

Curcumin Pretreatment Evokes Lympho Proliferative Responses and Macrophage Activation to Negate Chromate-Induced Immuno Suppression in Vitro

Sreepriya. M

Abstract—The effects of curcumin pretreatment to negate chromate (VI)-induced cytotoxicity and oxidative stress was evaluated in vitro on human peripheral blood lymphocytes and murine macrophages RAW 264.7. Chromate treatment at a dosage of 10µg/ml resulted in significant immunosuppression evident from the severe growth inhibition and cytotoxicity in both lymphocytes as well as macrophages which is revealed by MTT assay and LDH leakage. A decrease in the percentage phagocytosis, phagocytic index and depleted glutathione status was also observed in the macrophages subjected to chromate treatment. In addition to this, chromate treatment resulted in an increase in fragmented DNA (quantified by colorimetry) in the lymphocytes suggestive of severe cellular stress and apoptotic cell death. Curcumin pretreatment at a dose of 20µg/ml was able to protect both lymphocytes and macrophages against chromate induced toxic effects as evidenced by the increase in percentage survival and cell viability, increased percentage phagocytosis, phagocytic index and improved thiol status in the macrophages concomitant with a decrease in fragmented DNA in lymphocytes. This shows the influence of curcumin to ameliorate the altered immune responses and adverse changes induced by treatment with hexavalent chromium. The results of the study reveal the beneficial effects of curcumin supplementation to negate the toxic changes induced by chromate (VI).

Index Terms— Chromate-IV, Curcumin, Lymphocytes Macrophages, Phagocytosis.

I. INTRODUCTION

Chromium is a naturally occurring heavy metal found commonly in the environment in trivalent and hexavalent forms [1]. Hexavalent chromium is a potent occupational carcinogen [2] and is a widely used industrial chemical extensively used in paints, metal finishes, steel including stainless steel manufacturing, alloy cast irons, chrome and wood treatment. On the contrary, Chromium (III) salts such as chromium polynicotinate, chromium chloride and chromium picolinate are used as micronutrients and nutritional supplements and have been demonstrated to exhibit a significant number of health benefits in rodents and humans [3]. Dietary antioxidants are reported to be beneficial in combating the cellular stress and oxidative damage induced by many toxic compounds. Ascorbic acid, alpha

tocopherol, Vitamin A, gallic acid, ellagic acid, catechins are all examples of dietary antioxidants which have been reported to alleviate stress induced by toxicants including heavy metals like chromium[4]. Curcumin (Diferuloyl methane) is the active principle of *Curcuma longa* (Zingiberaceae) and is documented to possess several health benefits and medicinal properties. It is a well known anticancer agent [5], antioxidant [6], and anti-inflammatory agent [7]. Curcumin pretreatment was reported to prevent potassium dichromate-induced hepatotoxicity and oxidative stress in rats [8]. There are no previous reports about the effects of curcumin in influencing lymphoproliferative responses and macrophage activation during chromate (VI)-induced toxicity in vitro. Hence the current study was carried out to understand the effects of curcumin in attenuating chromate-induced damage thereby preventing cell death in human peripheral blood lymphocytes and murine macrophages cultured in vitro.

II. RESEARCH DESIGN AND METHODS

A. Isolation of Human peripheral blood lymphocytes: Fresh human peripheral blood was drawn from healthy volunteers (age group between 18-25) following the Helsinki protocol through the median cubital vein of the forearm. Lymphocytes were isolated from the blood by following the method of Boyum [9] applying the technique of density gradient centrifugation by using lymphocyte separation media (LSM 001-Hi Media, India). Lymphocyte preparations having >90% viability as assessed by trypan blue dye exclusion test was suspended in Dulbecco's Modified Eagles Medium (AL 007- Hi Media, India) supplemented with Antibiotic Antimycotic solution (A 002- Hi Media, India) and 10% fetal bovine serum (RM 1112- Hi Media, India). The cell count was adjusted to contain 5x10⁶ cells/ml and the cells were plated on to 96 well micro titer tissue culture plates (Hi Media, India) for the various assays.

A. Procurement and maintenance of RAW 264.7 cell line: The cell line was procured from National Center for Cell Science, Pune, India and cultured in DMEM/F12 medium supplemented with 10% Foetal Bovine Serum and antibiotics under standard conditions in a tissue culture flask. The cells on arriving at confluency were scraped from the substratum using cell scrappers and split at a ratio of 1:3. On arriving at confluency the cells were plated onto a 96 well micro titer tissue culture plates in

Sreepriya M, Assistant Professor, Department of Microbiology and Biotechnology, Bangalore University, Bangalore- 560 056, Karnataka, India.

