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Mollusc C-reactive protein crosses species barrier and reverses hepatotoxicity of lead in rodent models

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Achatina fulica C-reactive protein (ACRP) reversed the toxic effects of lead nitrate both *in vivo* in mice and *in vitro* in rat hepatocytes restoring the basal level of cell viability, lipid peroxidation, reduced glutathione and superoxides. Cytotoxicity was also significantly ameliorated in rat hepatocytes by *in vitro* pre-treatments with individual subunits (60, 62, 90 and 110 kDa) of ACRP. Annexin V-Cy3/CFDA dual staining showed significant reduction in the number of apoptotic hepatocytes pre-treated with ACRP. ACRP induced restoration of mitochondrial membrane potential was remarkable. ACRP pre-treatment prevented Pb-induced apoptosis mediated by caspase activation. The antagonistic effect of ACRP may be due to scavenging of reactive oxygen species which maintained the homeostasis of cellular redox potential as well as reduced glutathione status. The results suggest that ACRP crosses the species barrier and it may be utilized as a viable exogenous agent of cytoprotection against heavy metal related toxicity.

Keywords: *Achatina fulica,* Apoptosis, C-reactive protein, Lead toxicity, Liver, Oxidative stress, Rodents

The increasing load of environmental pollutants, majorly the heavy metals, poses a foremost threat to liver, the main detoxification centre of vertebrates. Position of lead as a severe hepatotoxic agent compromising the status of human health[1], especially children[2] has been reported. Vertebrate liver is affected deleteriously by several heavy metals among which Pb $(NO_3)_2$ occupies a significant position[3-5]. It is suggested that alteration in lead induced prooxidant/anti-oxidant balance could contribute to tissue injury via oxidative damage to critical biomolecules such as lipids, proteins, and DNA[6]. C-reactive protein (CRP), a prototypic acute phase reactant, is an evolutionary conserved cyclic pentameric protein[7] which maintains its basic properties in distantly related species. It is inducible in vertebrates but expressed constitutively in invertebrates[8]. Additionally, it is known to be a marker for various diseases[9,10] and synthesized in human liver in response to inflammatory stimuli[11]. In mammals, CRP plays an important role in modulation of inflammatory responses, although the mechanism of such an action has remained elusive[12,13]. Moreover, there are several reports on the potentiality of human CRP to inhibit superoxide (O_2^-) generation[14-16] and delay apoptosis in neutrophils[17]. Recently it has been reported that immunepotent CRP modulates antioxidant and anti-inflammatory effects in LPS-stimulated human macrophages[18]. Intravenous injection of human CRP was found to protect mice from death following administration of lethal numbers of *Streptococcus pneumoniae*[19].

Among the invertebrates the role of CRP as a main front-line innate immune molecule in Limulus, an arthropod, is known[20]. In the mollusc, *Achatina fulica* (Bowdich), concentration of endogenous CRP is also high (2-4 mg/mL) in the haemolymph[21]. Considering the evolutionary success of *Achatina* and its falling into disrepute as an agricultural pest in India, it is wondered whether the high level of CRP is the key to its effective survival in the environment. An attempt was made to delineate the anti-stress property of *Achatina* CRP (ACRP) firstly in mice which have a very low level of endogenous CRP (~2 µg/mL) even after an inflammatory stimulus[22]. In order to prove the

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hypothesis, lead nitrate, considered as a major ecological threat was administered intraperitoneally at an environmentally relevant dose in mice. Lead induced oxidative stress in the experimental mice was counteracted by exogenous administration of ACRP prior to Pb treatment. Further confirmation was obtained from *in vitro* studies in rat hepatocytes where the native protein and its subunits were tested for reversal of lead induced hepatotoxicity. A new functional role of ACRP crossing the species barrier as a cytoprotective agent in lead nitrate toxicity in rodents is proposed.

Materials and Methods

Chemicals and antibodies—Anti-C-reactive protein antibody (PA1-9579) and immobilized p-aminophenyl phosphoryl choline gel were purchased from Pierce Chemical Co. (Rockford, USA). Primary antibodies against FasL, caspase-8, Bid, caspase-9, caspase-3 and β-actin were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Mouse anti-rabbit ALP conjugated secondary antibody, Annexin V-Cy3/CFDA apoptosis detection kit (APOAC), Hoechst (Bisbenzimide H 33258), collagenase type-IV and 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT) were procured from Sigma Chemical Co. (St. Louis, MO, USA). DMEM (Dulbecco's Modified Eagle Medium) was obtained from GIBCO-BRL (Invitrogen Corporation, California, USA). FITC labelling kit (Cat.No.343210) was purchased from Calbiochem, EMD Biosciences, USA. JC-1 Mitochondrial Membrane Potential Assay Kit was purchased from Cayman Chemical Company, Ann Arbor, MI, USA. Reduced glutathione (GSH), 1-chloro-2, 4-dinitrobenzene (CDNB), H_2O_2, 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT), Nitro Blue Tetrazolium Salt (NBT) and lead nitrate [Pb $(NO_3)_2$] were purchased from Sisco Research Laboratory (SRL, Mumbai, India). All other chemicals used were of analytical grade.

Maintenance of animals—Adult, healthy, male albino rats of Sprague Dawley strain and albino mice of Wistar strain were maintained according to Inglis[23], under conditions prescribed by the Institutional Animal Ethics Committee. The giant African land snail *Achatina fulica* was maintained according to Bose and Bhattacharya[21].

Affinity purification of CRP from Achatina fulica (*ACRP*)—Haemolymph was collected as per Bose and Bhattacharya[21]. ACRP was affinity purified from haemolymph using p-aminophenyl phosphoryl choline conjugated agarose column[21]. The native and SDS PAGE analyses were carried out with purified ACRP along with the crude haemolymph. The purified ACRP was also checked by Western blot analysis with an anti-rat CRP antibody. Each of the subunits detected by SDS PAGE was eluted separately with elution buffer (50 m*M* Tris-HCl, 150 m*M* NaCl and 0.1 m*M* EDTA; *p*H 7.5) and stored at 4 °C for future use.

