Title: Exposure to As, Cd and Pb-mixture induces Aβ, amyloidogenic APP processing and cognitive impairments via oxidative stress-dependent neuroinflammation in young rats

Running title: Metal mixture induces early-onset AD-like pathology

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Abstract

Environmental pollutants act as risk factors for Alzheimer’s disease (AD), mainly affecting the aging population. We investigated early manifestations of AD-like pathology by a mixture of arsenic (As), cadmium (Cd) and lead (Pb), reported to impair neurodevelopment. We treated rats with As+Cd+Pb at their concentrations detected in groundwater of India, i.e., 0.38ppm, 0.098ppm and 0.22ppm or ten times of each respectively, from gestation-05 to postnatal day-180. We identified dose-dependent increase in amyloid-beta (Aβ) in frontal-cortex and hippocampus as early as post-weaning. The effect was strongly significant during early-adulthood, reaching levels comparable to an Aβ-infused AD-like rat model. The metals activated the pro-amyloidogenic pathway, mediated by increase in amyloid precursor protein (APP), and subsequent beta secretase (BACE) and presenilin (PS)-mediated APP-processing. Investigating the mechanism of Aβ-induction revealed an augmentation in oxidative stress-dependent neuroinflammation that stimulated APP expression through interleukin-responsive-APP-mRNA 5’-untranslated region. We then examined the effects of individual metals and binary-mixtures in comparison with the tertiary. Among individual metals, Pb triggered maximum induction of Aβ, whereas individual As or Cd had a relatively non-significant effect on Aβ despite enhanced APP, owing to reduced induction of BACE and PS. Interestingly, when combined the metals demonstrated synergism, with a major contribution by As. The synergistic effect was significant and consistent in tertiary mixture, resulting in the augmentation of Aβ. Eventually, increase in Aβ culminated in cognitive-impairments in the young rats. Together, our data demonstrates that exposure to As+Cd+Pb induces premature manifestation of AD-like pathology that is synergistic, and oxidative stress and inflammation-dependent.

Key words: Environment, heavy metals, early onset, synergistic, AD-like pathology
Introduction

Alzheimer’s disease (AD) is the most prevalent form of senile dementia, mainly affecting the aging population (Small et al., 1997, Fratiglioni et al., 1999). Majority of the AD cases (~90%) are sporadic, where environmental pollutants act as important risk factors (Dosunmu et al., 2007). Metals are ubiquitous contaminants present as mixtures; and particularly, a mixture of arsenic (As)-cadmium (Cd)-lead (Pb) is among the major toxic agents found in environment (Martin et al., 2011). Human studies find a correlation between ground water As exposure and poorer score in global cognition that reflect the earliest manifestations of AD (O’Bryant et al., 2011). An increase in plasma Cd levels is reported in patients with Dementia of the Alzheimer type (Basun et al., 1991), and epidemiologic studies indicate association between epigenetics, late-onset AD and Pb-exposure (Bakulski et al., 2012).

Pathological hallmarks in AD include the amyloid beta proteins, Aβ1-42 and less amyloidogenic Aβ1-40, generated through the processing of amyloid precursor protein (APP) by β-secretase (BACE) and γ-secretase in frontal cortex and hippocampus (Hardy and Selkoe, 2002, Selkoe and Schenk, 2003). Aβ accumulation in brain disrupts neuronal activity that evokes progressive loss of cognition (Dodart et al., 2000, Pozueta et al., 2013). Arsenic is reported to affect expression and processing of amyloid precursor protein (APP) in neuronal cells (Dewji et al., 1995, Zarazua et al., 2011). Cadmium reduces non-amyloidogenic processing of APP (Li et al., 2012), and Pb co-localizes within and facilitates amyloid plaque formation in transgenic mouse brain (Gu et al., 2012). Neonatal Pb exposure is also reported to promote late-onset amyloidosis in aging rodent and monkeys (Basha et al., 2005a, Basha et al., 2005b, Wu et al., 2008). However, none of the studies bring forth the effect of As+Cd+Pb-mixture on AD pathogenesis. In addition, effect of developmental exposure to these metals on the AD marker proteins at early-age remains un-investigated.
Inflammation plays a key role in onset and progression of sporadic AD (Rogers and Lahiri, 2004). The activated astrocytes and microglia over-express interleukin-1 (IL-1), promoting APP expression through an IL-responsive element of APP mRNA 5’- untranslated region (5’UTR) (Rogers et al., 1999). In addition to neuroinflammation, metal catalyzed oxidative stress caused by decrease in antioxidant enzyme activity and an increase in lipid peroxidation has been strongly associated with neurotoxicity in AD (Smith et al., 1991, Casado et al., 2008). Nevertheless, despite reports on AD-like pathology by As, Cd or Pb, the studies rarely relate to the role of neuroinflammation and oxidative stress.

We reported that gestation followed by postnatal exposure to a mixture of As, Cd and Pb at ground water relevant doses of India induced synergistic toxicity in neuroglia of developing rats (Rai et al., 2010, Rai et al., 2013b, Rai et al., 2014). Neuroglial degeneration is an important component of Aβ toxicity (Heneka et al., 2010, Alobuia et al., 2013), and therefore we hypothesized an early-onset AD-like neuropathology upon developmental exposure to As+Cd+Pb-mixture. We examined generation of Aβ, and effects on the APP-amyloidogenic pathway. We compared the pro-amyloidogenic effects of individual metals and binary mixtures in relation to the tertiary. We evaluated involvement of inflammation and oxidative stress in inducing APP and Aβ. Finally, we assessed whether the metal mixture affected cognitive performances in the rats. Overall, our report provides detailed characterization of early manifestations of AD-like pathology in young rats exposed to As+Cd+Pb-toxicant mixture.

Materials and Methods

Reagents and antibody
Reagents and kits

Sodium arsenite, cadmium chloride, lead acetate, protease inhibitor cocktail, poly-L-lysine, mammalian tissue protein extraction reagent, reagents required for oxidative stress assay, MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide], 3-Aminopropyl triethoxy-silane, Hoechst 33258 stain, and Triton X-100 were procured from Sigma Chemical Co (St. Louis, MO). Protein ladder for western blotting was purchased from Invitrogen (Carlsbad, CA). The supersignal west femto maximum sensitivity substrate for western blotting was purchased from PIERCE Biotechnology (Rockford, IL). Memantine (Brand name, Admenta) and donepezil (Brand name, Aricep 10) were purchased from Sun Pharma, Sikkim and Eisai Co. Ltd respectively. α-tocopherol was purchased from Sigma Chemical Co. (St. Louis, MO). Rat recombinant Aβ1-42 peptide was purchased from Tocris biosciences (Bristol, United Kingdom) and BACE enzyme activity kit was purchased from Abcam (Cambridge, MA). Rat recombinant IL-1 receptor antagonist (IL-1Ra) was purchased from R&D systems (Minneapolis, MN, USA). Diaminobenzidin tetrahydrochloride (DAB) substrate kit, Vectashield medium and Elite ABC kit were purchased from Vector Laboratories (Burlingame, CA). DMEM/F-12, Neurobasal media, antibiotic-antimycotic, fetal bovine serum (FBS) and Trypsin-EDTA were procured from Gibco (BRL, USA). Steady-Glo Luciferase Assay System was purchased from promega (Madison, USA).
Antibodies

