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Modulation of cadmium induced alterations in murine thymocytes by piperine: Oxidative stress, apoptosis, phenotyping and blastogenesis

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ABSTRACT

Piperine, a main component of Piper longum Linn. and Piper nigrum Linn., is a plant alkaloid with a long history of medicinal use in Indian medicine. It is known to exhibit a variety of biological activities which include anti-pyretic, anti-inflammatory, anti-depressant, hepatoprotective and antitumor. Its immunomodulatory role has so far been limited to humoral response. The influence of piperine on murine thymocytes, immunocompromised by cadmium has been reported by us in this investigation. The various biochemical parameters such as oxidative stress markers (ROS and GSH), Bcl-2 protein expression, mitochondrial membrane potential, caspase-3 activity, DNA damage, blastogenesis and T lymphocyte phenotypes were determined. Cadmium (25 μM) induced apoptosis earliest at 6 h. Alterations in ROS and GSH preceded mitochondrial membrane depolarization and caspase-3 activation followed by apoptosis. The phenotypic changes occurred at 18 h and blastogenesis at 72 h. Various conc. of piperine (1, 10 and 50 μg/ml) when added along with Cd (25 μM) from 1.5 to 72 h, caused a dose and time dependent amelioration in all the cellular events mentioned above. Modulation of oxidative stress has earlier been reported to reduce Cd induced apoptosis in murine lymphocytes. Inhibition of the ROS production and replenishment of GSH by piperine, may in part be responsible for the suppression of downstream cascade of events, i.e. apoptosis, blastogenesis and T lymphocyte phenotyping. The study clearly demonstrated the anti-oxidative, anti-apoptotic, and restorative ability against cell proliferative mitogenic response and phenotypic alterations by piperine, suggesting its therapeutic usefulness in immunocompromised conditions.

1. Introduction

Piper longum Linn. and Piper nigrum Linn., are traditional herbs, used in Indian medicine [1] and as spice throughout the world. It is commonly used as a good remedy for treating gonorrhea, menstrual pain, tuberculosis, sleeping problems, respiratory tract infection, chronic gut related pain and arthritic conditions. The anti-pyretic, anti-inflammatory and anti-depressent properties of piperine, the main alkaloid of piper species were reported by Parmar et al. [2].

Piperine is the *trans–trans* stereoisomer of 1-piperoylpiperidine. It is also known as (E,E)-1-piperoylpiperidine and (E,E)-1-[5-(1,3-benzodioxol-5-y1)-1-oxo-2,4-pentdienyl] piperidine. It is represented by the following chemical structure:

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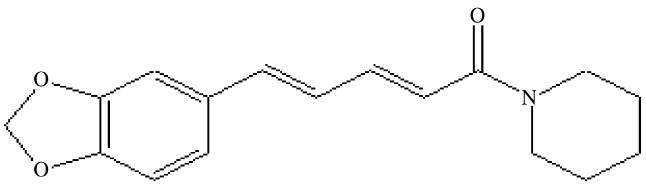

Current literature reveals a wide spectrum of biological activities of piperine. Piperine supplementation causes inhibition of Phase I and II enzymes, elevation of glutathione metabolizing enzymes, reduction in DNA damage and DNA protein cross-links in benzo(a)pyrene induced lung carcinogenesis in mice [3,4]. A decrease in lipid peroxidation, protein carbonyls, nucleic acid and polyamine synthesis was also shown by the same group [5], suggesting chemopreventive efficacy of piperine. The other potential activities of piperine studied both *in vitro* and *in vivo* include antidepressant [6], hepatoprotective [7], anti-metastatic [8], antithyroid [9], immunomodulatory and antitumor [10]. Constituents of piper species exhibit inhibitory activity on prostaglandin and leucotriene biosynthesis *in vitro* [11]. In addition, piperine also possesses bioavailability enhancing activity with various structurally and therapeutically diverse drugs [12].

Although, the exact mechanism underlying the biological efficacy of piperine remains elusive, the antioxidant characteristics likely to be involved was reported by Mittal and Gupta [13] and Vijayakumar et al. [14].

Regarding the immunomodulatory activity of Piperine, there is only one report which demonstrated an increase in the circulating antibody titre and antibody forming cells, reflecting stimulatory effect on the humoral arm of immune system. It also enhanced the bone marrow cellularity and α-esterase positive cells [10]. However, its influence on cell mediated immunity has not been studied so far.

Cadmium (Cd), a potent immunotoxicant, causes damage both to humoral immune response and cell mediated immunity [15,16]. Thymic atrophy in mice on Cd exposure was shown by Yamada et al. [17]. Divergent effects of Cd on the immune system has been demonstrated [18–21]. Cadmium induced B and T cell immune suppression possibly due to the apoptotic potential of Cd has been shown by Feng et al. [22]. In another report, susceptibility of different thymocyte subpopulation to Cd induced apoptosis revealed a marked decrease in $CD4^+/CD8^+$ ratio [23].

In our earlier studies, we demonstrated Cd induced apoptosis in murine splenocytes and thymocytes, following mitochondrial – caspase dependent pathway. The oxidative stress indices, i.e. ROS and GSH, acted as intracellular signals preceding mitochondrial membrane depolarization and caspase-3 activation [24,25].

Studies pertaining to identification of safe and active plant derived compounds for attenuation of cadmium (Cd) induced apoptosis and immune dysfunction may be important in the management of health risks associated with environmental and occupational Cd exposure. Since Cd is a potent apoptotic agent and piperine being an immunomodulatory agent, we considered worthwhile to investigate the role of piperine in modulating the oxidative and apoptotic effects of Cd. Its effect on blastogenesis and T lymphocyte phenotyping were also determined.

Experiments were therefore, planned to investigate the action of piperine at various check points: (a) its anti-oxidative efficacy was determined by monitoring ROS and GSH, (b) anti-apoptotic effect by mitochondrial membrane potential, Bcl-2 protein expression, caspase-3 activity and DNA damage, (c) its effect on blastogenesis was studied by cell proliferative mitogenic response and (d) its influence on T-phenotypes by $CD4^+/CD8^+$ ratio.

In the present study, we clearly demonstrated anti-oxidative, anti-apoptotic and anti-proliferative ability of piperine. The altered $CD4^+/CD8^+$ ratio was also markedly restored.

2. Materials and methods

2.1. Chemicals

All the chemicals were of highest grade purity available. Cadmium chloride ($CdCl_2$), RNase A, RPMI 1640, antibiotic–antimycotic solution, dulbecco's phosphate buffered saline (PBS), fetal bovine serum (FBS), agarose, 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescein diacetate (DCFH-DA), piperine, concanavalin A (Con A), FITC conjugated anti-rabbit IgG antibody and all other chemicals were purchased from Sigma–Aldrich, USA. Rhodamine 123 (Rh 123) and 5′-chloromethylfluorescein diacetate (CMF-DA) from Molecular Probes, propidium iodide (PI) from Calbiochem, rabbit anti Bcl-2 polyclonal antibody from Biovision, [^{3}H] Thymidine from BRIT, Bombay, FITC-conjugated anti-CD4 monoclonal antibody and PE-conjugated anti-CD8 monoclonal antibody from eBioscience. Cell Death Detection sandwich ELISA kit was purchased from Roche, Germany, caspase-3 fluorometric protease assay kit from Chemicon, USA and Annexin V-FITC Apoptosis Detection kit from Pharmingen (Becton Dickinson Company).