In vivo experiments in mice—Mice is an unusual species among mammals in which CRP levels remain <2 µg/mL, even after an acute phase response, whereas in humans mg/mL CRP levels is attainable after an inflammatory stimulus[9]. Therefore adult male albino mice weighing 20±2 g were selected for the *in vivo* experiments and maintained under standard conditions. They were segregated into 4 groups of 5 animals each. Group I, served as control and received intraperitoneal (ip) normal saline. Group II received ip 14.8 mg/kg body weight Pb $(NO_3)_2$ (1/10 LD_{50}) for 7 consecutive days. Group III received ip Pb $(NO_3)_2$ once daily for 7 days and from day 5 onwards ACRP (100 µg/mouse) was co-administered ip with Pb $(NO_3)_2$ until day 7. The mice were sacrificed under humane condition and the liver was dissected out, homogenized in 50 m*M* phosphate buffer, *p*H-7.4 and aliquots were used for biochemical assays as described below. All experiments were conducted under conditions laid down by the Institutional Animal Ethics Committee, Visva Bharati University.

Rat hepatocyte culture and treatment—Hepatocytes were isolated from adult male Sprague-Dawley rats following Ray *et al.*[24]. Isolated hepatocytes (1×10^6 cells/well) were treated with 20 µM of Pb for different time periods (0, 1, 2, 4 and 6 h) keeping a concurrent control under identical conditions. Treatment with 20 µM Pb was on the basis of its occurrence in the environment[25]. In different sets of experiments hepatocytes were pre-treated (pt) with ACRP (10, 20 and 40 µg/mL) and with 10 µg/mL of each of its subunits (S-60, S-62, S-90 and S-110 kDa) for 1 h prior to Pb treatment. On termination of incubation cells were lysed and subjected to various analyses as described below.

Determination of cell viability—Viability of Pb treated rat hepatocytes was checked by Trypan blue dye exclusion and cytotoxicity by MTT assay[26]. All values are expressed as mean ± SE. $P<0.05$ was considered significant.

Hoechst staining—Hoechst staining was performed in control and Pb treated rat hepatocytes by fixing the cells in ice-cold methanol for 10 min and washed with phosphate-buffered saline (PBS). Cells were stained with Hoechst-33258 (1 µg/mL) for 10 min in dark at room temperature (RT) followed by five washes with PBS. Apoptotic cells exhibiting condensed and fragmented nuclei were studied under a Zeiss Axio Scope A1 fluorescence microscope (Carl Zeiss, Germany) in randomly selected fields.

DNA fragmentation assay—Genomic DNA was isolated from control and treated rat hepatocytes by solvent extraction method[27] and loaded on 1% agarose gel containing 0.1% ethidium bromide (200 µg/mL). The gel was electrophoresed and visualized in a Bio-Rad Gel Documentation System using Quantity One software (Bio-Rad, USA).

AnnexinV- Cy3/CFDA staining and determination of apoptotic index—Annexin V-Cy3 staining was performed following the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO, USA) and studied under the fluorescence microscope. Annexin V-positive/6-CFDA positive cells were considered apoptotic; Annexin V-positive/6-CFDA negative cells were considered necrotic and Annexin V-negative/6-CFDA positive were considered as live cells. Apoptotic cells were counted by unbiased and random scoring method. About 100 cells per field were counted selecting at least 5 fields per incubation. All values are expressed as mean ± SE. $P<0.05$ was considered significant.

JC1 staining—JC-1 staining was performed using the JC-1 Mitochondrial Membrane Potential assay kit as per the manufacturer's protocol. Red fluorescence indicated higher mitochondrial potential or live cells as against green, which indicated necrotic or apoptotic cells.

Western blot analysis—Protein (60 µg) from the lysates of control and treated cells were resolved on 10% SDS-PAGE at a constant voltage (60 V) for 2.5 h, and then blotted onto a polyvinylidene fluoride (PVDF) membrane with the help of semi-dry trans blot apparatus (Bio-Rad Trans Blot® SD Cell, USA). The membrane were first incubated with primary antibodies at a dilution of 1:1000 over night at 4 °C, followed by 2 h incubation with corresponding ALP-conjugated secondary antibodies (Sigma-Aldrich, St. Louis, MO, USA) at 1:2000 dilutions with continuous rocking. The immunoreactive bands were detected by using 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium (BCIP/NBT). β actin served as loading control. Immunoblots are representative of five independent experiments.

Localization of Fluorescein isothiocyanate (FITC) labelled ACRP in hepatocytes—In order to detect whether ACRP is localized in the rat hepatocyte membrane it was labelled with FITC using the labelling kit (Calbiochem, EMD Biosciences, USA, Cat.No. 343210) as per the manufacturer's protocol.

Biochemical assays

Liver function tests— Serum from control and treated mice were subjected to liver function analysis[28] using serum glutamate pyruvate transaminase (SGPT) and serum glutamate oxaloacetate transaminase (SGOT) assay kits (Span Diagnostics, Surat, India). Results were expressed as IU/L.

Determination of reduced glutathione (GSH) and glutathione S-transferase (GST) activity—GSH was estimated from aliquots of protein free clear supernatant after trichloroacetic acid (TCA) extraction of homogenized hepatocytes. GSH was detected[29] by 5, 5′-dithiobis-2-nitrobenzoic acid (DTNB) at 405 nm in a Beckman DU 730 (Brea, CA, USA) spectrophotometer. Results were expressed in terms of µg GSH/mg protein. GST activity was measured according to Habig *et al*.[30] and results were expressed in terms of µM DNPG produced/min/mg protein. All data were derived from 5 independent experiments. All values are expressed as mean ± SE. Means were compared by a post hoc multiple range test. $P<0.05$ was considered as significant.

Thiobarbituric acid reactive substances (TBARS) assay—TBARS assay was performed according to the method of Buege and Aust[31]. Briefly, microsomal fraction was heated with TBA–TCA–HCl for 15 min and then centrifuged (Sigma centrifuge, Osterode am Harz, Germany) at 1000 *g* for 10 min. The absorbance of supernatants was measured at 535 nm and expressed in terms of nmoles MDA produced/mg protein. All data were derived from 5 independent experiments. All values are expressed as mean ± SE. $P<0.05$ was considered as significant.

Estimation of catalase activity and superoxide radicals—Catalase activity was determined according to Aebi[32] and expressed as µM H_2O_2 degraded /min/mg protein. The NBT reduction assay was performed to estimate the superoxide radicals[33] and results were expressed as percentage of superoxides generated in different conditions of exposure. All values are expressed as mean ± SE. Means were compared by a post hoc multiple range test. $P<0.05$ was considered as significant.

Statistical analysis—Student's *t*-test or one-way analysis of variance (ANOVA) was used to analyze the significance level of data. A probability of $P<0.05$ was considered as significant. Data are expressed as mean ± SE of 5 independent experiments.