- required numbers and were used for the different assays.
- B. Experimental setup: The cells were plated on to micro titer tissue culture plates. The cells were segregated into different groups viz., a) untreated control b) Chromate exposed cells (Potassium dichromate was dissolved in phosphate buffered saline and is added to the cells at a concentration of 10 μ g/ml) c) Curcumin dissolved in DMSO (final concentration < 0.1%) at a concentration of 20 μ g/ml prior to the exposure of the cells to hexavalent chromium d) cells treated only with curcumin.
- C. MTT assay: MTT assay was performed following the method of Mosman, 1983 [10]. After 72 hours of proliferation 25 μ l of MTT (5 mg/ml) in phosphate buffered saline (pH 7.4) was added to the wells. The plates were then incubated in a Carbon dioxide incubator for six hours. After six hours the formazan crystals produced were solubilized by adding 75 μ l of Dimethyl sulfoxide to each well followed by vigorous mixing. The intensity of the colour developed was read at 570nm in a micro plate reader (Bio Tek system, USA). The intensity of the colour developed is directly proportional to the number of viable cells present.
- D. LDH leakage: The activity of lactate dehydrogenase was assayed in the cell culture supernatant by using commercially available kits (Span Diagnostics, India) following the manufacturer's instructions. The leakage of LDH in the culture supernatant of the treated cells was compared with that of the untreated control cells.
- E. Determination of percentage phagocytosis and phagocytic index: Percentage phagocytosis and phagocytic index was determined as described earlier by Devi et al 2004 [11] and Tatarczuch et al 2002 [12]. Briefly, the macrophages were challenged with 5 μ l of heat-killed, opsonized yeast cells (macrophages to yeast, 1: 100 ratio) and incubated for 45 minutes. The unphagocytosed yeast cells were removed by washing the cells three times with 0.9% saline. The macrophages were then stained with Giemsa for 2 minutes and observed under light microscope and the number of macrophages that had phagocytosed the yeast cells were counted. A minimum of 400 cells were counted and the results were expressed as percent phagocytosis. Phagocytic index is the mean number of yeast cells phagocytosed or ingested per macrophage cell at the end of the treatment period.
- F. Estimation of total reduced glutathione: After treatment with curcumin and chromate the treated cells and untreated control cells were harvested and 0.5 ml of glacial acetic acid and 2.0 ml of 10% TCA was added mixed well. The mixture was then centrifuged (3000 rpm) at room temperature for 10 min to separate proteins. Supernatant was used for the estimation of total reduced glutathione by following the method of Moron et al., 1979 [13]. Briefly 0.5ml of the supernatant was mixed with 2 ml of Dithio nitro benzoic acid (DTNB) and the total volume was adjusted to 3ml with phosphate buffer.

The absorbance was read at 412nm. Results were expressed as n moles of GSH /mg protein.

- G. Estimation of fragmented DNA: This was performed following the method of Burton et al., 1956 [14]. Cells were cultured in a 6-well plate and treated with chromate (both in the presence and absence of curcumin) for 24 hours at 37°C in a Co2 incubator. Untreated cells served as the control. After incubation, both treated and untreated cells were lysed and homogenized in a lysis buffer (10mM Tris-Hcl, 1mM EDTA, 0.5% Triton X-100, and pH 8). Homogenates were centrifuged at 27,000g for 20 minutes to separate the high molecular weight chromatin from cleavage products. Pellet was suspended in TE buffer (10mM Tris, 1mM EDTA, pH 8). The resulting pellet and supernatant fraction were allowed to react with diphenylamine reagent and allowed to stand at room temperature for 16-20h, before spectrometric determination at 600nm. Percentage of fragmented DNA upon treatment with test compounds was compared with untreated control.

Percentage of fragmented DNA =

$$\frac{\text{OD of supernatant}}{\text{OD of supernatant} + \text{OD of pellet}} \times 100$$

- H. Statistical analysis: All the experiments were carried out in triplicate on atleast three different occasions and the mean of the replicate values were taken. Values were expressed as mean \pm SD. Statistical analysis of the data was determined by Student's-t-test and comparisons were made between the untreated control groups and the treated groups.