2.2. Preparation of thymocyte suspension

The BALB/c mice were maintained in our animal house under standard conditions. They were fed with standard rodent pellet and water *ad libitum*. Our animal house and breeding facility are registered with Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Government of India and CPCSEA guidelines were followed (IAEC approval obtained).

Thymus was dissected from male BALB/c mice (4–6-week old) and single cell suspension prepared under aseptic conditions. The suspension was passed through stainless steel mesh, centrifuged at $200 \times g$ at 4 °C for 10 min and resuspended in complete cell culture medium (RPMI 1640 containing HEPES and 2 mM glutamine, supplemented with 10% FBS and 1% antibiotic–antimycotic solution). The cell density was adjusted to ca. 1.5×10^6 cells/ml and the viability of the freshly isolated cells was always over 95% (trypan blue exclusion test).

For the monitoring of various parameters in the present investigation, we have used 25 μM conc. of Cd and 1, 10 and

50 μg/ml of piperine. The Cd conc. selected was based on our earlier studies [25].

2.3. Assessment of cell viability

The cell viability was measured by the MTT reduction method [26]. Cells in RPMI 1640 were seeded at a density of 1.0×10^4 cells in 96 well plate. Cadmium along with piperine were added for 18 h at 37 °C in a CO_2 incubator. Ten μl MTT (5 mg/ml PBS) was added to the wells, 4 h prior to the completion of incubation time. The plate was centrifuged at $1200 \times g$ for 10 min and 100 μl of DMSO was added after removing the supernatant, to dissolve the formazan formed. The absorbance was read at 530 nm after 5 min, in a microplate reader (Synergy HT of BIO-TEK International, USA)

2.4. Measurement of caspase-3 activity

Caspase-3 activity was measured by a commercial kit (Chemicon, USA). A population of 3.0×10^6 cells/ml was incubated with Cd and piperine for 1.5, 3 and 6 h at 37 °C in a CO_2 incubator. The cells were scraped and lysed on ice for 10 min using cell lysis buffer. The reaction buffer and DEVD-AFC substrate were then added and further incubated at 37 °C in dark for 2 h. The resultant fluorescence was measured at excitation and emission wavelengths of 400 and 505 nm respectively, on a microplate reader.

2.5. DNA fragmentation and cell death detection

The DNA fragmentation pattern (DNA ladder) was carried out by Agarose gel electrophoresis. An aliquot of 2 ml (1.5×10^6 cells/ml) was incubated with Cd and piperine for 6 and 18 h at 37 °C in a CO_2 incubator. At the end of incubation, cells were pelleted by centrifugation at $200 \times g$ for 10 min and the pellet lysed with 0.5 ml lysis buffer (10 mM Tris–HCl, pH 7.5, 20 mM EDTA, 0.5% Triton X-100) on ice for 30 min. The DNA in lysed solution was extracted with phenol/chloroform and precipitated with 3 M sodium acetate (pH 5.2) and cold ethanol. After repeated washings, the DNA was dissolved in TE buffer (10 mM Tris–HCl, pH 8.0: 1 mM EDTA). The purity of DNA at 260 and 280 nm absorbance ratio was between 1.7 and 1.9. DNA (2 μg) was then loaded on 0.7% agarose gel and electrophoresis carried out. The bands were visualized by ethidium bromide staining under UV light.

The DNA fragmentation (histone associated mono- and oligonucleosomes) was measured using Cell Death Detection Sandwich ELISA kit (Roche, Germany). A sample of 1.5×10^6 cells/ml was incubated with Cd (25 μM) along with piperine for 6 and 18 h at 37 °C in a CO_2 incubator, out of which 1×10^5 cells were transferred to a clear tube. After centrifugation at $200 \times g$ for 10 min, the cell pellet was suspended in the incubation buffer for 30 min at 15–25 °C for lysis. Following centrifugation at $20{,}000 \times g$ for 10 min, the supernatant was diluted 10-fold with incubation buffer and the nucleosomes in the sample solution were measured by ELISA. Modular microtitre plates were incubated overnight with 100 μl of the antihistone antibody (coating solution) under cold conditions. After aspirating the coating solution, 200 μl of incubation buffer was added and allowed to stand for 30 min at 15–25 °C. The plates were then rinsed with washing buffer (200 μl) thrice, 100 μl of the sample solution was added into each well and incubated for 90 min at 15–25 °C. After washing, 100 μl of conjugate solution (anti-DNA-peroxidase) was added to each well and the cells were further incubated for 90 min at 15–25 °C, followed by washing. A 100 μl of ABTS (2-2′-azino-di-[3-ethylbenzthiazoline sulfonate]) was then added and incubated for 10 min and the absorbance measured at 405 nm.

2.6. Flow cytometry analysis

All the following assays were carried out on thymocytes treated with Cd and piperine for different time intervals (1.5, 3, 6 and 18 h) at 37 °C in a CO_2 incubator. The flow cytometric analysis were done on BD-LSR flow cytometer. Cell debris, characterized by a low FSC/SSC was excluded from analysis. The data was analysed by Cell Quest software and mean fluorescence intensity was obtained by histogram statistics.

2.6.1. Apoptotic DNA analysis

The cells with hypodiploid DNA were determined by cell cycle studies. After the treatment period, the harvested cells were washed with PBS and fixed by drop-by-drop addition of ice cold 70% ethanol and stored at 4 °C overnight. The fixed cells were harvested, washed with PBS and suspended in 1 ml PBS. Phosphate citrate buffer (200 μl, pH 7.8) was added and the cells incubated for 60 min at room temp. After centrifugation, the cells were resuspended in 0.5 ml of PI stain (10 mg PI, 0.1 ml Triton-X 100 and 3.7 mg EDTA in 100 ml PBS) and 0.5 ml of RNase A (50 μg/ml) and further incubated for 30 min in dark. The PI fluorescence was measured through a FL-2 filter (585 nm) and 10,000 events were acquired [27].

2.6.2. Assessment of apoptotic and necrotic cells

The apoptotic and necrotic cell distribution was analysed by Annexin V binding and PI uptake. Positioning of quadrants on Annexin V/PI dot plots was performed and living cells (Annexin V^-/PI^-), early apoptotic/primary apoptotic cells (Annexin V^+/PI^-), late apoptotic/secondary apoptotic cells (Annexin V^+/PI^+) and necrotic cells (Annexin V^-/PI^+) were distinguished [28]. Therefore, the total apoptotic proportion included the percentage of cells with fluorescence Annexin V^+/PI^- and Annexin V^+/PI^+. Briefly, after the treatment period (6 and 18 h), the harvested cells were suspended in 1 ml binding buffer (1×). An aliquot of 100 μl was incubated with 5 μl Annexin V-FITC and 10 μl PI for 15 min in dark at room temperature and 400 μl binding buffer (1×) was added to each sample. The FITC and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired.

2.6.3. Assesssment of T-lymphocyte phenotyping

The phenotyping of treated thymocytes was conducted to analyze the effect of piperine in Cd altered subpopulations based on CD4 and CD8 surface molecules. Positioning of quadrants on FITC/PE dot plots was performed and $CD4^+$, $CD8^+$, $CD4^+CD8^+$ (double positive: DP) and $CD4^-CD8^-$ (double negative: DN) subpopulations were distinguished. Briefly, after incubation time (6 and 18 h), the harvested cells were

resuspended in 1 ml PBS. An aliquot of 100 μl was incubated with 5 μl FITC-conjugated anti-CD4 monoclonal antibody and 5 μl PE-conjugated anti-CD8 monoclonal antibody for 30 min in dark at room temperature, after which 400 μl PBS was added to each sample. The FITC and PE fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm) respectively, and 10,000 events were acquired. Appropriate electronic compensation was adapted among the two fluorescence channels to overcome residual spectral overlap.