Results

In vivo hepatoprotective action of ACRP in mice—Affinity purification of *Achatina* CRP (ACRP) was done through a phosphoryl choline affinity column (Fig. 1a) and purity of the protein was checked in the native gel (Fig. 1b) and immunoblot with anti-rat CRP antibody that showed cross reactivity with ACRP (Fig. 1c). In the present investigation, ameliorating effect of ACRP was checked *in vivo* in Pb treated mice. Mice were treated without (control) or with Pb $(NO_3)_2$ or Pb $(NO_3)_2$ plus ACRP for 7 days. Liver function indices such as SGPT and SGOT ($P<0.05$) were significantly elevated in Pb $(NO_3)_2$ treated mice in comparison to the control (Fig. 2a). Interestingly, ACRP co-treatment in Pb $(NO_3)_2$ treated mice ($P<0.05$) significantly restored the SGPT and SGOT levels.

Administration of Pb $(NO_3)_2$ effected marked depletion of liver glutathione and elevation of lipid peroxidation products. Concomitant activity of the antioxidant enzymes, glutathione-s-transferase (GST) and catalase also decreased significantly ($P<0.05$) in Pb $(NO_3)_2$ treated mice as against the control. As observed in respect of liver function indices (Fig. 2a), ACRP co-treatment not only reversed liver glutathione and TBARS levels (Fig. 2b) but also significantly restored {($P<0.05$ vs Pb $(NO_3)_2$} catalase (Fig. 2c) and GST (Fig. 2d) activities.

ACRP and ACRP subunits prevented Pb induced cell death in rat hepatocytes—Isolated rat hepatocytes incubated with 20 µM Pb showed a gradual decrease in cell viability recording highest mortality at 6 h (70.02±4%) as estimated by Trypan blue dye exclusion test. Pre-treatments with ACRP at different doses of 10, 20 and 40 µg/mL followed by Pb treatments reduced the rate of cell death significantly ($P<0.05$). Since 20 µg/mL of ACRP was the most potent dose in ameliorating Pb induced damage (data not shown) further experiments were performed with 20 µg/mL of ACRP. Moreover, pre-treatments with individual subunits of 60, 62, 90 and 110 kDa (each at 10 µg/mL) for 1 h followed by Pb treatments also reduced (Fig. 3a) the rate of cell death significantly ($P<0.05$, vs Pb). MTT assay demonstrated 68.3±1.3% of cell viability in Pb treated and 78.36±1.5% in ACRP pre-treated hepatocytes. Pre-treatments with individual subunits of 60, 62, 90 and 110 kDa also resulted in 79±1.8, 80±1.3, 83.25±1.0 and 82.59±1.2% cell viability respectively at 6 h suggesting a protective role of both ACRP and its subunits (Fig. 3b).

Assessment of ROS mediated cell damage in Pb treated hepatocytes— Level of intracellular pool of GSH was estimated in Pb and ACRP (pt) plus Pb treated hepatocytes. A sharp depletion in GSH (45%) occurred between 0-6 h of incubation with Pb alone as compared to control ($P<0.05$). ACRP pre-treatment significantly restored (Fig. 4a) the Pb induced depletion in GSH pool ($P<0.05$). Profile of lipid peroxidation in Pb treated hepatocytes demonstrated a significant time dependent increase in MDA

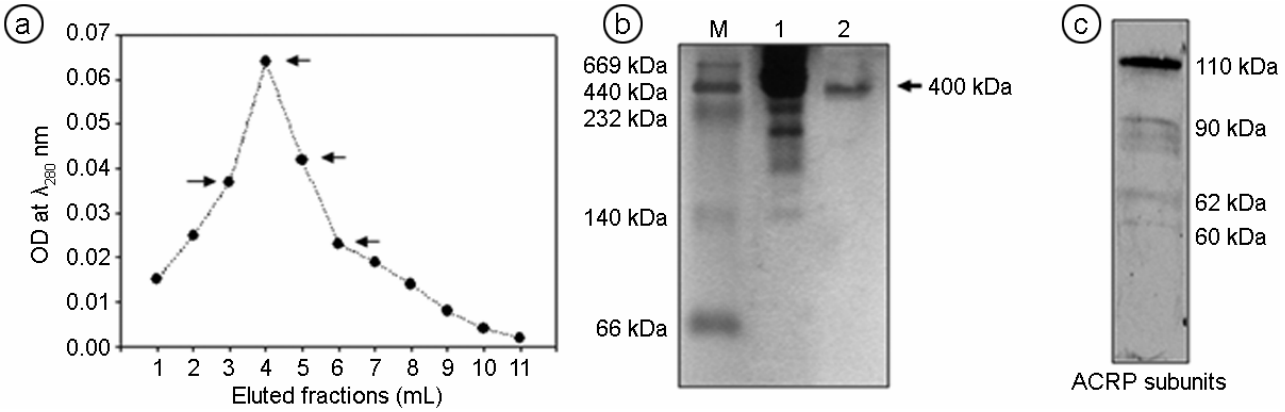

Fig. 1—Affinity purification of ACRP (a) haemolymph was collected from *Achatina fulica* and affinity purification was carried out through phosphorylcholine agarose column. Eluted fractions were collected (arrows) in tubes and pooled for further experiments, (b) pooled fractions were dialyzed and subjected to native-PAGE. Gel was stained with Coomassie and arrow indicates the affinity purified band of ACRP, (c) affinity purified protein was then loaded on to SDS-PAGE and immunoblotted with anti-rat CRP antibody. Corresponding bands of four distinct ACRP subunits could be detected in the blot [M= marker, 1= crude heamolymph, 2= affinity purified CRP].

