuring the level of mRNA expression of the inflammatory marker Ccl5. Various authors have found significant decrease or genetic deficiency of antitrypsin serum level in subjects with a range of cancers, including liver. Our study demonstrated that IQ administration also significantly inhibits the level of serum antitrypsin. Immunoglobulins are major components of the adaptive immune system. IQ administration reduced the level of IgG which is restored by curcumin co-administration, it is worthy mentioned that curcumin interacts with immunoglobulins. The effect of curcumin in blood serum proteins as obtained in the current study is also in good agreement with other such reports.

In the present study, a significant decrease in lymphocytes and monocytes percentage as well as a significant increase in the neutrophils percentage was recorded after IQ toxicity. This effect is probably due to the myelotoxic effect of IQ on bone marrow leads to bone marrow hypoplasia and apoptosis.

Similarly, in our results, curcumin administration increased the levels of IL-1 and TNF-α in IQ-treated mice. Cho et al. also observed a significant reduction of inflammatory cytokines; TNF-α, IL-1 and IL-6 in neuro-inflammation treated with curcumin. In addition, it has been shown that curcumin antagonizes inflammation by suppressing the functions of neutrophils, and by tampering with the synthesis of various eicosanoids that play a role in the development of inflammation. However, our results differ from the results obtained previously by other researchers which could be attributed to difference in drug treatment, cells or organs used in the study.

Here, the results of this study have shown the dietary curcumin to be a powerful agent against IQ-induced hepatic damage on the immunologic, apoptotic and serologic levels. Further, the curcumin also inhibited IQ-induced inflammation of liver.

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