2.6.4. *Mitochondrial membrane potential and Bcl-2 protein expression*

For the detection of mitochondrial membrane potential, Cd and piperine treated cells were incubated with Rh 123 (5 μg/ml final conc.) for 60 min in dark at 37 °C, harvested and suspended in PBS. The mitochondrial membrane potential was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells [29].

For Bcl-2 protein expression, Cd and piperine (50 μg/ml) treated cells were harvested and suspended in 1 ml RPMI buffer (containing 2% FBS and 0.1% sodium azide). An aliquot of 200 μl was incubated with 5 μl of 0.2 mg/ml stock solution of rabbit anti Bcl-2 polyclonal antibody for 20 min at 2–8 °C. The excess antibody was removed by washing cells twice with RPMI buffer. After washing, the cells were resuspended in 200 μl RPMI buffer and binding of unlabelled antibodies was visualized by adding 5 μl of 50 μg/ml stock solution of FITC conjugated anti-rabbit IgG antibody for 20 min at 2–8 °C. After incubation, cells were washed with RPMI buffer, then with PBS and resuspended in 400 μl PBS. The FITC fluorescence was measured through FL-1 filter (530 nm) and 10,000 events were acquired.

2.6.5. *Reactive oxygen species (ROS) measurement*

The generation of ROS was detected by DCF fluorescence. Thymocytes were incubated with Cd and piperine and DCFH-DA (100 μM final conc.) was added simultaneously to the medium. The cells were harvested, suspended in PBS and ROS generation was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells [30].

2.6.6. *Glutathione (GSH) measurement*

The cellular level of GSH in thymocytes was monitored by CMF-DA. After treatment, the cells were incubated with CMF-DA (1 μM final conc.) for 30 min in dark at 37 °C. After harvesting, the cells were suspended in PBS and GSH was measured by the fluorescence intensity (FL-1, 530 nm) of 10,000 cells [31].

2.7. *[^{3}H] Thymidine incorporation measurement*

To measure the proliferation, 1.0×10^4 cells were seeded in 96-well plates in 200 μl of complete medium with or without one or more reagents (2.5 μg/ml Con A, Cd and piperine) and incubated for 24 and 72 h at 37 °C in a CO_2 incubator. [^{3}H] Thymidine (2 μCi) was added to the wells, 18 h prior to the completion of incubation time. The cells were collected with cell harvester and incorporated radioactivity was measured in a liquid scintillation counter (Hewett Packard).

2.8. *Statistical analysis*

Significance of mean of different parameters between the treatment groups were analysed using one way analysis of variance (ANOVA) after ascertaining the homogeneity of variance between the treatments. Pairwise comparisons were done by calculating the least significant difference.

3. Results

3.1. *Effect of piperine on Cd-induced cell viability*

Increase in cell viability, on the addition of piperine to Cd treated thymic cells was dose dependent (Fig. 1). Cadmium (25 μM) caused ~54% loss in cell viability. Even with 1 μg/ml piperine, there was a mild change ($p < 0.05$). On increasing the conc. to 50 μg/ml, the cell viability was almost similar to that observed in control cells. Piperine alone did not result in any cytotoxicity.

3.2. *Effect of piperine on Cd-induced apoptosis*

3.2.1. *DNA damage*

Cadmium induced apoptotic DNA (sub-G1) population at 6 and 18 h was suppressed by piperine. The apoptotic population (18.7%) decreased with the advancing dose of piperine and was time related (Fig. 2). A 2.5-fold lowering in the sub-G1 population at 6 h became 3.5-fold by the highest piperine conc. at 18 h (Table 1). Annexin V binding data coincided with the results of apoptotic DNA as depicted in Fig. 3. The total number of apoptotic cells (early + late) by 50 μg/ml piperine was 5.2% as compared to 19.7% in Cd treated cells at 18 h. There was a drastic fall in the percentage of necrotic cells (PI^+ cells) as well. From 5.4% in the Cd loaded cells, it decreased to 0.6% with Cd and piperine (50 μg/ml) co-treatment as shown in the figure. Piperine causes a reduction not only in apoptotic, but also in the number of necrotic cells.

The DNA ladder (internucleosomal fragmentation) induced by Cd was also diminished with the various doses of piperine

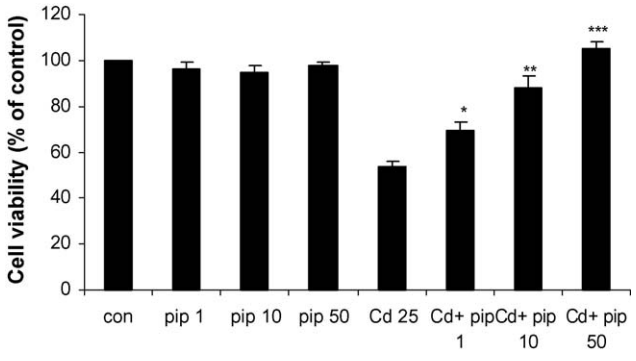

Fig. 1 – Effect of piperine on Cd altered viability in murine thymocytes. Freshly isolated thymocytes (1.5×10^4) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 18 h. Absorbance was measured at 530 nm. Each bar represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