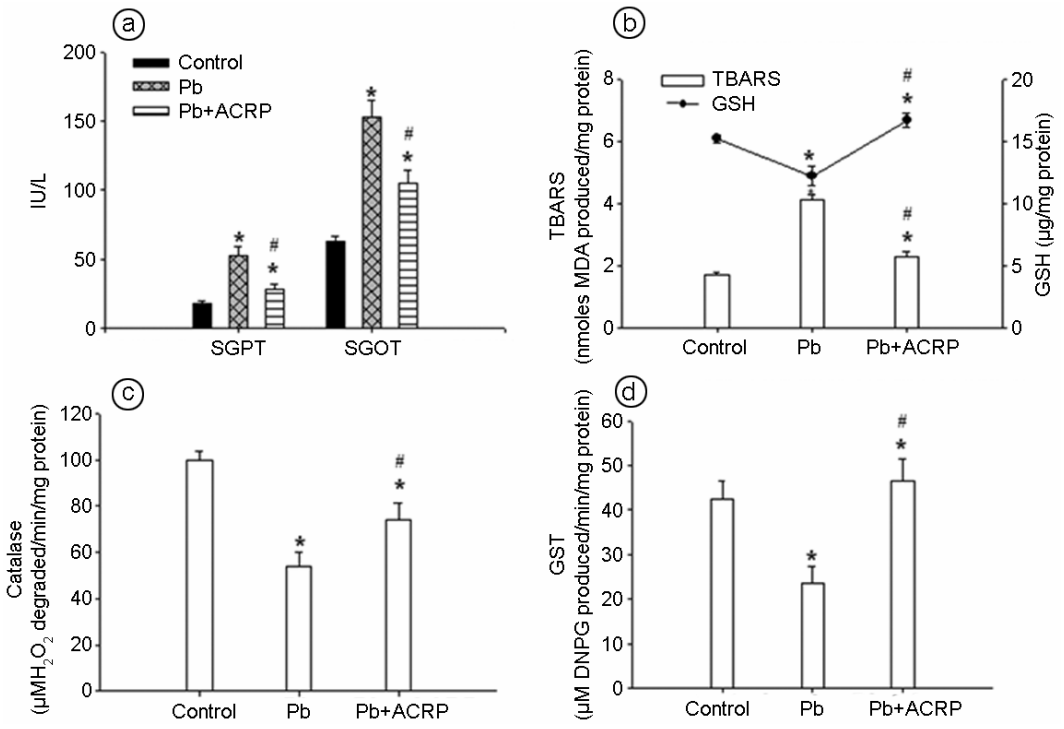

Fig. 2—Effect of ACRP in mice exposed to Pb $(NO_3)_2$. (a) Serum SGPT and SGOT levels in mice without (control) or treated with Pb $(NO_3)_2$ or Pb $(NO_3)_2$ co-treated with ACRP after 7 days of exposure, (b) GSH and TBARS in the liver of untreated (control) and Pb $(NO_3)_2$ treated or Pb $(NO_3)_2$ and ACRP co-treated mice, (c) catalase activity in the liver of untreated (control) and Pb $(NO_3)_2$ treated or Pb $(NO_3)_2$ and ACRP co-treated mice, (d) GST activity in the liver of untreated (control) and Pb $(NO_3)_2$ treated or Pb $(NO_3)_2$ and ACRP co-treated mice. [Values are mean ± SE of three independent experiments. **P*<0.05 represents comparison between control and Pb $(NO_3)_2$ treated groups. #*P*<0.05 represents comparison between Pb $(NO_3)_2$ and ACRP plus Pb $(NO_3)_2$ co-treated mice. n=5 for each group]

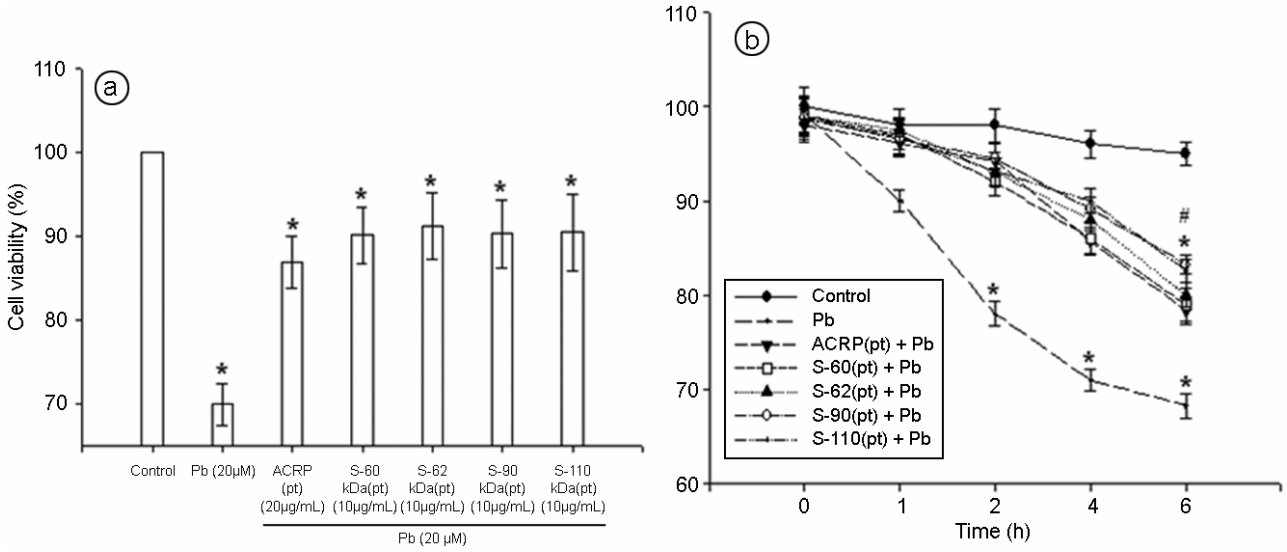

Fig. 3—Determination of cell viability in rat hepatocytes. (a) hepatocytes were treated for 6 h without (control) or with Pb (20µM) or pre-treated with 20 µg/mL ACRP or 10µg/mL of each of the four ACRP subunits (S-60, S-62, S-90 and S-110) followed by Pb treatment. Cells were incubated with ACRP or the subunits 1 h prior to Pb treatment and viability was assessed by Trypan blue dye exclusion test, (b) cell viability checked by MTT assay: cells were incubated without (control) or with Pb (20 µM) or pre-treated with 20 µg/mL ACRP or 10µg/mL of each of the four ACRP subunits (S-60, S-62, S-90 and S-110) followed by Pb treatment for 0 ,1 ,2 ,4 , and 6 h. Values are mean ± SE of 5 independent experiments. **P*<0.05 represents comparison between control and Pb $(NO_3)_2$ treated groups. #*P*<0.05 represents comparison between Pb $(NO_3)_2$ and ACRP plus Pb $(NO_3)_2$ co-treated mice. n=5 for each group.

production (P<0.05) as compared to the control (Fig. 4b). A significant reduction in MDA level was noted in ACRP pre-treated cells at 4 and 6 h (P<0.05) compared to Pb treated cells again indicating a protective role of ACRP. Pb induced generation of superoxide anions in hepatocytes was assessed by NBT formazan assay. The changes in NBT formazan deposition and its content in the hepatocytes demonstrated a time dependent increase (from 0-6 h) in the Pb treated hepatocytes (Fig. 4c) which was significantly reduced (about 30% at 6 h; P<0.05) by ACRP pre-treatment.