Rabbit polyclonal antibodies to Aβ1-42 (cat: ab10148), APP (cat: ab15272), IL-1α (cat: ab7632) and IL-1β (cat: ab2105) were purchased from Abcam (Cambridge, MA). Rabbit polyclonal antibody to presenilin-2 (PS2; cat: 2192S), rabbit monoclonal antibody to PS1 (cat: 5643S) or rabbit monoclonal antibody to microtubule associated protein -2 (MAP2; cat: 4542S), rabbit polyclonal antibody to tumor necrosis factor alpha (TNF-α; cat: 3707S) and rabbit monoclonal antibody to interleukin-6 (IL-6; cat:12912S) were purchased from Cell Signaling Technology (Danvers, MA). Rabbit polyclonal antibody to interleukin-1 receptor-1 (IL-1R1; cat: sc-689) was purchased from Santa Cruz Biotechnology (Dallas, texas). Mouse monoclonal antibody to cluster of differentiation molecule 11B (CD11b; cat: 101201), was procured from Biolegend (San Diego, CA). Mouse monoclonal antibody to glial fibrillary acidic protein (GFAP; cat: MAB360) was obtained from Millipore (Temecula, CA). Mouse monoclonal antibody to β-actin (cat: A5441), horseradishperoxide (HRP)-conjugated secondary antibodies, anti-rabbit IgG (cat: A0545) and antimouse IgG (cat: A9044) was from Sigma Chemical Co. (St Louis, MO). Alexa Fluor® 488 goat anti-rabbit IgG (cat: A11008), Alexa Fluor® 546 goat anti-rabbit IgG (cat: A11010) and Alexa Fluor® 546 goat anti-mouse IgG (cat: A11003) were from Invitrogen (Carlsbad, CA).

Animals and treatments

Rat ethical approval

All animal-handling procedures were carried out in accordance with the current regulations of the Institutional Animal Ethics Committee, and with its prior approval for using the animals.

Metal treatment in animals
Pregnant female Wistar rats were housed in a 12-h day and light cycle environment with ad libitum chow diet and Reverse Osmosis (RO) water. The rats were divided into nine groups (Table 1) and daily gavage-treated with vehicle (Group 1), tertiary mixtures (Groups 2-3), binary mixtures (Groups 4-6) or individual metals (Groups 7-9) dissolved in RO water. The treatment of the dams started from gestation day 5 (G-05) until the pups weaned (postnatal day-21, P-21), and the pups from P-22 were directly treated with the metals until P-180. 1X (Group 2) denotes the most frequently occurring concentration of As, Cd and Pb in ground water sources of India (Jadhav et al., 2007, Rai et al., 2010, Abbas et al., 2013). The baseline (1X) and 10X-metal mixture doses were adjusted according to the comparable rat weights (Yang and Rauckman, 1987). To eliminate confounding consequences of the female reproductive cycle, only male off-springs were used for our study.

Recombinant Aβ1-42 treatment using stereotaxic technique

To compare 10X-metal mixture-induced Aβ and APP levels at P-90 with that of an Aβ1-42 peptide-infused AD-like rat model of the same age (Yamada et al., 1999b, Wu et al., 2014), we performed intracerebroventricular (i.c.v.) injection of recombinant Aβ1-42 using stereotaxic technique. Briefly, rats were anesthetized with intraperitoneal injection of ketamine and xylazine (60 and 20 mg/kg body weight, respectively). The rats were placed in the stereotaxic frames (Stoelting Co. USA), skull exposed and disinfected with betadine. An incision was made into the scalp and drilled, and recombinant Aβ1-42 (1 μg in 5 μl PBS in each i.c.v.) with slight modification (Colaianna et al., 2010, Cetin et al., 2013) was bilaterally injected slowly with a 10 μl Hamilton syringe, once, at P-90 in un-treated rats, and dissected after ten days. The vehicle control rats for these groups were treated with sterile PBS that followed the same procedure.

Memantine and donepezil treatment
We investigated whether the drugs used for palliative treatment of AD, memantine (Minkeviciene et al., 2004) and donepezil (Easton et al., 2013), had any effect on metal mixture-mediated alterations in Aβ and APP levels. For this, we first treated rats with the 10X dose until P-90. From P90, we gavage-treated memantine (conc. 10 mg/kg body weight in water) or donepezil (conc. 1.5 mg/kg body weight in water) along with 10X for ten days. We kept a vehicle and 10X group in parallel. Tissues from vehicle, 10X and 10X+memantine/donepezil were dissected and analyzed for Aβ and APP on the same day.

Antioxidant treatment

To determine whether oxidative stress participates in 10X-metal mixture-mediated changes in IL-1, Aβ and APP levels, we treated the antioxidant, α-tocopherol, reported to prevent chronic oxidative stress-induced pathologies, including AD (Sung et al., 2004, Tucker and Townsend, 2005). For this, we first treated the rats with 10X until weanling. From P-22, we daily gavage treated α-tocopherol (100mg/Kg body weight) (Yamada et al., 1999a), along with 10X until P-60. We kept a vehicle and 10X-treated group in parallel. Tissues from vehicle, 10X and 10X+α-tocopherol were dissected and analyzed for IL-1α, IL-1β, Aβ and APP.

Recombinant IL-1Ra treatment using stereotaxic technique

To detect whether 10X-mediated alterations in Aβ and APP was neuroinflammation-dependent, we performed intracerebroventricular injection of recombinant IL-1Ra (5 µl of 350 ng/ml) using stereotaxic technique (Schmid et al., 2009), following the protocol mentioned above for Recombinant Aβ1-42 treatment, once, at P-90 in 10X rats, and dissected
after ten days. The vehicle control rats for these groups were treated with sterile PBS that followed the same procedure.

The overall treatment of the animals is depicted by a timeline (Suppl. 1).

*Western blot analysis*

Tissues of the cerebral cortex and hippocampus from five to seven postnatal Wistar rats were harvested, snap-frozen in liquid nitrogen, and stored at -80°C until further investigation. The cortical and hippocampal tissues were washed with PBS and suspended in tissue lysis reagent and kept on ice for 20 min. The tissues were homogenized using a Teflon homogenizer and centrifuged at 15,000 rpm for 30 min at 4°C, and the supernatant was collected. Protein content in the tissue samples was quantified using Nano-drop spectrophotometer. SDS-PAGE and western blotting on equal amount of protein samples was performed following an optimized protocol (Rai et al., 2013a). The blots were probed with 1:1000 dilutions (in PBS plus 0.2% Tween 20, PBST) of APP, Aβ1-42, PS1, PS2, IL-1α, IL-1β, IL-1R1, IL-6 and TNF-α antibodies followed by secondary antibodies with intermittent washings. The working dilutions for secondary anti-rabbit IgG conjugated to horseradish peroxidase were 1:2000 in PBST. For mouse monoclonal antibody of β-actin, the primary antibody dilution was 1:10,000 and secondary anti-mouse IgG was 1:20,000. The samples were detected by chemiluminescence with super signal west femto max substrate. Relative expression of each protein was determined by densitometric quantification of blots using VersaDoc Gel Imaging System (BioRad, Hercules, CF).