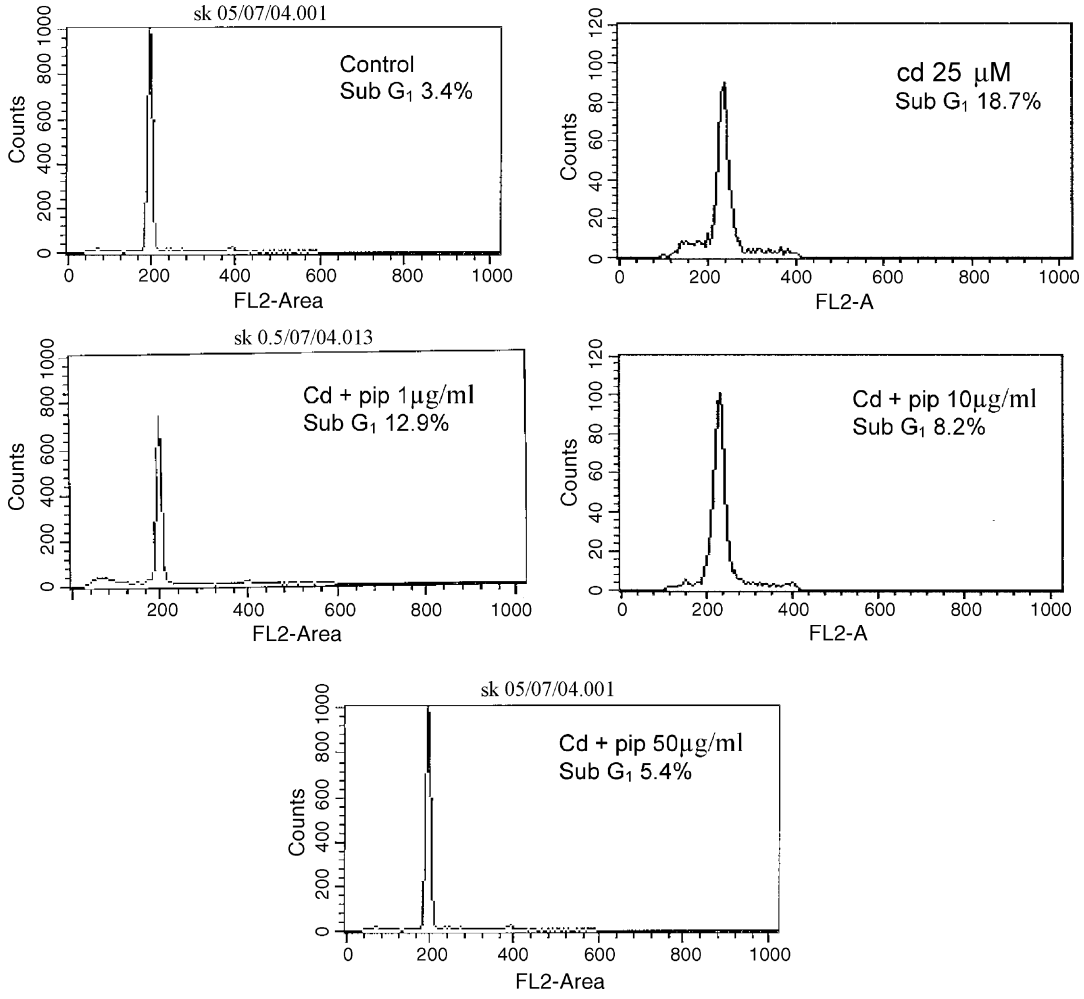

Fig. 2 – Effect of piperine on Cd induced apoptotic DNA. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 18 h. The propidium iodide fluorescence was measured using a flow cytometer with FL-2 filter. Results were expressed as histogram representing the percentage of sub-G1 population (18 h).

Table 1 – Effect of piperine on Cd induced apoptotic DNA

Groups	% of apoptotic cells		
	3 h	6 h	18 h
Control	2.4 ± 0.6	2.9 ± 0.5	3.4 ± 1.6
pip 1	2.6 ± 0.4	3.1 ± 1.2	3.6 ± 2.1
pip 10	2.8 ± 0.8	3.9 ± 0.5	4.2 ± 1.6
pip 50	2.9 ± 0.3	4.3 ± 1.1	3.8 ± 1.8
Cd 25 μM	2.2 ± 0.7	8.2 ± 1.4	18.7 ± 4.5
Cd + pip 1	3.6 ± 1.2	8.6 ± 3.2	12.9 ± 5.3**
Cd + pip 10	3.4 ± 1.4	5.2 ± 2.4*	8.2 ± 4.6***
Cd + pip 50	3.5 ± 2.1	3.3 ± 1.5**	5.4 ± 3.5***

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 18 h. The propidium iodide fluorescence was measured using a flow cytometer with FL-2 filter. Results were expressed as the percentage of apoptotic cells obtained from the histogram statistics. Each value represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

at 18 h as shown in Fig. 4, corroborating our flow cytometer data. Piperine alone, did not induce apoptosis, when added to the control cells. The sandwich ELISA method for histone associated DNA fragments in cell lysate, another assay for DNA damage determination, showed a dose dependent decrease in the Cd enhanced absorbance at 405 nm by piperine, as depicted in Table 2. Although, no distinct DNA ladder was evident by Cd and Cd + piperine at 6 h, the other apoptotic assays reveal anti-apoptotic effect of the higher dose of piperine even at 6 h. Consequent to establishing the anti-apoptotic potential of piperine, we further explored its influence on caspase-3 and mitochondrial membrane potential, the two early markers of apoptosis along with Bcl-2 protein expression. To understand its antioxidant activity, the two indices of oxidative stress (ROS and GSH) were also ascertained.

3.2.2. *Mitochondrial membrane potential ($\Delta\psi$), Bcl-2 protein and caspase – 3 activity*

Mitochondrial $\Delta\psi$, being an early marker of apoptosis, was monitered at 1.5, 3 and 18 h. Significant mitochondrial

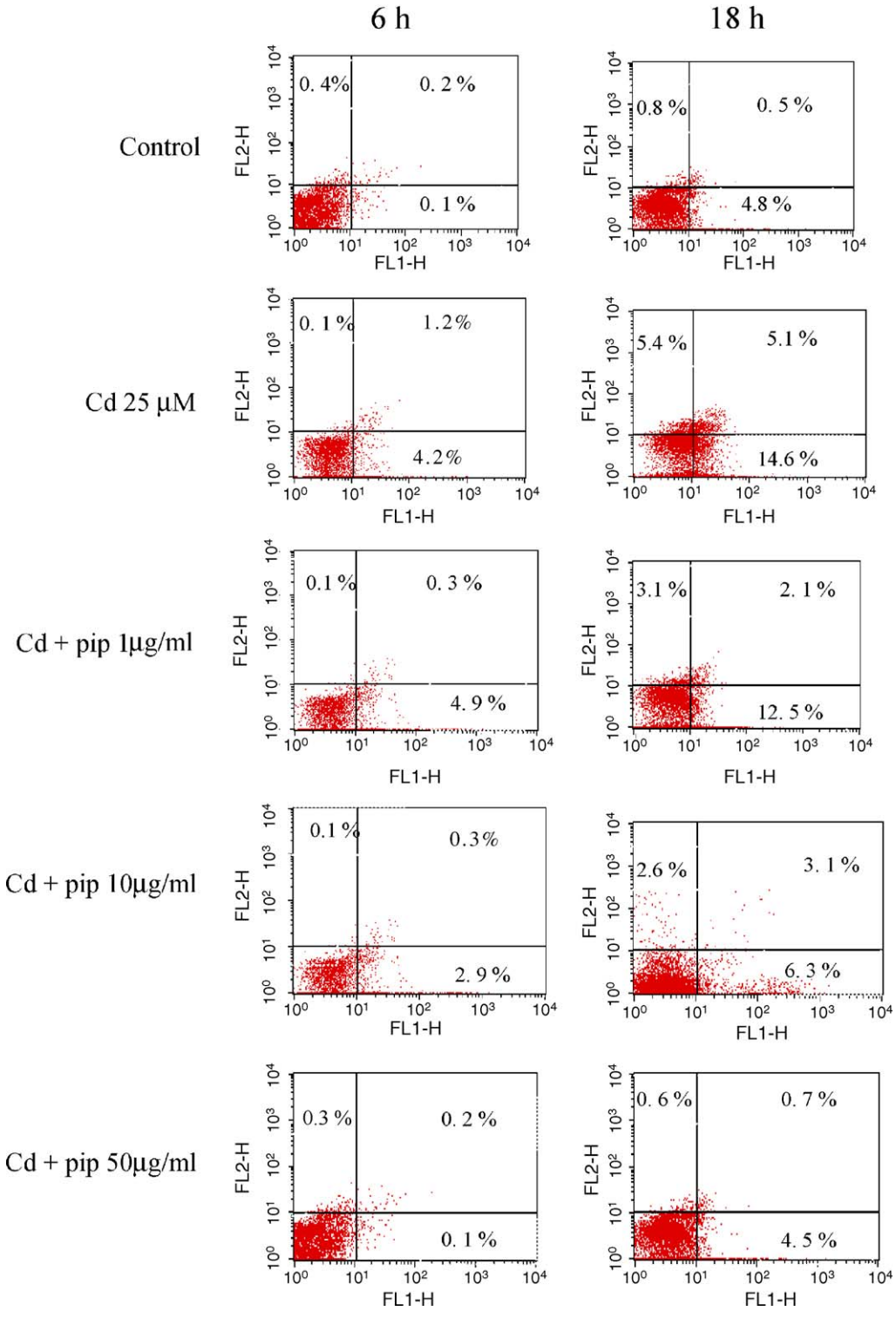

Fig. 3 – Effect of piperine on Cd altered apoptotic and necrotic cell distribution. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) and the cell distribution was analysed using Annexin V binding and PI uptake. The FITC and PI fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plot representing as one of the three independent experiments. LL: living cells (Annexin V^-/PI^-); LR: early/primary apoptotic cells (Annexin V^+/PI^-); UR: late/secondary apoptotic cells (Annexin V^+/PI^+); UL: necrotic cells (Annexin V^-/PI^+).