In the present investigation, localization of FITC labelled ACRP in rat hepatocytes was studied to address whether ACRP enters inside the cells. It was clearly depicted that FITC labelled ACRP is localized in hepatocyte membrane and cytosol (presented as green fluorescence) and counter staining with propidium iodide (showing orange fluorescence) demonstrated the intact nucleus (Fig. 4d).

In vitro amelioration of Pb induced oxidative damage by ACRP subunits—Level of intracellular pool of GSH was estimated in Pb treated and ACRP

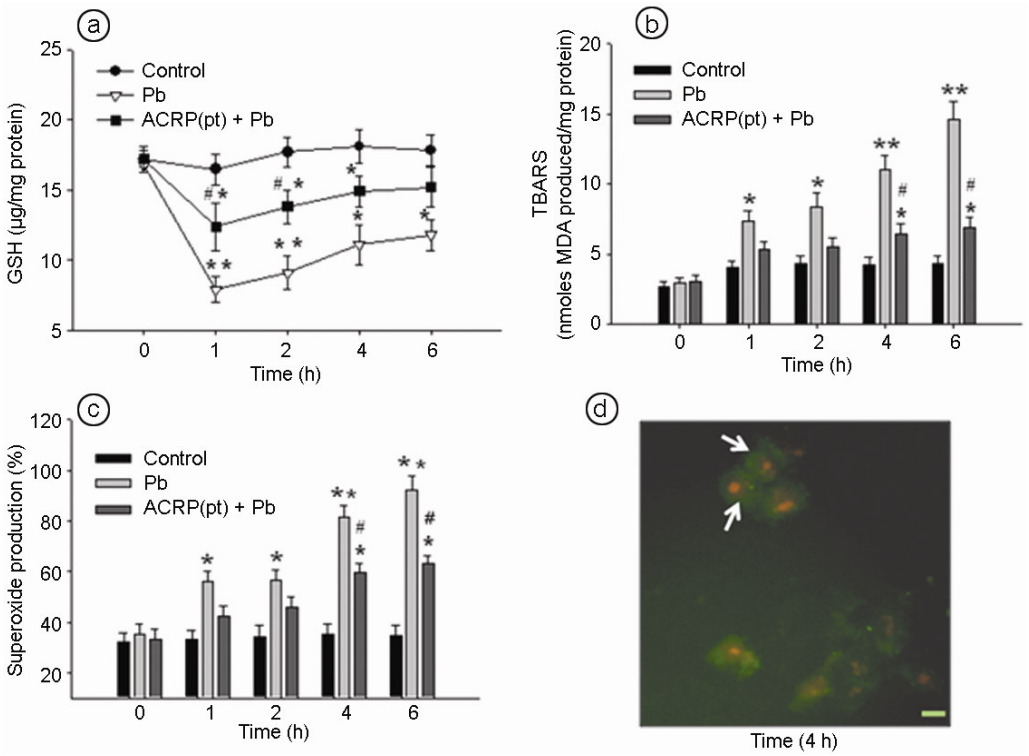

Fig. 4—Levels of GSH, TBARS and superoxide anion and localization of ACRP in rat hepatocytes. (a) GSH content (µg GSH/mg protein) in control or Pb treated (20 µM) or 20 µg/mL ACRP (pt) followed by Pb treatment, (b) TBARS measured in terms of MDA concentration (nmoles MDA produced/mg protein) in control or Pb treated (20 µM) or 20 µg/mL ACRP (pt) followed by Pb treatment, (c) percentage of superoxide anion generation was determined by NBT assay in control or Pb treated (20 µM) or 20 µg/mL ACRP (pt) followed by Pb treatment. Values are mean ± SE of 5 independent experiments. P values: *<0.05, **<0.05 (vs control), #<0.05(vs Pb), (d) ACRP (20µg/mL) was labelled with FITC following the manufacturer's protocol. After 4h intracellular localization of ACRP was studied under a fluorescence microscope (arrows indicate localized ACRP in the hepatocytes). Bar = 20 µm.

Table 1— GSH level (µg GSH/mg protein) in rat hepatocytes at different intervals (h) of incubation with Pb (20µM) alone and in hepatocytes pre-treated for 1 h with 10µg of each ACRP subunit

[Values are mean ± SE of 5 independent experiments]

System	0	1	2	4	6
Untreated	19.50±2.0	20.71±1.9	20.12±2.0	21.84±2.3	21.02±1.9
Pb treated	16.90±1.6	07.91±0.9	08.50±1.1	10.93±1.3	13.61±1.6
ACRP S-60 (pt) +Pb	17.20±1.9	14.02±1.5	16.06±1.8	17.02±1.8	17.42±1.9
ACRP S-62 (pt)+ Pb	16.50±1.8	15.85±1.7	17.86±1.6	18.03±1.6	18.72±1.7
ACRP S-90 (pt)+ Pb	16.80±1.9	16.62±1.9	18.35±1.9	18.62±1.9	19.32±1.8
ACRP S-110 (pt)+Pb	17.50±1.8	15.01±1.8	17.54±1.6	18.83±1.8	19.05±1.6

subunit (pt) plus Pb treated hepatocytes. Intracellular GSH level significantly decreased with time, recording the lowest level at 1 h of incubation against control ($P<0.05$). Pre-treatment with ACRP subunits restored GSH level towards the control (Table 1). Depletion of GSH pool leads to a significant time dependent increase in MDA production in Pb treated hepatocytes ($P<0.05$) as compared to the control (Table 2). A significant reduction in MDA level was noted in ACRP subunits pre-treated cells from 2 h onwards ($P<0.05$) compared to Pb treated cells indicating a protective role of the subunits against Pb induced cell damage. Generation of O_2^- was also measured by NBT assay where it was found that NBT formazan deposition in the hepatocytes demonstrated a time dependent increase (from 0-6 h) in the Pb treated hepatocytes (Table 3) which was significantly reduced ($P<0.05$) by pre-treatment with each of the ACRP subunits.