*Aβ1-40 ELISA of cortical and hippocampal tissues*

Cortex and hippocampus from Wistar rats were micro-dissected and quickly homogenized in extraction buffer. Homogenates were centrifuged (45 min) at 4°C and 40,000 rpm. The supernatant extract was collected and protein content in the tissue samples was quantified using Nano-drop spectrophotometer. The samples were then analyzed for Aβ1-40 using a
specific ELISA kit (cat. 27720) as per manufacturer’s instructions (Immuno-Biological Laboratories (Gunma, Japan)). Briefly, equal amount of protein samples (100 μg) in the Buffer (provided with the kit) were incubated overnight in antibody-coated plates at 4°C. After washing, the plates were incubated with labelled antibody for 1 hr at 4°C in dark, washed and incubated with chromogen (30 min). The reaction was then stopped with stop solution and assayed at 450 nm using a FLUOstar Omega - Multi-mode microplate reader (BMG LABTECH, Ortenberg, Germany). The Aβ₁₋₄₀ levels of the samples were then determined from the curve obtained from the kit-based standards (0, 1.56, 3.12, 6.25, 12.5, 25, 50 and 100 pg/ml) run in parallel with the samples. The Aβ₁₋₄₀ levels in the tissues were represented as pg/mg of total tissue protein.

**Immunohistochemistry**

Five wistar rat pups from five separate litters were anaesthetized, brain was perfusion-fixed and cryoprotected as previously described (Rai et al., 2010). Briefly, five to 10-µm cryostat sections were made from the cerebral cortex and hippocampus using cryomicrotome (Microm HM 520, Labcon, Germany) and mounted on (3-Aminopropyl) triethoxy-silane coated slides.

**DAB Staining**

For DAB staining, a standard procedure was followed (Rai et al., 2010). Sections were blocked in 10% normal donkey serum/0.1M PBS, incubated with APP (1:100) or Aβ₁₋₄₂ (1:100) antibodies at 4°C overnight. Following 0.1M PBS rinse, sections were incubated with secondary antibody (1-2 hr), incubated with ABC reagent (30 min), rinsed and DAB stained, counterstained with haematoxylin, washed, mounted in DPX mounting-medium and visualized under Nikon Eclipse E600 microscope (Tallahassee, FL). Images were imported into Image-J 1.43q (http://rsb.info.nih.gov/ ij/; developed by Wayne Rasband, National Institutes of Health, Bethesda, MD) for quantifying the intensity, size and number of Aβ₁₋₄₂-stained peptides in vehicle and 10X-metal mixture-treated brain sections.
Immunofluorescence staining

For detecting expression of APP, Aβ_{1-42}, CD11b or GFAP, sections were immunolabelled with anti-rabbit APP, Aβ_{1-42} or anti-mouse CD11b or GFAP antibodies at 1:100 dilution at 4°C for overnight. Following 0.1M PBS rinse, sections were incubated with Alexa Fluor 488 goat anti-rabbit IgG conjugate (1:200) or Alexa Fluor 546 goat anti-mouse IgG conjugate (1:200) for 60 min, counterstained with hoechst 33258 (0.2 mM), mounted in vectashield medium and visualized under fluorescence microscope (Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan).

BACE enzyme activity assay

BACE enzyme activity was detected spectrofluorometrically, as per manufacturer’s instructions. Protein was extracted from tissue using ice-cold extraction buffer, incubated on ice for 20 minutes and centrifuged at 15000 rpm for 15 minutes at 4°C. The supernatant was collected. Hundred μg protein samples were added to black 96-well micro plates, followed by 2X-secretase reaction buffer and BACE substrate provided with the kit. The plates were covered, tapped, kept at 37°C (1-2 hr), and fluorescence read using excitation (335-355 nm) and emission (495-510 nm) using a FLUOstar Omega-Multi-mode microplate reader (BMG LABTECH, Ortenberg, Germany). The BACE activity was determined as relative fluorescence unit/μg of protein sample. The relative fluorescence unit was calculated as,

\[
RFU = 100 \times \frac{[\text{fluorescence} - \text{avg neg. control}]}{[\text{avg. pos. control} - \text{avg. neg. control}]}
\]

where average negative control is the average fluorescence from wells containing BACE inhibitor in extraction buffer, and average positive control is the average fluorescence from wells containing reconstituted active BACE in extraction buffer. Data were shown as the relative increase in BACE activity.

Measurement of oxidative stress
To investigate whether the 10X-metal mixture affected the antioxidant status in frontal cortex and hippocampus, we assessed lipid peroxidation, superoxide dismutase (SOD), catalase activity and glutathione-S-transferase (GST) activity (Chaudhary and Parvez, 2012, Safhi et al., 2014) at P-90, i.e. the time point showing strongly significant Aβ induction. LPO was measured by estimating malondialdehyde (MDA) levels (Boehme et al., 1977). In brief, cortex and hippocampal homogenates prepared in phosphate buffer were incubated at 37°C, reaction terminated with 10% trichloroacetic acid, and centrifuged at 3000 rpm. Supernatant was boiled with 0.67% thiobarbituric acid, and absorbance measured at 532 nm in FLUOstar Omega-Multi-mode microplate reader. Results are expressed as nmoles MDA formed/min/mg protein.

Total SOD was measured in cortical and hippocampal tissues following a previously described method (Kakkar et al., 1984). In brief, reaction was initiated by addition of NADH, followed by incubation at 37°C for 90s in an assay mixture consisting of sodium pyrophosphate buffer (0.082 M, pH 8.3), phenazine methosulphate (PMS, 186 µM), nitrobluetetrazolium (300 µM), NADH (780 µM) and 10% brain tissue homogenate (prepared in 0.1 M phosphate buffer). Reaction was stopped with glacial acetic acid, vigorously shaken with n-butanol, kept for 10 min, centrifuged at 3000 rpm (10 min) and upper-layer of butanol separated, and absorbance recorded at 560 nm using FLUOstar Omega-Multi-mode microplate reader. The SOD activity is expressed in nmol formazan formed/minute/mg protein.

Catalase activity was determined as previously described (Farmand et al., 2005). Briefly, changes in absorbance (240 nm) were recorded using FLUOstar Omega-Multi-mode microplate reader after addition of hydrogen peroxide (0.019 M) in assay mixture of cortex and hippocampus brain homogenates (10% w/v) in phosphate buffer (0.05 M, pH 7.0). Enzyme activity is expressed as µmol H₂O₂ consumed/min/mg protein.
GST activity was determined as previously described (Habig et al., 1974). Absorbance was recorded at 340 nm and enzyme activity was calculated as nmol CDNB conjugate formed/(min/mg protein).

**In vitro experimental procedure**

**Primary Neuronal culture**

Control pregnant Wistar rats were sacrificed by cervical dislocation and embryos were removed on 16th day of gestation as described (Ray et al., 1993). Embryonic brain tissues were mechanically dissociated in dissection media containing glucose (1M), sucrose (1 M), HEPES Buffer (1M) and Hank’s salt (1X), cells centrifuged (1,500 rpm, 10 min), resuspended in neurobasal medium containing B-27 supplement and N2 supplement (Invitrogen, Carlsbad, CA), L-glutamine (0.5 mM), penicillin (100 units/ml) and Streptomycin (100 μg/ml) and plated. Culture medium was changed every two days. Neurons (>90%) were confirmed through immunostaining with MAP-2 antibody.

**Primary astrocyte culture**

Briefly, prefrontal cortices of 0-2 day-old control Wistar rats were dissected out and cultured as described previously (Maurya et al., 2012). Cultures were maintained in DMEM/F-12 containing 10% heat-inactivated FBS, 100 U/ml penicillin, and 100 mg/ml streptomycin. Confluent cells were rinsed with sterile PBS, detached with 0.25% trypsin with EDTA, and sub-cultured, which reached confluence at 3rd day. Astrocytes (>95%) were confirmed through immunostaining with GFAP antibody.