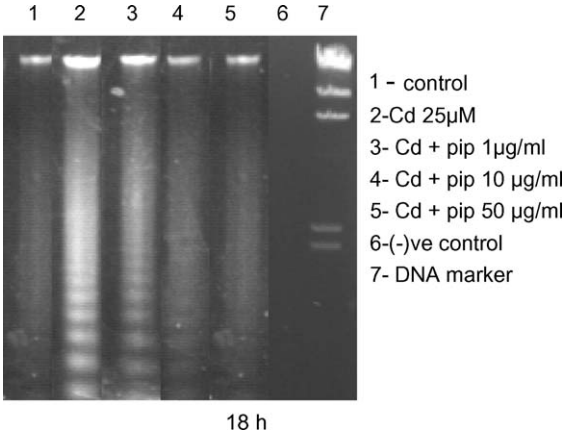

Fig. 4 – Effect of piperine on Cd induced DNA fragmentation by (0.7%) agarose gel electrophoresis. Freshly isolated thymocytes (3.0×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 18 h.

membrane depolarization at 3 and 6 h by 25 μM Cd was markedly restored by all the doses of piperine, the highest conc. of piperine exhibiting maximum affect (Fig. 5). At 3 and 6 h, the intensity of the Rh 123 fluorescence in presence of 50 μg/ml piperine was almost close to the control fluorescence, suggesting piperine's ability to mitigate the depolarizing effect of Cd.

Since the function of permeability transition pore and the release of intermembrane contents of mitochondria are under regulatory control of Bcl-2 family proteins [32,33], the cells exposed to Cd and piperine (50 μg/ml) showed a remarkable restoration of the Bcl-2 protein. Cadmium alone caused almost three-fold lowering in the protein expression (Fig. 6) indicating facilitation of the cells to release mitochondrial apoptogenic signaling molecules. As caspase-3 activation is linked to mitochondrial membrane depolarization [24,25], piperine addition to the Cd exposed cells also caused significant reduction in the protease activity (Table 3). Maximum amelioration by piperine was exhibited at 3 h, when caspase-3 activity was highest.

Table 2 – Effect of piperine on Cd induced DNA fragmentation (mono- and oligonucleosomes)

Groups	O.D. 405 (6 h)	O.D. 405 (18 h)
Control	100 ± 12.5	100 ± 13.8
Cd 25 μM	120 ± 8.6	156 ± 18.8
pip 1	104 ± 5.6	112 ± 11.2
pip 10	98 ± 6.4	111 ± 7.8
pip 50	107 ± 6.8	103 ± 14.5
Cd + pip 1	121 ± 11.6	133 ± 8.6*
Cd + pip 10	112 ± 8.4	117 ± 17.9***
Cd + pip 50	104 ± 18.8**	98 ± 11.0***

Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 6 and 18 h at 37 °C. DNA fragments were determined by Cell Death Detection ELISA. Each value represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

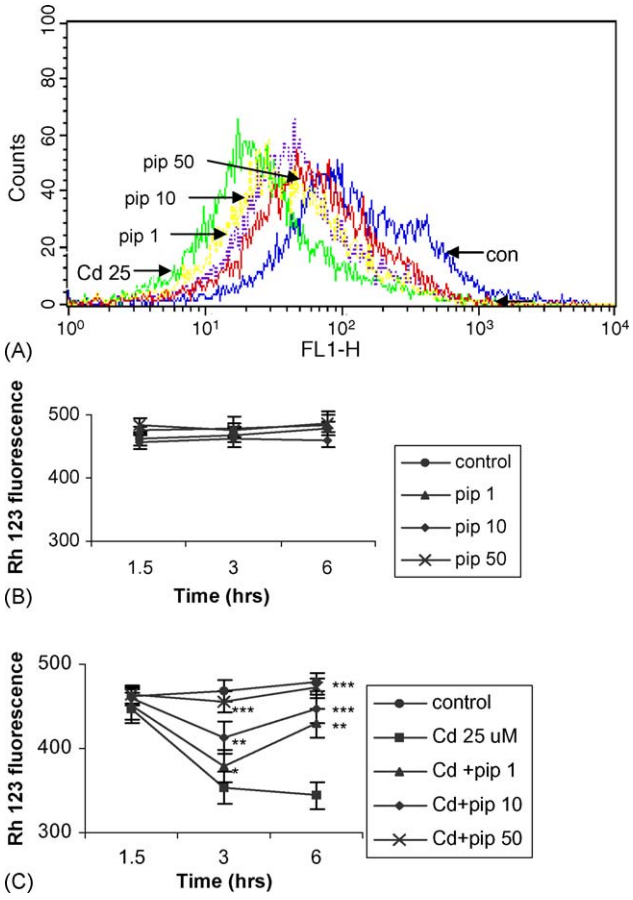

Fig. 5 – Effect of piperine on Cd induced mitochondrial membrane depolarization. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 1.5, 3, 6 and 18 h at 37 °C. Rh 123 was added and incubated for 60 min.The fluorescence was measured using a flow cytometer with FL-1 filter. Results were expressed as a representative histogram (A, 3 h) and mean fluorescence obtained from the histogram statistics (B and C). Each bar represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

As described above, the results on apoptosis, mitochondrial $\Delta\psi$, Bcl-2 protein and caspase activity, suggest that attenuation of early markers of apoptosis by piperine could lead to the suppression of Cd induced apoptotic cell death.

3.3. *Effect of piperine on ROS generation and intracellular GSH*

To further investigate the role of piperine on ROS and GSH levels, the cells were stained with DCFH-DA, the fluorescent probe for determination of wide range of ROS, including species derived from H_2O_2 such as OH° [34]. The DCF fluorescence, proportionate to the ROS levels in the cells, was monitored on flow cytometer (Fig. 7). Suppression of Cd induced DCF fluorescence by piperine was dose and time dependent. The earliest inhibition by 50 μg/ml piperine at 1.5 h, became more intense with the passage of time. Even the