Pattern of DNA fragmentation and apoptosis—Nuclear damage was assessed by Hoechst staining of control, Pb treated and ACRP (pt) plus Pb treated hepatocytes at 6 h. There was no DNA damage in untreated cells. Contrastingly, nuclear degradation was prominent in Pb treated cells, which was reversed by ACRP pre-treatment (Fig. 5a). The same pattern was revealed by characteristic genomic DNA fragmentation in Pb treated hepatocytes at 4 h and 6 h. In untreated or ACRP (pt) plus Pb treated cells no DNA fragmentation or ladder-type pattern was observed (Fig. 5b).

Apoptotic and non-apoptotic cells were distinguished by Annexin V-Cy3/CFDA dual staining. Increased number of apoptotic cells was visualized in Pb treated cells as against the control or ACRP (pt) plus Pb treated hepatocytes, where lesser number of apoptotic cells and higher number of CFDA positive cells (live cells) were observed (Fig. 5c). Apoptotic index was calculated by unbiased and random scoring method. A significant number of apoptotic cells were found in Pb treated hepatocytes ($P<0.05$) compared to the ACRP pretreated or control cells (Fig. 5d).

Mitochondrial membrane potential maintained by ACRP treatment—Mitochondrial membrane potential is a critical parameter to assess mitochondrial function which reflects cellular health. Changes in the mitochondrial membrane potential were determined by JC1 staining. In untreated cells, intense red fluorescence indicated normal membrane potential, while in Pb treated cells loss of mitochondrial membrane potential was denoted by the appearance of green fluorescence. ACRP pre-treatment maintained the membrane integrity as demonstrated by enhanced number of red fluorescent cells as compared to the hepatocytes treated with Pb alone (Fig. 6a).

ACRP inhibited Pb-induced apoptosis, mediated by both extrinsic and intrinsic apoptotic pathways. The level of FasL progressively increased in Pb treated cells over time showing maximum rise at 6 h. However, in control and ACRP pre-treated cells, the level of FasL did not differ and remained almost unchanged over the entire incubation period (Fig. 6b).

Table 2— TBARS level (nmoles MDA produced/mg protein) in rat hepatocytes at different intervals (h) of incubation with Pb (20µM) alone and in hepatocytes pre-treated for 1 h with 10µg of each ACRP subunit

[Values are mean ± SE of 5 independent experiments]

System	0	1	2	4	6
Untreated	2.43±0.50	2.31±0.40	2.60±0.43	2.69±0.31	2.60±0.36
Pb treated	2.67±0.44	5.89±0.97	7.53±0.68	7.93±0.62	9.61±0.91
ACRP S-60 (pt) +Pb	2.69±0.76	4.57±0.79	4.70±0.46	4.99±0.52	5.42±0.59
ACRP S-62 (pt)+ Pb	2.71±0.61	4.61±0.35	4.83±0.52	5.53±0.67	5.72±0.72
ACRP S-90 (pt)+ Pb	2.44±0.65	3.81±0.43	4.35±0.66	4.92±0.54	5.36±0.61
ACRP S-110 (pt)+Pb	2.56±0.80	4.67±0.80	4.54±0.52	4.56±0.52	6.05±0.60

Table 3— NBT assay (% of superoxides) in rat hepatocytes at different intervals (h) of incubation with Pb (20µM) alone and in hepatocytes pre-treated for 1 h with 10µg of each ACRP subunit

[Values are mean ± SE of 5 independent experiments]

System	0	1	2	4	6
Untreated	21.93±2.80	23.19±2.00	26.43±2.43	26.33±2.11	26.98±3.60
Pb treated	45.80±4.10	57.96±5.11	75.23±7.11	79.38±7.02	96.20±9.11
ACRP S-60 (pt) + Pb	45.21±4.31	45.50±4.29	47.17±4.36	49.75±4.12	54.13±4.92
ACRP S-62 (pt)+ Pb	44.23±4.11	46.13±4.15	48.44±4.52	50.30±4.67	57.01±5.27
ACRP S-90 (pt)+ Pb	36.40±4.00	38.23±3.43	44.05±4.16	49.20±4.54	53.27±4.61
ACRP S-110 (pt)+Pb	38.10±3.90	46.07±4.30	45.46±4.12	45.78±3.82	60.26±5.80