**MTT assay**

We performed MTT assay to determine neuron and astrocyte viability upon treatment with individual As, Cd and Pb as described before (Rai et al., 2010). The assay was performed to detect IC50 values of the metals for their treatments in luciferase assays. For this, neurons and astrocytes were grown to around 70-80% confluence, pre-incubated in reduced serum (0.5%
FBS) medium for 2 h, treated with As, Cd or Pb, in the form of NaAsO₂, CdCl₂ or Pb(C₂H₃O₂)₂ respectively, at concentrations ranging from 0.01 to 200 µM for 18 h, and kept in a humidified tissue culture incubator at 37°C with 5% CO₂-95% air. Cells were then incubated with MTT reagent, incubated (4 h) to convert water-soluble MTT to insoluble formazan, treated with 50% dimethylformamide and 20% SDS to solubilize formazan, incubated at 37°C overnight and absorbance measured at 595 nm, with background subtraction at 655 nm. IC₅₀ of metals on neurons and astrocytes was analyzed by the GraphPad Prism 3.0 software.

Transfection and luciferase assay

Using Turbofect transfection reagent (Fermentas, PA, USA), cultured neurons and astrocytes were transfected with pIRES(APP mRNA 5’UTR) construct (Bandyopadhyay et al., 2006), having a luciferase reporter under the translational control of the 146-nucleotide APP mRNA 5’UTR and a green fluorescent protein (GFP) as the internal specificity control (Bandyopadhyay et al., 2006). Transfection efficiency of around 70% was determined by GFP assay using a fluorescent microscope (Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan) (Suppl. 2). The cells were then trypsinized, harvested, resuspended in culture medium and plated at equal number. They were then pre-incubated in reduced serum, and treated with individual metals or with a mixture of 1/3rd of IC₅₀ of the metals, MM (Group 1-2 of Table 2) in phenol red-free medium, and luminescence assay performed using Steady Glo luciferase substrate in GloMax microplate luminometer (Promega, Madison, USA). The luciferase activity was calculated as:

\[
\text{% increase} = 100 \times \frac{\text{[luminescence-avg neg. control]}}{\text{[avg. pos. control- avg. neg. control]}}
\]

where avg. neg. control is the average luminescence from wells with un-transfected cells and avg. pos. control is the average of luminescence from wells containing transfected cells.
without any treatment. The negative controls took into account the background effects. To confirm specific effects on APP 5'UTR, we counter-treated MM on the prion-protein construct pIRES(PrP mRNA 5'UTR) that served as another rigorous negative control (Bandyopadhyay et al., 2006).

The APP-5'UTR bears an IL-1 responsive element (Rogers et al., 1999), and therefore, to determine possible participation of IL-1, we co-treated the transfected cells with MM and IL-1Ra (10 ng/ml) and performed luciferase assay.

Determination of Combination index (CI)

To characterize the combinatorial impact of As, Cd and Pb for their effects on Aβ₁-42, Aβ₁-40, APP, BACE activity, PS1, PS2 and IL-1 levels in the tertiary mixtures, the animals were treated with individual metals and their mixtures, and the CI values were calculated (Rai et al., 2010, Rai et al., 2013b). The CI values less than, equal to, or more than 1 indicated synergism, additivity, or antagonism, respectively (Zhao et al., 2004).

Immunocytochemistry

Cells were treated with a mixture of As, Cd and Pb (metal mixture, MM) at 1/3rd of their IC₅₀. Neurons and astrocytes were grown to 80% confluence, pre-incubated in reduced serum for 2 h, treated with a mixture of As, Cd, and Pb (Group 2 of Table 2) in reduced serum medium for 16 h, and incubated in a humidified tissue culture incubator at 37°C with 5% CO₂-95% air. Cells were fixed with 4% PFA (1 hr) at room temperature (Rai et al., 2010), pre-incubated with PBS containing 0.3% Triton X-100 (Sigma) and 2% normal horse serum (Gibco-BRL), incubated overnight with antibodies for APP (1:100) and Aβ₁-42 (1:100), and diluted in PBS containing 0.3% Triton X-100 and 2% normal horse serum at 4°C. Following a rinse in 0.1M PBS, cells were incubated with Alexa Fluor 546 goat anti-rabbit IgG conjugate (1:200) for 60 min, mounted with vectashield medium and visualized under a
fluorescence microscope (Nikon Instech Co. Ltd., Kawasaki, Kanagawa, Japan). Cell fluorescence intensity was quantified using Image-J 1.43q software.

Behavioral study

All behavioral experiments were carried out between 10 a.m. and 12-noon with V-P90 and 10X-P90 rats.

Y-maze test

Y-maze test was carried out as described previously (Rai et al., 2010) using Y-maze (Techno, India). The Y-maze training apparatus contained three arms with electrifiable grid-floored and 15-W light bulb at the end of the arm. During training, one arm with the light on (light arm) was a safe arm without footshock, and the two other arms with the lights off (dark arms) were unsafe and with electric footshock (1–5 mA). The safe and unsafe arms were randomly shifted during training. The training session was of 30 trials per animal. Running by the rats into the dark arm of the Y-maze was counted as an error (E). Retention of light discrimination was determined after 24 h, 48 h and after 7 days of initial learning using a 30-trial relearning session. The performance of rats in relearning was considered as test for memory and expressed as percent (%) saving.

\[
\text{% saving} = \frac{(E_{\text{training}} - E_{\text{test}}) \times 100}{E_{\text{training}}}
\]

Passive avoidance test

The rats were subjected to the passive avoidance test by placing in a compartment with light at an intensity of 8 [scale from 0 to 10 (brightest)] in a computerized shuttle box (Techno, India) as previously described (Yadav et al., 2011). The light compartment was isolated from the dark compartment by an automated guillotine door. After an acclimatization period of 30s, the guillotine door was opened and closed after entry of the rat into the dark
compartment. The subject received a low-intensity foot shock (0.5mA; 10 s) in the dark compartment. Infrared sensors monitored the transfer of the animal from one compartment to another, which was recorded as transfer latency time (TLT) in seconds. The 1st trial was for acquisition and retention (R1) was tested in a 2nd trial (1st retention), given 24h after the 1st trial. The duration of a trial was 300s. Further, 2nd and 3rd trial (R2 and R3) were given on alternate days to test retention in the metal mixture treated rats. The shock was not delivered in the retention trials to avoid reacquisition. The criterion for learning was taken as an increase in the TLT on retention (2nd or subsequent) trials as compared to acquisition (1st) trial.

Statistical Analysis
All statistical analyses were performed with SPSS-9.0 software (SPSS Inc., Chicago, IL). Comparisons between two groups of independent samples were made with two-tailed, unpaired t-tests, and for more than two groups by ANOVA using Student–Newman–Keuls post-hoc-test.

Results
Effect of As+Cd+Pb-mixture on Aβ
We orally fed developing rats (G05 to P-180) with As+Cd+Pb-mixture at doses, 1X and 10X (refer to Table 1), and examined Aβ$_{1-42}$ peptide levels at post-weaning (P-24) and adulthood (P-60, P-90 and P-180) through western blotting. We observed a dose-dependent and time-dependent increase in Aβ$_{1-42}$ in the tissues of frontal cortex (Fig. 1A) and hippocampus (Fig. 1B); the effect being evident even at P-24. Treatment with 10X-metal mixture until early adulthood i.e. 10X-P90, caused a strongly significant increase in Aβ$_{1-42}$ (Fig. 1A-1B), and therefore, we compared Aβ$_{1-42}$ expression of 10X-P90 with an Aβ$_{1-42}$-infused AD-like rat model of the same age. The increase in Aβ$_{1-42}$ peptide levels was comparable in the
hippocampus and greater in the cortex for 10X-P90 (Fig. 1C). (Treatment of the metal mixture up to P-180 failed to demonstrate a more significant change than P-90 compared to their respective vehicles (data not shown), and thus, we performed our study majorly on 10X-P90). Immunostaining for Aβ1-42, which showed a significant increase in intensity, count and size of the peptide (Fig. 1D), corroborated the increase in Aβ1-42 for 10X-P90.