III. RESULTS

A. Effect of curcumin on cell growth and viability during chromate (VI)-induced immunotoxicity on lymphocytes and macrophages- MTT assay:

Fig 1 A and 1B shows the effect of curcumin pretreatment on cell viability as assessed by MTT assay in both human peripheral blood lymphocytes and macrophages RAW 264.7. Results indicate that there was significant growth inhibition and decrease in percentage survival of the cells exposed to chromate (VI) treatment in both lymphocytes and macrophages. This was found to be statistically significant as compared to untreated control (P<0.001). Curcumin pretreatment significantly prevented the drop in cell survival and elicited lymphoproliferative responses and macrophage activation which was evidenced by the increase in cell survival, growth and viability of the curcumin pretreated lymphocytes and macrophages exposed to chromate as compared to chromate only treated cells. This reveal the cytoprotective and immunomodulatory properties of curcumin and its ability to negate chromate (VI) induced

immunotoxicity. The curcumin only treated cells did not showed nonsignificant changes as compared to the untreated control thereby indicating the absence of adverse toxic effects of curcumin on lymphocytes and macrophages.

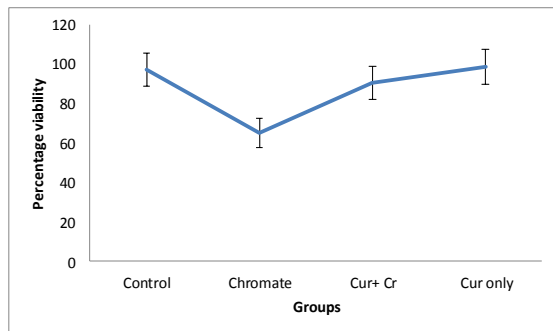


Fig 1A: Effect of curcumin on the percentage viability of lymphocytes during chromate (VI)- induced immunosuppression – MTT assay Values were expressed as mean \pm SD. Comparisons were made between untreated control and the treated groups. *** P < 0.001; ** P < 0.01; * P < 0.05.

decreased leakage of LDH in the curcumin treated cells as compared to the untreated cells and chromate (VI)-treated cells. The results obtained with LDH leakage is in agreement with the results obtained with the MTT assay. In tandem with MTT assay, the curcumin only treated cells showed nonsignificant changes in the activity of LDH as compared to untreated control. This implicate that curcumin treatment did not adversely influence the membrane integrity in both lymphocytes and macrophages.

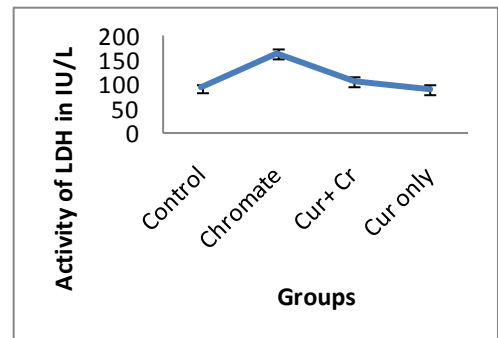


Fig 2A: Effect of curcumin on LDH leakage during chromate (VI)- induced immunosuppression in human peripheral blood lymphocytes. Values were expressed as mean \pm SD. Comparisons were made between untreated control and the treated groups. *** P < 0.001; ** P < 0.01; * P < 0.05.

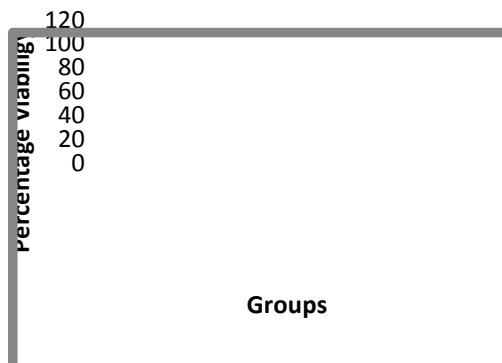


Fig 1B: Effect of curcumin on the percentage viability of RAW 264.7 Macrophages during chromate (VI)- induced immunosuppression – MTT assay Values were expressed as mean \pm SD. Comparisons were made between untreated control and the treated groups. *** P < 0.001; ** P < 0.01; * P < 0.05.

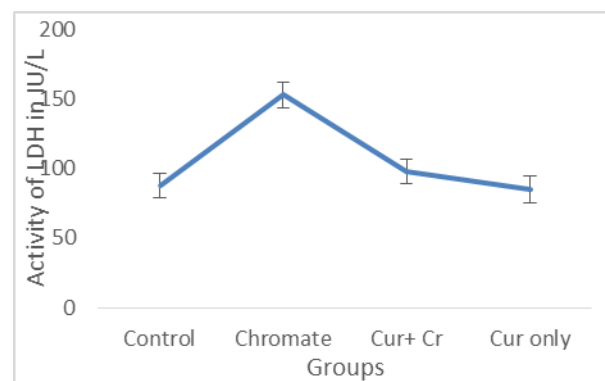


Fig 2B: Effect of curcumin on LDH leakage during chromate (VI) - induced immunosuppression in RAW macrophages 264.7. Values were expressed as mean \pm SD. Comparisons were made between untreated control and the treated groups. *** P < 0.001; ** P < 0.01; * P < 0.05