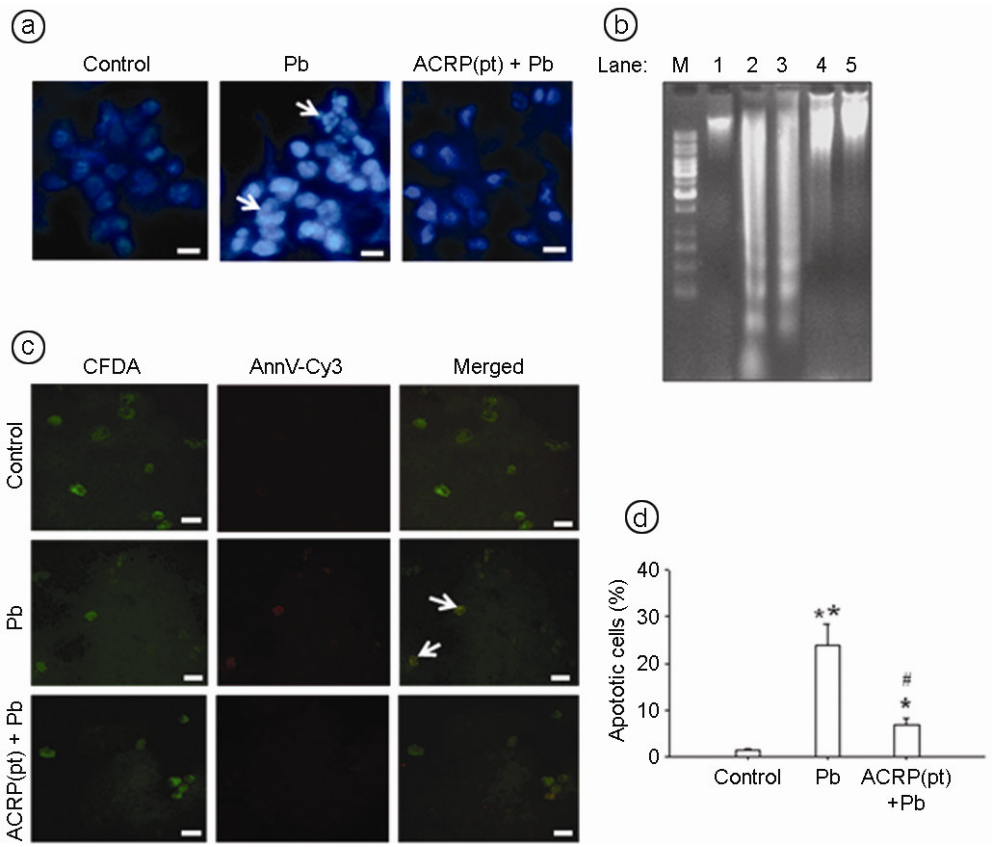

Fig. 5—Nuclear breakdown, DNA fragmentation, and apoptosis in rat hepatocytes. (a) hepatocytes were incubated without (control) or with Pb (20 µM) or 20 µg/mL ACRP (pt) followed by Pb for 6 h. Cells were fixed in 4% paraformaldehyde followed by Hoechst staining. White arrows indicate disintegrated nuclei in Pb treated hepatocytes. Bar = 20 µm, (b) DNA ladder formation in treated and untreated rat hepatocytes, (c) control and treated hepatocytes (as mentioned in the figure) were subjected to AnnexinV-Cy3-CFDA dual staining. CFDA positive cells were considered as live, AnnexinV-Cy3 positive cells as necrotic and AnnexinV-Cy3/CFDA dual positive cells as apoptotic. Arrows indicate apoptotic cells in the merged image of Pb treated cell population. Bar = 20 µm, (d) cells were counted by unbiased and random scoring method. About 100 cells per field were counted selecting at least 5 fields per incubation. Values are mean ± SE of 5 independent experiments. P values: *<0.05, **<0.05 (vs control), #<0.05(vs Pb), Lanes: M=marker, 1= control, 2= Pb (4h), 3= Pb (6h), 4=ACRP (pt) + Pb (4h), 5= ACRP (pt) + Pb (6h).

Caspase-8, the initiator caspase in death receptor signalling pathway was not activated by Pb treatment within 1 h of incubation and did not vary much from the control. However, by 2 h a clear expression of caspase-8 was noted followed by a sharp elevation at the end of the incubation indicating that caspase-8 was fully activated. Contrastingly, the expression of active caspase-8 in ACRP pre-treated cells was significantly diminished as compared to cells treated with Pb alone (Fig. 6b). Activated caspase-8 can cleave Bid, a 22-kDa proapoptotic protein that translocates to the mitochondria for further processing of apoptosis[34]. The results of the present study depicted that processing of intact Bid to truncated Bid (tBid) occurred in Pb treated cells from 1 h onwards that reached its peak at 6 h indicating activation of mitochondrial apoptotic pathway. In contrast, in ACRP pre-treated cells, level of intact Bid was unchanged and tBid was almost undetectable (Fig. 6b). Caspase-9, which is activated by truncated Bid, was detectable at 1 h and subsequently increased during the incubation which was directly correlated to the Pb-induced reduction of procaspase-9. Substantial processing of procaspase-9 was evidenced at 2 h with the detection of the active caspase-9 that continued to exist at 6 h. (Fig. 6c). Caspase-3 is an executioner caspase that can be activated by a mitochondrial pathway involving caspase-9 or a death receptor pathway involving caspase-8[35]. The immunoblot data showed activation of procaspase-3 to form caspase-3 only from 2 h onwards reaching the highest level at 6 h. No detectable bands of activated caspase-9 and caspase-3 were noted on ACRP pre-treatment, clearly indicating absence of apoptosis (Fig. 6c).

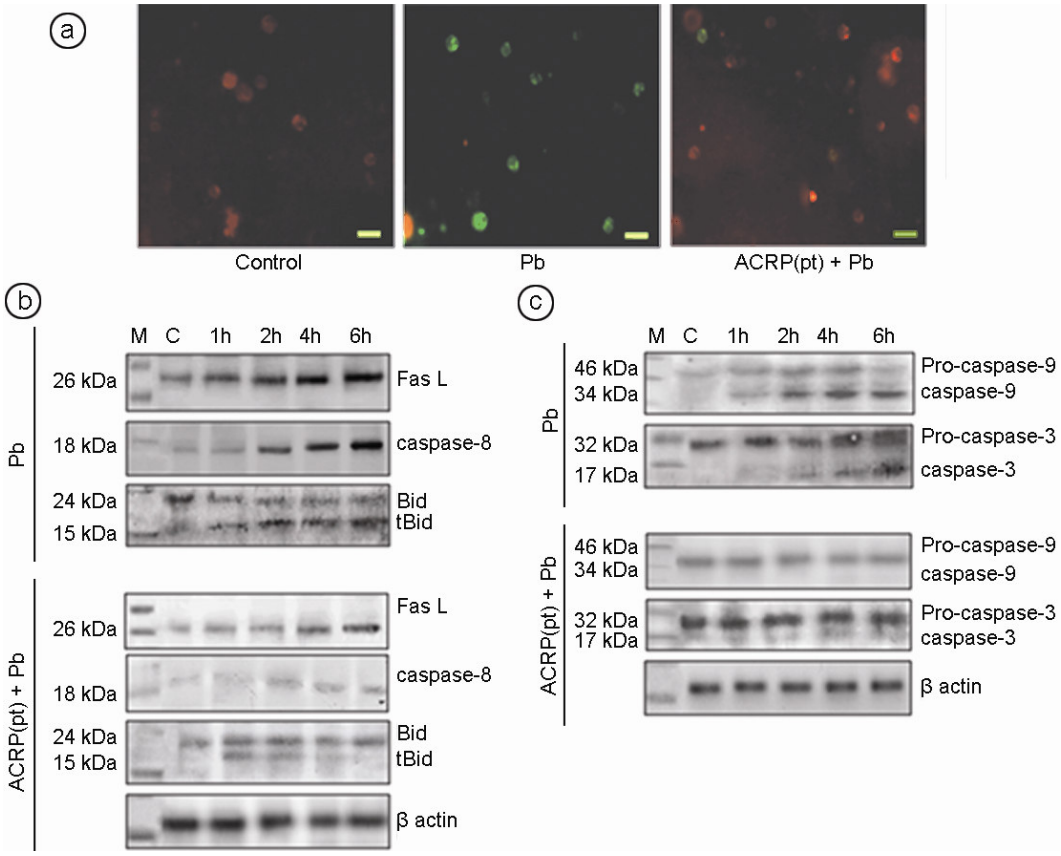

Fig. 6—Mitochondrial permeability transition assay and caspase activation in rat hepatocytes. (a) hepatocytes were treated without (control) or with Pb (20 µM) or 20 µg/mL ACRP(pt) followed by Pb for 4 h. Intensity of JC-1 staining was followed to differentiate the level of mitochondrial membrane disruption in different cells. Untreated (control) cells showed red fluorescence indicating normal hepatocytes, Pb treated apoptotic cells emitted green fluorescence and ACRP pre-treated cells showed significant number of live cells similar to the untreated set which emitted red fluorescence. Bar = 20 µm, (b) hepatocytes were incubated without (control) or with Pb (20 µM) or 20 µg/mL ACRP (pt) followed by Pb for 6 h. Lysates were prepared and subjected to immunoblot analyses of Fas L, caspase-8, and Bid using the respective antibodies, (c) cells from the same incubations as stated above were used for immunoblotting with anti-caspase-9 and anti-caspase-3 antibodies. β actin served as loading control. Immunoblots are representative of 5 independent experiments.