We examined the levels of the less amyloidogenic, Aβ1-40 peptide. ELISA revealed a dose- and time-dependent induction of Aβ1-40 by the metal mixture in tissues of both frontal cortex (Fig. 1E) and hippocampus (Fig. 1F).

We then validated the expression of Aβ1-42 and Aβ1-40 peptides by checking the effect of known AD drugs, memantine and donepezil. The drugs could attenuate the levels of both Aβ1-42 (Fig. 1G) and Aβ1-40 (Fig. 1H) in 10X-metal mixture exposed rat brain.

**Effect of individual metals, binary and tertiary mixtures of As, Cd and Pb on Aβ**

We compared the effects of individual metals and binary and tertiary mixtures on Aβ at P-90 (refer to Table 1). Among individual metals, the increase in Aβ1-42 (Fig. 2A, Suppl. 3) as well as Aβ1-40 (Fig. 2B, Suppl. 3) was as Pb>Cd>As. For Pb that showed the highest effect, the cortical increase in Aβ1-42 and Aβ1-40 was 84±8% and 40±5%, and hippocampal increase was 54±6% and 34±4% respectively (Fig. 2A-2B, Suppl. 3). For As, the increase in Aβ1-42 and Aβ1-40 was non-significant, and for Cd, the increase was in the range of 12-18% (Fig. 2A-2B, Suppl. 3).

Although individual As failed to induce Aβ, binary mixture of As and Pb (As+Pb) demonstrated a cortical increase of 140±15% and 77±8%, and hippocampal increase of 96±12% and 59±7% for Aβ1-42 and Aβ1-40 respectively, that were significantly above Pb alone.
(Fig. 2A-2B, Suppl. 3). Noticeably, despite the non-significant or small induction by individual As or Cd respectively, As+Cd showed an increase in Aβ1-42 (cortex: 100±10%, hippocampus: 100±7%) and Aβ1-40 (cortex: 38±4%, hippocampus: 51±6%) that were significantly above Cd alone (Fig. 2A-2B, Suppl. 3). Therefore, despite the non-significant effect of As alone, when combined with Pb or Cd, a greater-than-additive increase was observed for both Aβ1-42 and Aβ1-40. However, for Cd+Pb, the increase in Aβ1-42 and Aβ1-40 was consistent with the summed values of their individual increase (Fig. 2A-2B, Suppl. 3). In tertiary mixtures, the increase in Aβ1-42 (cortex: 295±25%, hippocampus: 199±10%) and Aβ1-40 (cortex: 199±15%, hippocampus: 175±12%) was greater than the binary mixtures (Fig. 2A-2B, Suppl. 3). More specifically, the increase in Aβ1-42 and Aβ1-40 by tertiary mixture was significantly greater (P<0.001) than the summed values of their individual increase, or of binary mixtures and the third (Suppl. 4). To verify a greater-than-additive combinatorial effect in the tertiary mixture, we calculated CI values for combination of individual metals, as well as for binary mixtures and the third. We found the CI values to be prominently less than 1, suggestive of synergism (Table 3). Thus, As+Cd+Pb-mixture induced greater-than-additive effect on Aβ1-42 and Aβ1-40 peptides.

**Effect of As, Cd and Pb on APP levels and APP-processing**

We examined whether As+Cd+Pb-mixture affected the APP-amyloidogenic pathway of AD. For this, we first assessed the expression levels of APP. We observed an increase in APP, and the effect was strongly significant for 10X-P90 (Fig. 3A). Immunostaining data corroborated the increase in APP, especially in the pre-frontal cortex and dentate gyrus of the hippocampus (Fig. 3B). Supportive of Aβ data, memantine and donepezil could reduce the metal mixture-induced APP (Suppl. 5).
We then measured BACE activity, and expression levels of the γ-secretase components, PS1 and PS2. The cortical BACE activity and PS1 and PS2 expression levels for 10X-P90 increased by $2.28 \pm 0.13$-fold ($P<0.001$), $2.18 \pm 0.09$-fold ($P<0.001$) and $2.32 \pm 0.15$-fold ($P<0.001$) respectively compared to vehicle (Suppl. 6). The hippocampal BACE activity and PS1 and PS2 expression levels for 10X-P90 was $2.24 \pm 0.10$-fold ($P<0.001$), $2.20 \pm 0.12$-fold ($P<0.001$) and $2.26 \pm 0.15$-fold ($P<0.001$) respectively compared to vehicle (Suppl. 6).

We then assessed the effect of individual metals, and binary and tertiary mixtures on APP (Fig. 3C), BACE (Fig. 3D), PS1 (Fig. 3E) and PS2 (Fig. 3F). Consistent with Aβ data, the effect of individual Pb was significantly high for all (Fig. 3C-3F, Suppl. 3). Individual As demonstrated a significant increase in APP but failed to induce BACE and PS (Fig. 3C-3F, Suppl. 3), thereby justifying its non-significant effect on Aβ (refer to Fig. 2). For individual Cd, the induction of APP and PS (other than hippocampal APP) were significantly less than Pb (Fig. 3C-3F, Suppl. 3), thereby rationalizing a lower increase in Aβ compared to Pb (refer to Fig. 2).

The binary mixtures (As+Cd, As+Pb and Cd+Pb) showed an increase in APP that was in the range of the summed values of individuals (Fig. 3C, Suppl. 3). Specifically, for As+Cd and As+Pb, the summed effects of the individuals were $43 \pm 5\%$ and $75 \pm 9\%$ in cortex and $43 \pm 4\%$ and $56 \pm 5\%$ in the hippocampus as compared to an increase of $50 \pm 5\%$ and $68 \pm 7\%$ in cortex and of $51 \pm 5\%$ and $52 \pm 6\%$ in hippocampus respectively for binary mixtures (Suppl. 3). However, for the APP-processing proteins, more prominently for PS, despite a non-significant increase by As alone, the binary mixture of As and Cd (As+Cd) showed a significant increase above that of individual Cd (Fig. 3E-3F, Suppl. 3). Therefore, despite the additive impact on APP, the effect on the APP-processing proteins led to a greater-than-additive result on Aβ in binary mixtures (refer to Fig. 2).
Consistent with the effect of tertiary mixtures on Aβ, the increase in APP, BACE and PS was significantly greater (P<0.001) than the summed effects of the individuals or of binary mixtures and the third (Suppl. 4). Furthermore, CI values less than 1 towards the tertiary mixture during combination of individual metals or binary mixtures and the third strongly suggests synergism for APP, BACE and PS (Table 3). Thus, As+Cd+Pb-mixture induced greater-than-additive effect on APP, BACE and PS, and thereby Aβ expressions.