B. Effect of curcumin on cell membrane integrity during chromate (VI)-induced immunotoxicity on lymphocytes and macrophages- LDH leakage assay:

Fig 2 shows the effect of curcumin pretreatment in maintaining the cell membrane integrity during chromate (VI)-induced immunotoxicity in lymphocytes and macrophages. Chromate treated group of cells elicited a significant increase in the leakage of LDH (P<0.001) in the culture supernatant as compared to the untreated control cells and the curcumin pretreated cells exposed to chromate. This implicate that upon exposure to chromate there is an alteration in membrane permeability and integrity thereby making the membrane leaky resulting in the increased activities of LDH into the culture supernatant. Curcumin pretreatment protected the cells against the altered membrane permeability and integrity which is witnessed by the

C. Effect of curcumin pretreatment on percentage phagocytosis and phagocytic index during Chromate (VI)-induced toxicity in RAW macrophages 264.7:

Table 1 shows the effect of curcumin on percentage phagocytosis and phagocytic index of the macrophages during chromate (VI) exposure. Chromate treatment resulted in significant drop in percentage phagocytosis and phagocytic index as compared to untreated control whereas the curcumin pretreated cells showed an increase in percentage phagocytosis and phagocytic index thereby confirming its

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effects in activating macrophages and counteract chromate (VI) induced immunosuppressive conditions in vitro.

Table 1: Effects of curcumin on percentage phagocytosis and phagocytic index during chromate (VI)-induced immunosuppression in RAW 264.7 macrophages

S.No	Groups	Percentage phagocytosis	Phagocytic index
1	Control	86.33± 7.2	1.92 ±0.2
2	Chromate	39.11± 4.3 ^{***}	1.02 ±0.1 ^{***}
3	Curcumin + Chromate	79.54± 6.2 [*]	1.63 ±0.4 [*]
4	Curcumin	87.92± 8.4 ^{NS}	1.98 ±0.7 ^{NS}

Values were expressed as mean ± SD. Comparisons were made between untreated control and the treated groups. ^{***} P< 0.001; ^{**} P <0.01; ^{*} P < 0.05. Percentage phagocytosis is the number of macrophage cells that has phagocytosed yeast cells (for every 100 cells). Phagocytic index is the mean number of yeast cells phagocytosed per macrophage cell.

D. Effect of curcumin pretreatment on the levels of total reduced glutathione and fragmented DNA during Chromate (VI)-induced –immunotoxicity on lymphocytes and macrophages.

Table 2 shows the influence of curcumin treatment on the levels of total reduced glutathione in the macrophages and the levels of fragmented DNA in the lymphocytes during Chromate (VI)-induced immunotoxicity. Chromate treatment resulted in significant depletion in the levels of total reduced glutathione (P<0.001) as compared to the untreated control cells. Curcumin pretreatment at a dose of 20µg/ml for 30 minutes prior to chromate exposure was able to protect the cells against the depletion of glutathione implicating its ability to replenish the thiol status of the cells probably by stimulating the antioxidant machinery of the cells. Chromate treatment resulted in severe damage to the DNA of the cells which was evidenced by the increase in the levels of fragmented DNA (P<0.001) in the chromate exposed lymphocytes as compared to the untreated control cells. Curcumin pretreatment could protect the cells against damage inflicted to the DNA by chromate treatment which is supported by the appreciable decrease in the levels of fragmented DNA in curcumin pretreated cells exposed to chromate as compared to the chromate exposed cells.

Table 2: Effects of curcumin on the levels of total reduced glutathione (GSH) in RAW 264.7 cells and percentage of fragmented DNA in the lymphocytes during Chromate (VI)-induced immunosuppression.

S.No	Groups	GSH	Fragmented DNA (%)
1	Control	0.73± 0.05	20.32± 1.3
2	Chromate	0.28± 0.03 ^{***}	61.75± 3.9 ^{***}
3	Curcumin + Chromate	0.52± 0.05 [*]	29.39 ±3.5 [*]
4	Curcumin	0.76±0.06 ^{NS}	20.19± 2.2 ^{NS}

Values were expressed as mean ± SD. Comparisons were

made between untreated control and the treated Groups. ^{***} P< 0.001; ^{**} P <0.01; ^{*} P < 0.05. Units for GSH- n moles/ mg protein.