Discussion

Mammalian CRP is a multifunctional plasma protein which increases rapidly during acute phase response by as much as 1000 fold above the normal concentration following tissue injury or infection precipitating immunological responses[36]. CRP was found to be one of the major components of the haemolymph of *A. fulica* with a normal level of approximately 2 mg/mL haemolymph[37]. CRP from *Achatina fulica* has been purified earlier exploiting its phosphorylcholine binding property as found in higher vertebrates[21]. Some of the functions of the ACRP can be linked to the higher vertebrate CRPs which play significant role in removal of necrotic tissue, damaged chromatin material[38] and in host defence mechanism[39]. It may therefore be presumed that CRP originated as a multifunctional protein and retained its basic function in invertebrates and vertebrates[40]. The role of *Achatina* CRP as a cytoprotective agent in the mammalian system is reported here. The *in vivo* results in mice correlated well with the *in vitro* findings in rat where ACRP was able to counter Pb induced toxicity. ACRP being a large protein a question arose whether it enters the cell to trigger cellular activity as recorded in the present study. Accordingly, it has been confirmed by FITC labelling that localization of ACRP is restricted to the cytoplasm of the hepatocytes.

It is probable that a dramatic drop in intracellular GSH pool in Pb treated hepatocytes triggered the cells towards oxidative damage accelerating apoptosis[41]. The depletion in intracellular GSH, being one of the indices of oxidative stress, indicates profound scavenging of O_2^- radicals; however, utilization of

GSH beyond a critical level has also been found to drive the cells towards apoptosis[42]. It was noted that both native ACRP and its subunits revert Pb induced free radical generation *in vitro* by lessening the fall in the intracellular GSH pool and protecting the cells from subsequent oxidative damage. As evidenced by Trypan blue dye exclusion and MTT assay, present data reveal that ACRP and its subunits induce a remarkable increase in the rate of cell viability in Pb treated hepatocytes. In line with enhanced formation of ROS, the rate of lipid peroxidation, as assessed by the level of MDA production, was significantly enhanced in Pb treated hepatocytes which too was remarkably reduced by ACRP pre-treatment.

The intracellular level of superoxide anions was also measured by NBT formazan assay. The results clearly depicted that ACRP and ACRP subunits exert significant inhibitory influence on O_2^- generation when hepatocytes were pre-incubated with ACRP and its subunits prior to Pb treatment. However, there are conflicting reports on the effect of human CRP on O_2^- generation[43].One report indicates that human CRP decreases the production of O_2^- in different cell types, while others demonstrated an increase in O_2^- and H_2O_2 production after prolonged exposure to CRP[44,45]. However, it has also been found that CRP at a concentration higher than 10 µg/mL could suppress superoxide generation[14]. The present findings provided corroborative evidence that 20 µg/mL of ACRP suppresses superoxide generation in Pb treated hepatocytes. Moreover, it has been demonstrated that anti-inflammatory and antioxidant activities of immunepotent CRP are mediated by its capacity to modulate inflammatory and oxidant metabolites through IκB/NF-κB pathway in LPS stimulated human macrophages[18]. It is thus opined that during inflammation and tissue injury CRP may have an anti-oxidant action limiting the effect of free radicals.

The restoration of GSH pool and removal of oxidative stress by the molluscan CRP in the mammalian system could be a receptor mediated endocytotic event as reported in human macrophages[46]. Earlier reports demonstrated lysosomal degradation of CRP to small peptides which play a major role in scavenging superoxides[47]. Moreover, localization of ACRP in the cytoplasm of rat hepatocytes as evidenced in the present study indicates that ACRP might be degraded into small peptides or subunits that ultimately scavenge Pb induced superoxides. However, the exact mechanism of such an action of ACRP warrants further research.

DNA fragmentation being a hallmark of caspase mediated apoptosis was also checked and monitored for any trace of nuclear fragmentation or DNA damage inflicted by Pb treatment. The present results on DNA fragmentation assay along with Hoechst staining clearly demonstrated structural degradation of nucleus along with subsequent fragmentation of genomic DNA due to Pb which was strongly inhibited by ACRP. AnnexinV-Cy3/CFDA dual staining showed increase in the ratio of live (CFDA positive)/apoptotic (dual stained) cells thereby confirming its cytoprotective role.

It is reported here that ACRP suppressed Pb induced extrinsic and intrinsic pathways of apoptosis effectively. It is well known that Fas induced apoptosis depends on the activation of caspase cascades along with the expression of death-domain receptors[42]. In the present study an enhanced expression of FasL with Pb treatment and subsequent activation of caspase-3, the final executioner of apoptosis, via caspase-9 and caspase-8 was found. FasL is known to be firmly associated with activation of caspase-8 and cleavage of Bid into tBid. tBid results mitochondrial pore formation and subsequent release of cytochrome c that initiates the process of apoptosis[48]. Interestingly, present findings clearly indicate that ACRP pre-treatment significantly reduces FasL expression in Pb treated rat hepatocytes and appreciably inhibits the caspase-8 and Bid mediated death signal.

Human CRP can reduce the bacterial load in mice which clearly indicates that CRP can successfully retain its protective function crossing the inter species barrier[49, 50, 19]. The present findings strengthen the anti stress property of an invertebrate CRP (ACRP) against Pb induced oxidative damage in mammalian hepatocytes. In conclusion, our findings clearly indicate the versatility of ACRP action against ROS mediated stress that otherwise affects cell mortality. Adequate availability of this protein, in *Achatina*, designated as a pestiferous snail, may be exploited further for functional characterization and to explore the underlying mechanism of its cytoprotective function.

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