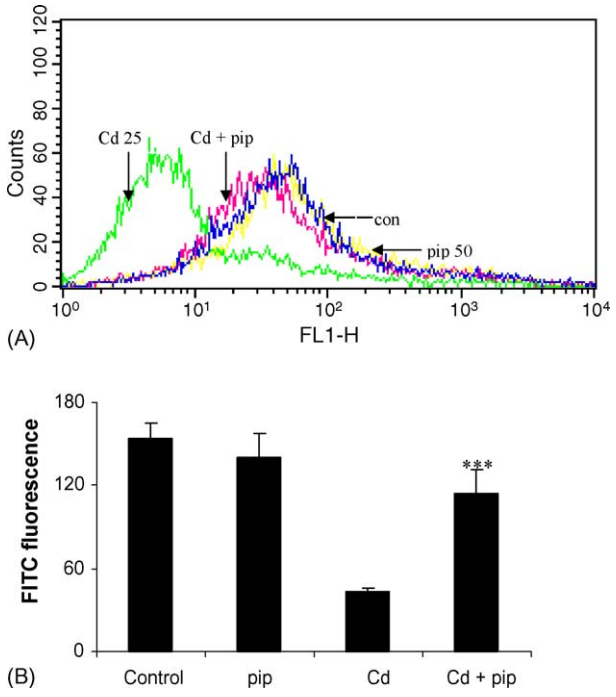

Fig. 6 – Effect of piperine on Cd altered Bcl-2 protein. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (50 μg/ml) for 18 h at 37 °C. Bcl-2 protein was measured using rabbit anti Bcl-2 polyclonal antibody and FITC conjugated anti-rabbit IgG antibody. FITC fluorescence was measured using a flow cytometer with FL-1 filter. Results were expressed as a representative histogram (A) and mean fluorescence obtained from the histogram statistics (B). Each bar represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$ as compared to Cd treated group, using one-way ANOVA.

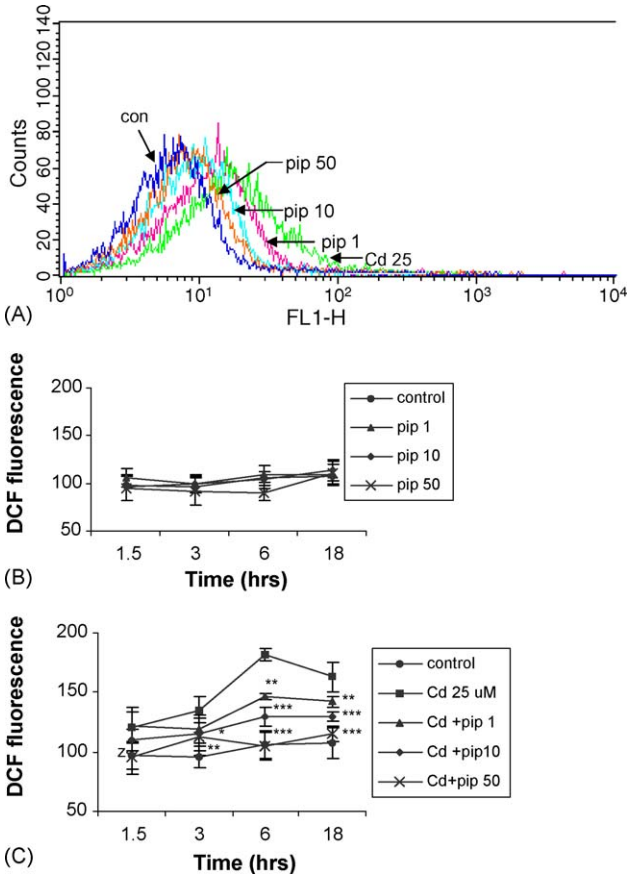

Fig. 7 – Effect of piperine on Cd induced ROS generation. Freshly isolated thymocytes (1.5×10^6) were incubated with DCFH-DA (100 μM), Cd (25 μM) and piperine (1–50 μg/ml) for 1.5, 3, 6 and 18 h at 37 °C. DCF fluorescence was measured using a flow cytometer with FL-1 filter. Results were expressed as a representative histogram (A, 6 h) and mean fluorescence was obtained from the histogram statistics (B and C). Each bar represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

lowest dose of piperine (1 μg/ml) caused inhibition in the fluorescence intensity at both 6 and 18 h ($p < 0.01$) as shown in Fig. 7A. Piperine (50 μg/ml) was able to almost completely abolish Cd-induced ROS generation at both time points, suggesting its free radical scavenging property [13]. There was a non-significant lowering in the DCF fluorescence at 3 and 6 h, when piperine (50 μg/ml) alone was added to the cells (Fig. 7B).

Table 3 – Effect of piperine on Cd induced caspase-3 activity

Groups	Fluorescence (% of control)		
	1.5 h	3.0 h	6.0 h
Cd 25 μM	175	310	175
pip 1	102	96	98
pip 10	98	99	101
pip 50	103	98	99
Cd + pip 1	164	294	160
Cd + pip 10	128	143	123
Cd + pip 50	106	98	106

Freshly isolated thymocytes (3.0×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 1.5, 3 and 6 h at 37 °C. The enzyme activity was determined by Fluorometric Protease Assay Kit. Fluorescence was measured at Ex: 400 nm and Em: 505 nm. The value represents mean of two independent experiments.

For GSH measurement on flow cytometer, the intensity of CMF fluorescence has been shown to be well correlated with biochemically estimated content of GSH [35]. As shown in Fig. 8, decreased CMF fluorescence by Cd observed at 3 h onwards, was significantly enhanced by piperine in a dose dependent manner. Piperine (50 μg/ml), effectively raised the GSH levels almost close to control at 6 and 18 h, thereby replenishing the GSH levels. Piperine alone, when added to the cells, did not alter the CMF fluorescence (Fig. 8B).

3.4. *Effect of piperine on Cd altered thymocyte proliferation*

A significant inhibition in the (^{3}H) thymidine uptake in Con A stimulated, Cd (25 μM) treated thymocytes was observed at

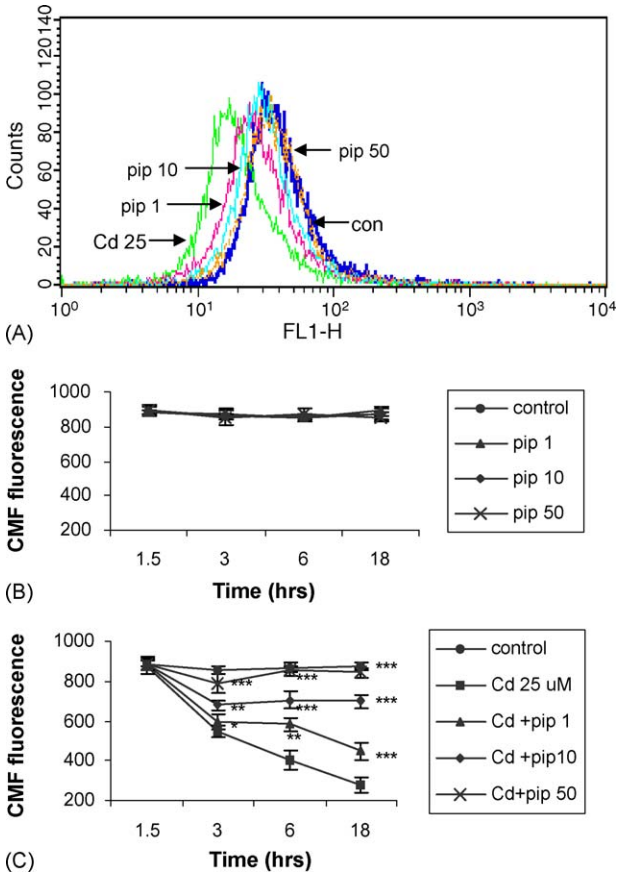

Fig. 8 – Effect of piperine on Cd altered GSH levels. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) for 1.5, 3, 6 and 18 h at 37 °C. CMF-DA was added and incubated for 30 min. CMF fluorescence was measured using a flow cytometer with FL-1 filter. Results were expressed as a representative histogram (A, 6 h) and mean fluorescence obtained from the histogram statistics (B and C). Each bar represents mean ± S.D. ($n = 3$). $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$ as compared to Cd treated group, using one-way ANOVA.