**Effect of As, Cd and Pb on neuroinflammation and oxidative stress**

Neuroinflammation and oxidative stress play a key role in AD pathogenesis (Rogers and Lahiri, 2004), and hence we examined their involvement in As+Cd+Pb-mixture-mediated induction of Aβ. We assessed generation of inflammation by measuring IL-1α, IL-1β and their receptor, IL-1R1, and verified oxidative stress by measuring LPO, SOD, catalase and GST. We observed an increase in IL-1α, IL-1β and IL-1R1, suggestive of neuroinflammation, which was supported by an increase in IL-6 and TNF-α (Table 4, Suppl. 7). We also observed increased oxidative stress, characterized by enhanced LPO (indicated by MDA), and a fall in SOD, catalase and GST (Table 5). We then verified which of the two (IL-1 or oxidative stress) was proximally activated by the metal mixture. To identify whether oxidative stress was upstream of inflammation, we co-treated 10X with the anti-oxidant, α-tocopherol, and measured IL-1. We observed a suppression in 10X-induced IL-1α (Fig. 4A) and IL-1β (Fig. 4B), indicating an oxidative stress-dependent induction of IL-1. On the other hand, intracerebroventricular insertion of an IL-1R1 antagonist, IL-1Ra, failed to alter 10X-induced oxidative stress (data not shown), implying oxidative stress to be up-stream of IL-1. Therefore, As+Cd+Pb-mixture proximally activated oxidative stress that induced neuroinflammation in the frontal cortex and hippocampus.
We then examined direct involvement of IL-1 in As+Cd+Pb-mixture-induced Aβ and APP. We observed that IL-1Ra suppressed 10X-induced Aβ (Fig. 4C) and APP (Fig. 4D), very prominently in the pre-frontal cortex and dentate gyrus region of hippocampus (RHS of Fig. 4C-4D), implying IL-1-dependent Aβ and APP induction. An increased expression of the microglial and astrocyte activation marker, CD11b and GFAP respectively, very close to Aβ1-42 (Fig. 4E) and APP (Fig. 4F) corroborated the neuroinflammation-dependent induction of Aβ1-42 and APP and. (We also confirmed oxidative stress to be leading to the AD-like pathology by detecting attenuation in Aβ and APP in α-tocopherol and 10X-co-treated rats (Suppl. 8).

A well-known concept in the IL-1-mediated induction of APP is that a 146-nt-APP-5’UTR, that bears an IL-1 responsive element, drives APP translation (Rogers et al., 1999). Here we examined whether the APP-5’UTR participated in the IL-1-mediated induction of APP. For this, we first generated stable transfectants of neurons and astrocytes that expressed luciferase under the translational control of APP-5’UTR, and treated the transfectants with a non-toxic concentration of metal mixture (refer to method). We observed a profoundly significant increase in luciferase activity (Fig. 4G), indicating a 5’UTR-mediated translation of APP by the metal mixture. ((Metal mixture had no effect on the internal specificity control, GFP, and PrP-5’UTR that served as the negative control (data not shown)). Comparing the increase in luciferase activity of the individual metals (data not shown) with that of the metal mixture revealed a CI-value of around 0.61 and 0.71 for neurons and astrocytes respectively, suggesting a synergistic induction in the APP 5’UTR-mediated APP expression. We further observed that co-treating IL-1Ra with metal mixture in the APP-5’UTR-transfected cells attenuated the increase in luciferase activity (Fig. 4G), thereby suggesting an IL-1-dependent APP translation via the IL-1 responsive element of APP-5’UTR. Therefore, this provides support to the concept of IL-1-mediated APP induction by the metal mixture.
To further relate the synergistic APP induction with IL-1, *in vivo*, we compared the effects of individual metals and binary and tertiary mixtures on IL-1α and IL-1β in the tissues of frontal cortex and hippocampus. We observed induction of IL-1 by all three metals, with Pb showing the maximum effect (Fig. 4H, Suppl. 3); the cortical increase of IL-1α and IL-1β being 42±5% and 42±4%, and hippocampal increase being 39±4% and 41±5% respectively for individual Pb (Fig. 4H, Suppl. 3). Consistent with APP data, we observed that the binary mixtures (As+Cd, As+Pb and Cd+Pb) showed an increase in IL-1 that was in the range of the additive values of individuals. In tertiary mixtures, the increase in IL-1α and IL-1β was 147±14% and 108±10% in cortex, and 124±13% and 102±12% in hippocampus, respectively (Fig. 4H, Suppl. 3), which was greater than the summed effects of individual metals or of binary metals and the third in almost all combinations (Suppl. 4). Furthermore, supporting our previous data, the CI values towards the tertiary mixture during combination of individual metals or of binary mixtures and the third was prominently less than 1 for IL-1 (Table 3), indicating synergism. Thus, As+Cd+Pb-mixture induces greater-than-additive effect on IL-1α and IL-1β that led to synergistic APP and thereby Aβ expressions.

**Effect of As, Cd and Pb-mixture on learning-memory performance**

To detect whether significant increase in Aβ by As+Cd+Pb-mixture leads to abnormalities in learning-memory performance, we carried out Y-Maze and passive-avoidance tests. In Y-Maze Test, 10X-P90 rats exhibited errors during training, and loss in learning-memory at 24h, 48h, and 7th day post-training (Fig. 5A). In passive-avoidance test, 10X-P90 rats showed a less significant increase in TLT of the retention trials weighed against acquisition trial, signifying reduced learning memory ability (Fig. 5B).

**Overall effect of As, Cd and Pb-mixture on early AD-like pathology**

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It could be construed that As+Cd+Pb-mixture at environment relevant doses stimulate oxidative stress-dependent IL-1 to promote APP expression via the APP-5’UTR that bears an IL-1 responsive element. APP then undergoes pro-amyloidogenic processing by BACE and PS to induce Aβ in synergism. The Aβ accumulation culminates in cognitive impairments at an early age (Fig. 6).

**Discussion**

We used a rat model of human exposure to chronic heavy metal (As, Cd, and Pb) mixture and characterized manifestations of early-onset AD-like pathology. Our findings are important because they demonstrate that the metals at their ground water doses invoked greater-than-additive up-regulation of the pro-amyloidogenic proteins at an early age. More specifically, the metals stimulated a dose and time-dependent generation of Aβ, mediated by the pro-amyloidogenic processing of APP. The APP expression was induced by oxidative stress-dependent neuroinflammation via APP mRNA 5’UTR that bears an interleukin-1 responsive element. Eventually, the premature induction of Aβ-peptides culminated in cognitive impairments in young rats (Fig. 6).

Environmental influences occurring during brain development drive amyloidogenesis that result in Aβ expression later in life, suggesting an environmental trigger and a developmental origin of AD (Basha and Reddy, 2010, Bihaqi et al., 2014). Supporting the concept, infantile exposure to Pb is reported to cause late-age over-expression of APP and Aβ (Wu et al., 2008, Bihaqi et al., 2013, 2014), and long-term ground water As exposure induces earliest signs of AD in the elderly (O’Bryant et al., 2011, Tyler and Allan, 2013). However, these studies had been conducted only on single metals, and none of them cited manifestations of early-onset AD-like pathology. Our data demonstrate that drinking water exposure to As, Cd and Pb-
mixture up-regulated Aβ in young adults, to the extent of a non-transgenic AD-like model, suggesting strong neurodegenerative action that appears to be independent of age. (The higher cortical Aβ levels in the 10X-P90 rats compared to the AD-like model may have resulted from a less significant effect on the cortical Aβ through intracerebroventricular infusion process while generating the latter). It also underscores that in a real world situation, the combinatorial toxic effects may be such that manifestation of AD-like pathological hallmark is noticeable in young adults. The increased effect is relatively less prominent during late adulthood (P-180), probably because of enhanced Aβ levels during normal aging (Fukumoto et al., 1996).