IV. DISCUSSION

Hexavalent chromium is widely used in many industries and hence human exposure to them is unavoidable. Exposure to hexavalent chromium compounds have been reported to result in severe immunosuppression, inflammatory changes in the lungs, and increases the risk of cancer [15, 16]. Dietary antioxidants have been reported to be beneficial to combat the toxicity and oxidative stress induced by many toxic compounds including hexavalent chromium compounds. Antioxidants like ascorbic acid has been proved to be extremely beneficial in counteracting the toxic effects of chromate [17]. Curcuma longa belonging to the Zingiberaceae family is one of the spices that is extensively used in Indian culinary. In addition to its use in food products, the plant is also widely used in Indian traditional system of medicine like Ayurveda, Siddha and Unani for treating various diseases and ailments [18]. Most of the medicinal properties of the plant is attributed to its excellent anti-inflammatory and antioxidant properties. Curcumin is the active principle of Curcuma longa. Several studies report the beneficial effects of curcumin in boosting the immune responses and protecting the immune cells against various noxious agents. But there are no previous reports available with respect to the influence of curcumin to negate the immunotoxicity induced by exposure to hexavalent chromium on immune cells like lymphocytes and macrophages and hence the current study undertaken.

The results of MTT assay and the LDH leakage test demonstrate the ability of curcumin to protect the cells against chromate VI-induced membrane permeability changes and alterations. This could be probably due to the preconditioning effect of curcumin on the immune cells thereby counteracting the changes induced by chromate. Phagocytosis is an important innate immune response exhibited by macrophage cells to eliminate an antigen. Decreased phagocytosis imply suppressed immune responses and severe immunotoxicity which could culminate in increased vulnerability to harmful antigens. Increased phagocytosis in curcumin pretreated cells is an evidence for its immune response boosting effects which could be mediated through the upregulation in the expression of few cytokines involved in the activation of macrophages thereby resulting in increased phagocytic response. Further detailed studies involving the quantitative estimation of the cytokine levels will have to be undertaken to clearly understand this hypothesis.

Total reduced glutathione is an important nonenzymic antioxidant involved in several detoxification processes in the human and animal systems. Depletion of glutathione levels indicate poor antioxidant status, reduced detoxification mechanisms, and induction of oxidative stress in the cells which could be detrimental. Replenishment in the thiol status through an improvement in the levels of total reduced glutathione in the curcumin treated cells implicate the ability of the compound to correct the altered balance between the



prooxidant- antioxidant status. This could have resulted in improved antioxidant status and increased scavenging of free radicals thereby could have contributed to the observed beneficial effects of curcumin. Increased levels of fragmented DNA is considered as a hall mark feature of apoptotic cell death. Several reports earlier indicate that Chromate (VI) exposure resulted in programmed cell death as apoptosis in cells [19]. Curcumin pretreatment elicited significant antiapoptotic effects as evidenced by the decrease in the percentage of fragmented DNA in the curcumin treated cells as compared to untreated control of the chromate (VI)-treated cells [20]. Curcumin is an excellent compound which has the capability to act as a prooxidant or antioxidant depending upon the conditions prevailing. It has been observed that during malignant transformation curcumin induces the death of the transformed cells by inducing apoptotic death which is mediated through an upregulation in the expression of P 53 and other tumour suppressor genes. This is normally accompanied by a shift to prooxidant status thereby resulting in the production of free radicals culminating in the death of cancer cells. Alternatively, when the immune responses are affected by inhibition of growth of lymphocytes and macrophages, the compound induces lymphoproliferative responses and macrophage activation thereby acting as a very good immunomodulator. The observed protective effects of curcumin in the current study, could be attributed to its antioxidant property, and its influence in controlling the mitochondria mediated intrinsic pathway of apoptosis.

Further studies are ongoing in the laboratory, to clearly understand the mechanisms involved behind the immunoprotective effects of curcumin during chromate (VI)-induced immunosuppression in vitro on immune cells.

V. CONCLUSION

Results of the current study implicate the immunomodulatory effects of curcumin and its effects in evoking lymphoproliferative responses and macrophage activation in vitro during chromate (VI)-induced immunosuppression. This also implicate that curcumin could influence the outcome of both innate as well as acquired immune responses in an individual. Supplementation of curcumin in the diet of workers employed in industries utilizing hexavalent chromium compounds might be beneficial in preventing the toxic changes and immunosuppression inflicted by the heavy metal.

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Corresponding Author: Dr.M.Sreepriya, M.Sc., M.Phil and Ph.D in Biochemistry, currently Faculty in Biotechnology, Department of Microbiology and Biotechnology, Bangalore University, Bangalore- 560 056, Karnataka, India. Specialization: Experimental therapeutics (Cancer and osteoporosis), preclinical trials on in vitro and in vivo model systems.