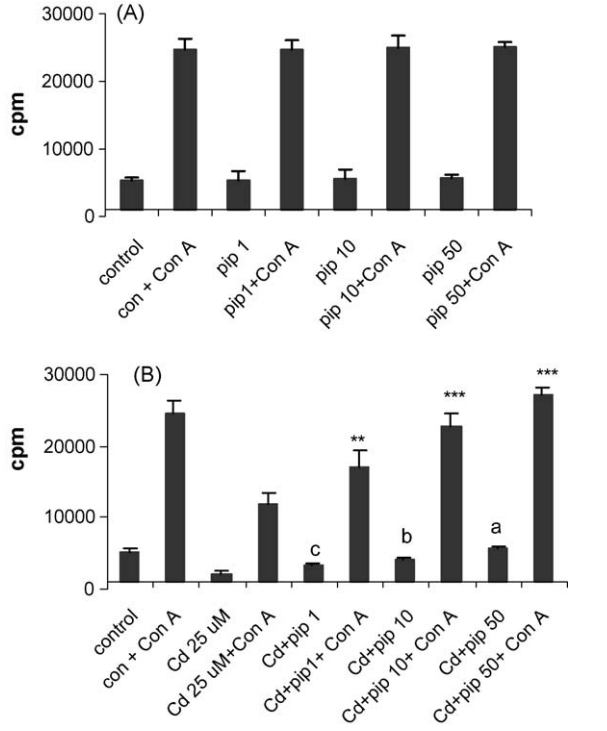

Fig. 9 – Effect of piperine on Cd altered blastogenesis. Freshly isolated thymocytes (1.0×10^4) were treated with Cd (25 μM), piperine (1–50 μg/ml) and Con A (2.5 μg/ml) for 72 h at 37 °C. [^{3}H] Thymidine (2 μCi) was added, 18 h prior to completion of incubation time. The radioactivity was measured in a liquid scintillation counter and the results were expressed as cpm (A) Con A and piperine, (B) Con A, Cd and piperine. Each bar represents mean ± S.D. ($n = 3$). $^{a}p < 0.001$, $^{b}p < 0.01$, $^{c}p < 0.05$ compared to Cd treated group and $^{***}p < 0.001$, $^{**}p < 0.01$ as compared to Cd + Con A treated group, using one-way ANOVA.

72 h. The (^{3}H) thymidine incorporation into cellular DNA is a measure of the proliferative capacity of the cells towards antigenic response. Con A stimulated thymocytes incorporated almost five times more radioactivity than the untreated cells at 72 h (Fig. 9). At 24 h, the (^{3}H) thymidine uptake was ~1.9-fold as compared to the unstimulated thymic cells. Cadmium and piperine in combination or alone, did not influence the proliferative capacity of the Con A stimulated cells at 24 h (data not shown). At 72 h, Cd caused two-fold inhibition in (^{3}H) thymidine uptake in mitogenically stimulated cells. Even the non-stimulated cells, on exposure to Cd, incorporated less thymidine. Thymocyte blastogenesis under both the conditions, was inhibited by Cd to the same degree. Cadmium proliferative response to Con A was abolished by piperine in a concentration dependent fashion. With the highest dose of piperine (50 μg/ml), the (^{3}H) radioactivity measured was slightly above that of Con A treated control cells, i.e. the Cd inhibited mitogenic proliferative response was completely attenuated by piperine. Even the (^{3}H) thymidine incorporation in the logarithimically growing cells, inhibited by Cd was also reversed by piperine and at the highest dose, the inhibitory effect of Cd was completely neutralized.

3.5. *Effect of piperine on Cd altered phenotypic changes*

After establishing significant protection by piperine against Cd induced apoptosis, oxidative stress and blastogenesis, we further investigated the influence of piperine on various T-lymphocyte subpopulations.

Cadmium (25 μM) treatment altered the thymocyte surface marker expression, leading to distinct phenotypic changes which were evident at 18 h and not at 6 h, as shown in Fig. 10. The percentage of $CD4^+$ cells decreased from 10.5 to 4.0 and there was a significant increase in the $CD8^+$ cells. The $CD4^+/CD8^+$ ratio fell from 1.8 in control to 0.17 in the Cd loaded cells. The $CD4^+CD8^+$ (DP) also declined, whereas $CD4^-CD8^-$ (DN) registered a substantial increase (Fig. 9). These phenotypic changes caused by Cd were nullified by piperine. A gradual dose dependent rise in the altered $CD4^+/CD8^+$ ratio was exhibited. The $CD4^+/CD8^+$ ratio from 0.17 in Cd treated cells was elevated to 2.3 in the Cd + piperine (50 μg/ml) loaded cells.