Our study shows that the metal mixture promoted a typical pro-amyloidogenic processing of APP through sequential activation of BACE and PS, and generated Aβ. This is a step ahead of earlier reports, lacking the effects of these metals on PS (Wu et al., 2008, Zarazua et al., 2011, Bihaqi et al., 2014).

The current investigation brings forth the effect of individual As, Cd and Pb, and of their binary and tertiary mixtures on AD marker proteins. The data identifies that individual Pb generated APP, BACE and PS maximally, leading to a prominent augmentation in Aβ. This data strengthens the hypothesis that Pb exposure may be associated with the formation of amyloid plaques (Gu et al., 2012), and claims Pb to be a risk factor for AD not only in the elderly population as reported earlier (Wu et al., 2008, Bihaqi et al., 2014), but also in the young following infantile exposure. Moreover, it proposes that other than the epigenetic concept that defines the relationship between Pb exposure and late-onset AD (Bakulski et al., 2012), gene alterations inducing early-onset AD-like pathology by Pb needs exploring. Our data shows that individual Cd, though less significant than Pb, was capable of inducing Aβ. To the best of our knowledge, this is the first study revealing stimulation of the pro-amyloidogenic pathway by Cd; an earlier report showing suppression of the non-
amyloidogenic pathway leading to Cd-induced Aβ (Li et al., 2012). Therefore, our data
strengthens the hypothesis that Cd, known to directly interact with Aβ (Notarachille et al.,
2014), may be a risk factor for AD. Corroborating a previous in vitro study (Zarazua et al.,
2011), our data verifies in vivo induction of APP by individual As. However, the inability of
individual As to activate BACE and PS may have led to the unaltered Aβ levels observed in
our study.

Arsenic, despite having no effect on Aβ alone, influenced Aβ induction by Pb or Cd in binary
mixtures. Probably, As-induced APP undergoes processing by Cd- and Pb-induced BACE
and PS, generating enhanced Aβ in binary mixtures. In a tertiary mixture, the effect is more
prominent, where each metal influenced the effect of the other two components through
synergism.

The three metals, individually and in mixtures could induce APP, the effect being most
prominent in tertiary mixture. This may be explained from the increase in IL-1, known to up-
regulate APP induction (Rogers et al., 1999). Noticeably, despite the non-significant effect of
As alone, As+Cd and As+Pb induced higher amyloidogenic processing (depicted by
enhanced BACE and PS) compared to individual Cd or Pb respectively. Inactivation of the
acetylcholinesterase (AChE) by arsenite through its binding with functional Tyr residues of
the enzyme (Page and Wilson, 1985) may be a reason towards the inability of individual As
to modulate APP-amyloidogenic processing, known to be influenced by the AChE activity
(Silveyra et al., 2008, Silveyra et al., 2012). However, in binary or tertiary mixtures, Cd and
Pb may have influenced the interaction of As and Tyr, thereby hindering the inactivation of
AChE and allowing enhanced BACE and PS activity. Nevertheless, this aspect calls for
further confirmation. Thus, in support of our earlier reports showing synergistic toxicity in
glial cells (Rai et al., 2010, Rai et al., 2013b), the present data verify that As, Cd and Pb
influenced the toxicity of one another in inducing the AD-like hallmarks. Thus this metal
mixture in synergism is predicted to hamper brain development in children (from our earlier reports) and evoke neurodegeneration, including AD-like pathology in young and matured adults.

The present investigation points to the induction of IL-1 as an important driving force for APP and thereby Aβ generation. Via the APP mRNA 5’UTR that bears an IL-1 responsive element (Rogers et al., 1999), an enhanced IL-1 by the metal mixture promoted the translation of APP in neurons and astrocytes in synergism (Suppl. 9, showing enhanced APP and Aβ in neurons and astrocytes), resulting in elevated APP levels in brain tissue. Other significant markers, such as activated microglia and astrocytes close to Aβ and APP confirmed the presence of local inflammatory events. Here too we found that among individual metals, increase in IL-1 by Pb was the highest. Furthermore, consistent with the trend observed for APP, the increase in IL-1 was additive for binary mixtures and synergistic for the tertiary, supporting a direct correlation of IL-1 with APP. Therefore, our observation on elevated APP and thereby Aβ synthesis by IL-mediated mechanism is in agreement with epidemiological evidence linking the use of anti-inflammatory drugs to treat AD pathology (Rogers et al., 1999).

While there is an abundance of data implicating IL-1 in AD, to the best of our knowledge, there are very few reports that describe the mechanism that causes aberrant expression of the cytokine (McCarthy et al., 2013). Our data clearly demonstrates an oxidative-stress-mediated induction of IL-1 by the metal mixture. Oxidative stress-induced premature senescence in the astrocytes may be responsible for their generation of pro-inflammatory mediators, as reported earlier (Bhat et al., 2012). This is strongly supported by our earlier report demonstrating enhanced reactive oxygen species-induced astrocyte degeneration by As+Cd+Pb-mixture in developing rat brain (Rai et al., 2010, Rai et al., 2013b).
Our data reveals that As+Cd+Pb-mixture caused learning-memory impairments in the young rats. APP misprocessing and hippocampal Aβ deposition that hamper neurogenesis and compromise synaptic plasticity (Jellinger and Attems, 2013) justify the cognitive impairments. Moreover, enhanced Aβ in association with damaged astrocytes and axons, reported by us earlier (Rai et al., 2010, Rai et al., 2013b), may have altered normal glia-neuron interactions leading to reduced synaptic efficacy (McCall et al., 1996) in the metal mixture-exposed young rats. Thus, our As+Cd+Pb-exposed rats bear the essential features of AD-like pathology, viz. enhanced hippocampal and cortical APP-amyloidogenic processing, increased Aβ deposition and loss in cognitive memory. The APP and Aβ levels induced by 10X dose match that of a non-transgenic AD model, thereby providing insight into developing our metal mixture-exposed rats as suitable animal models mimicking early-onset Alzheimer’s-like amyloid pathology. In addition, attenuation of Aβ and APP by the blockers of N-methyl D-aspartate (NMDA) receptor and acetylcholinesterase, memantine (Minkeviciene et al., 2004) and donepezil (Easton et al., 2013) respectively, may well claim our metal mixture-exposed rats as a potential model for screening AD-drugs. However, detailed study on the model concept is in progress, where we hypothesize excitotoxic consequence via the modulation of NMDA and cholinergic functions upon exposure to our metal mixture.

**Conclusion**

Taken as a whole, our study reinforces our earlier reports that unrestrained use of As, Cd and Pb evokes synergistic neurodevelopmental aberrations. Extrapolating present findings to humans with temporal metal mixture exposure predicts that young-adult men are vulnerable to suffer from the early manifestations of AD-like pathology. Our study further underscores the importance of considering interactive effects of metals at environment relevant
concentrations, and provides regulatory agencies with enough impetus to reconsider the health risks of chemical mixtures.

**Disclosure statement**

None of the authors has a conflict of interest to declare in relation to the present research.