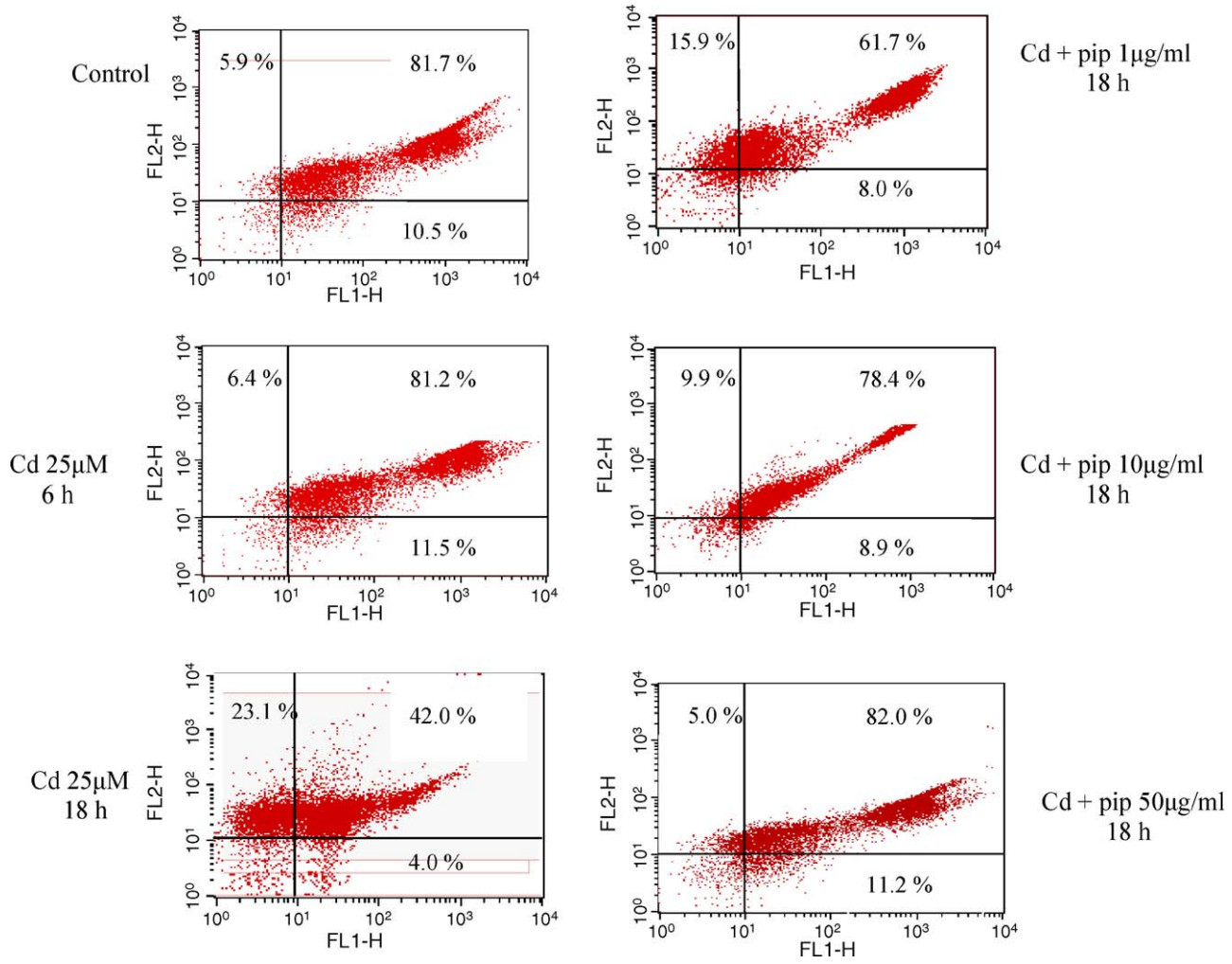

Fig. 10 – Effect of piperine on Cd altered T-cell phenotypic changes. Freshly isolated thymocytes (1.5×10^6) were treated with Cd (25 μM) and piperine (1–50 μg/ml) and the cells were stained with FITC-conjugated anti-CD4 monoclonal antibody and PE-conjugated anti-CD8 monoclonal antibody. The FITC and PE fluorescence were measured using flow cytometer with FL-1 and FL-2 filters, respectively. Results were expressed as dot plot representing as one of the three independent experiments. LL: $CD4^-$ $CD8^-$ cells ($FITC^-/PE^-$); LR: $CD4^+$ cells ($FITC^+/PE^-$); UR: $CD4^+$ $CD8^+$ cells ($FITC^+/PE^+$); UL: $CD8^+$ cells ($FITC^-/PE^+$).

Similarly, the restoration in $CD4^+CD8^+$ (DP) and $CD4^-CD8^-$ (DN) population was also dose related. The Cd-induced phenotypic changes were completely alleviated by the highest dose of piperine (Fig. 10). Piperine could successfully mitigate the Cd-induced phenotypic changes.

Our data clearly indicates that piperine can counteract the deleterious immune effects of Cd such as oxidative stress, apoptosis, blastogenesis and phenotypic alterations.

4. Discussion

Although Cd is not a Fenton metal, increasing evidence suggests that its toxicity is mediated by oxidative stress-induced apoptosis. ROS, acting as intracellular signals prior to mitochondrial membrane depolarization and caspase-3 activatioin has been studied by us in murine lymphocytes [24,25].

This study demonstrates that piperine, the major alkaloid of piper species has potent inhibitory effects on Cd induced apoptosis in murine thymocytes. The anti-apoptotic property of piperine may be attributed to its antioxidant potential, reported both *in vivo* and *in vitro* conditions. Simultaneous supplementation with black pepper or piperine in rats fed high fat diet lowered TBARS and conjugated dienes levels and maintained SOD, CAT, GPX, GST and GSH levels close to controls in rats [14].

Piperine is also shown to exert significant protection against *tert*-butyl hydroperoxide and carbon tetrachloride hepatotoxicity by reducing both *in vitro* and *in vivo* lipid peroxidation, enzymatic leakage of GPT and AP, and preventing the depletion of GSH and total thiols in the intoxicated mice [7]. On the contrary, Panda and Kar [9] observed that 15 days oral administration of 0.25 and 2.5 mg/kg piperine in mice, failed to alter hepatic LPO and the activity of endogenous antioxidants: SOD and CAT. The direct antioxidant activity of

piperine against various free radicals, hydroxyl as well as superoxide, was explored under *in vitro* conditions by Mittal and Gupta [13]. The critial role of ROS and GSH in the apoptogenic potential of Cd shown by us [24,25] and their modulation by antioxidants [36–38] could provide valuable leads in understanding the anti-apoptotic action of piperine in the present study. Suppression of ROS generation by piperine (50 μg/ml) as early as 1.5 h and the restoring trend in GSH depletion at 3.0 h, could be attributed to its antioxidant property. Piperine as such, is non cytotoxic to the thymic cells (MTT assay).

Recently, Sunila and Kuttan [10] demonstrated the immunomodulatory and antitumor activity of *Piper longum* and piperine activity in ascites tumor bearing mice. Piperine showed an increase in bone marrow cellularity and α-esterase positive cells. It also enhanced the circulating antibody titre and plaque forming cells, reflecting stimulatory humoral response. In the present study, the enhancing effect of piperine on thymic functions, i.e. mitogenic response could be implicated as a result of its anti-apoptotic activity. This proposition is not defined at present since inhibition of apoptosis does not necessarily be translated into an increased survival of cells. Lock and Stribinskiene [39] reported that Bcl-2 expressing HeLa cells showed a marked inhibition of etoposide induced apoptosis, but no significant increase in survival when assessed by a colony forming assay.

Apoptosis by Cd as evident in this study, involving down regulation of Bcl-2 protein, lowering of mitochondrial $\Delta\psi$ followed by caspase activation, all suggest Bcl-2 dependent mitochondrial caspase pathway. Similar results were demonstrated in Cd treated rat fibroblast cells [40] and human leukemia cells [41]. Piperine was very successful in ameliorating these events, linked possibly to its anti-oxidant property.

In this work, we observed cadmium induced apoptosis at 6 h followed by phenotypic changes at 18 h. The differential effect on T-subsets by Cd are consistent with the findings of Dong et al. [23]. Various other immunotoxicants such as dioxin and arsenic trioxide are also reported to cause phenotypic alterations in T lymphocytes [42,43]. Thymocytes undergoing apoptosis upregulate a number of cell surface markers [44]. Evident apoptosis observed in the Cd treated thymocytes at 6 h, with no significant phenotypic changes, suggest that such phenotypic alterations could be a result of apoptosis. Cd exposure caused a significant reduction in $CD4^+$ and DP cells and increased proportions of $CD8^+$ and DN cells. Cadmium is therefore, capable of altering thymocyte development and cell phenotypes through the induction of apoptosis in thymocytes [23]. Alleviation of altered phenotypes ($CD4^+$, $CD8^+$, DP and DN) as well as markers of apoptosis by piperine could be suggestive of its anti-apoptotic potential being responsible for restoration of altered T subsets.

In general, protection against acute and chronic Cd toxicity by reducing oxidative stress has been demonstrated by several groups [45–49]. It appears that the various intracellular parameters studied in this investigation, such as ROS and GSH preceding mitochondrial membrane depolarization with concomitant caspase-3 activation followed by DNA damage at 6 h, phenotypic changes at 18 h and blastogenesis not before 72 h, are all interlinked and any modulation of early signals may eventually lead to altered apoptosis, thereby affecting immune function. Such a possibility may exist and the antioxidant property of piperine could be one of the main triggering factors, responsible for mitigation of all subsequent events.

In conclusion, this study clearly demonstrated the antioxidative, anti-apoptotic, and restorative ability against cell proliferative mitogenic response and phenotypic alterations by piperine, suggesting its therapeutic usefulness in immunocompromised conditions.

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