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Figure legend

Figure 1: As, Cd and Pb-mixture induces dose and time-dependent Aβ induction

Cortical and hippocampal tissues from vehicle (V), 1X and 10X-metal mixture-treated rats were immunoblotted for Aβ1-42 (15 kDa) and β-actin (42 kDa) at P-24, P-60 and P-90. Representative western-blot and densitometry showing dose and time-dependent increase in Aβ1-42 normalized with β-actin in (A) frontal cortex and (B) hippocampus. Fold change is calculated relative to V-P24. Data represent means ± SE of five-seven pups from five-seven litters. ***P<0.001, **P<0.01 and *P<0.05 indicate significant difference compared to vehicle (V) of same time point, or as indicated; ns indicates non-significant.

C. Cortical and hippocampal tissues from vehicle (V-P90), 10X-P90, vehicle for AD-like rat model (V-Aβ1-42-infusion) and Aβ1-42-infused AD-like rat model (Aβ1-42-infusion) were immunoblotted for Aβ1-42 and β-actin. Representative western-blot and densitometry showing Aβ1-42 normalized with β-actin. Data represent means ± SE of five pups from five litters. *** P<0.001 and **P<0.01 indicate significant difference compared to respective vehicles.

D. Five-µm-thick cryostat sections of cortex and hippocampus from vehicle (V-P90) and 10X-P90 rats were immunostained for Aβ1-42 using peroxidase conjugate and DAB...
chromogen. Representative photomicrograph (LHS) of immunostained A\(\beta\)\(1-42\) (shown by arrow), and relative intensity, total count and size (RHS) of A\(\beta\)\(1-42\) immunostained spots in cortex and hippocampus. The sections are representatives of five pups from five litters. ***\(P<0.001\) indicates significant difference compared to V-P90.

E-F. Dose and time-dependent increase in A\(\beta\)\(1-40\) levels (determined through ELISA) in the cortical and hippocampal tissues from vehicle (V), 1X and 10X-metal mixture-treated rats at P-24, P-60 and P-90. Data represent means \(\pm\) SE of five pups from five litters. ***\(P<0.001\) and **\(P<0.01\) indicate significant difference compared to vehicle (V) of same time point, or as indicated; ns indicates non-significant.

G-H. Cortical and hippocampal tissues from vehicle (V), 10X, 10X+memantine (Mem) or 10X+donepezil (Don)-treated rats were immunoblotted for A\(\beta\)\(1-42\) and \(\beta\)-actin, or assayed by ELISA for A\(\beta\)\(1-40\). Representative western-blot and densitometry of A\(\beta\)\(1-42\) (G) normalized with \(\beta\)-actin, and ELISA for A\(\beta\)\(1-40\) (H). Data represent means \(\pm\) SE of five pups from five litters. ***\(P<0.001\) indicates significant difference compared to V or as indicated.

Figure 2: As, Cd and Pb-mixture induces greater-than-additive effect on A\(\beta\)

Cortical and hippocampal tissues from vehicle (V), individual metal, binary mixture and tertiary mixture-treated 10X-P90 rats were assayed for A\(\beta\)\(1-42\) through western blotting. A. Representative western blot and densitometry showing A\(\beta\)\(1-42\) and \(\beta\)-actin in cortex (LHS) and hippocampus (RHS) relative to \(\beta\)-actin. B. Relative A\(\beta\)\(1-40\) levels, determined through ELISA in vehicle (V), individual metal, binary mixture and tertiary mixture-treated 10X-P90 rats in cortex (LHS) and hippocampus (RHS). Data represent means \(\pm\) SE of five pups from five litters. ***\(P<0.001\), **\(P<0.01\) and *\(P<0.05\) indicate significant difference compared to V or as indicated, and ^\(P<0.001\) indicates significant difference compared to all.
Figure 3: As, Cd and Pb-mixture increases APP levels and amyloidogenic processing in young rats

A. Cortical and hippocampal tissues from vehicle (V) and 10X-treated rats at P-24, P-60 and P-90 were immunoblotted for APP (87 kDa) and β-actin (42 kDa). Representative western-blot and densitometry of APP normalized with β-actin in cortex (LHS) and hippocampus (RHS). Data represent means ± SE of five pups from five litters; ***P<0.001 and *P<0.05 indicate significant difference compared to vehicle (V) of same time point, or as indicated.

B. Five-µm-thick cryostat sections of cortex and hippocampus from vehicle (V-P90) and 10X-P90 rats were immunostained for APP (shown by arrow) using peroxidase conjugate and DAB chromogen. Representative cortical (upper panel) and hippocampal (lower panel) photomicrographs and intensity (RHS) of APP expression in vehicle (V-P90) and 10X-P90 rats. The sections are representatives of five pups from five litters. ***P<0.001 indicates significant difference compared to vehicle (V).

C-F. Cortical and hippocampal tissues from vehicle (V), individual metal, binary mixtures and tertiary mixture-treated 10X-P90 rats were assayed for APP, BACE, PS1 and PS2. C. Representative immunoblot and densitometry showing APP in cortex (LHS) and hippocampus (RHS) relative to β-actin. D. Relative BACE activity, determined through enzymatic assay, in cortical (LHS) and hippocampal (RHS) tissues. E-F. Representative immunoblot and densitometry showing PS1 (E), PS2 (F) in cortex (LHS) and hippocampus (RHS) relative to β-actin. Data represent means ± SE of five pups from five litters. ***P<0.001, **P<0.01 and *P<0.05 indicate significant difference compared to V or as indicated, and cP<0.001 indicates significant difference compared to all.

Figure 4: As, Cd and Pb-mixture induces Aβ and APP via oxidative stress-dependent neuro-inflammation
**A-B** Cortical and hippocampal tissues from vehicle, 10X, 10X+α-tocopherol-treated rats were immunoblotted for IL-1α (17 kDa) (A) or IL-1β (17 kDa) (B) and β-actin (42 kDa). Representative western blot and densitometry of IL-1α and IL-1β in frontal cortex (LHS) and hippocampus (RHS) normalized with β-actin. Data represent means ± SE of five pups from five litters. ***P<0.001 and **P<0.01 indicate significant difference compared to V or as indicated.

**C-D.** Cortical and hippocampal tissues from vehicle, 10X, PBS or IL-1Ra (diluted in PBS)-infused rats were immunoblotted for Aβ1-42 or APP and β-actin (42 kDa). Representative western blot and densitometry of Aβ1-42 (C) and APP (D) in cortex and hippocampus normalized with β-actin. Data represent means ± SE of five pups from five litters. ***P<0.001 indicates significant difference compared to V or as indicated.

Cortical and hippocampal sections from vehicle, 10X, PBS or IL-1Ra (diluted in PBS)-infused rats were immunofluorescence labeled for Aβ1-42 (C-RHS) or APP (D-RHS) and co-stained with nuclear hoechst. Representative merged photomicrograph (10x-magnification) of Aβ1-42 (green fluorescence) or APP (green fluorescence), nucleus (blue fluorescence) in the same field.

**E-F.** 10-μm-thick cryostat sections of cerebral cortex (coronal section) from vehicle (V-P90)- and 10X-P90 treated rats were co-immunolabelled for CD11b or GFAP, and Aβ1-42 (E) or APP (F), and then labelled with nuclear Hoechst. Representative photomicrograph (20x-magnification) of CD11b (red fluorescence) or GFAP (red fluorescence), Aβ1-42 (green fluorescence) or APP (green fluorescence), nucleus (blue fluorescence), and the three merged in the same field. The sections are representatives of five pups from five litters. **G.** pIRES(APP mRNA 5’UTR) construct-transfected cultured neurons (upper panel) and astrocytes (lower panel) were treated with metal mixture (MM) or IL-1Ra + MM, and
assayed for luciferase activity. Data represent means ± SE of five independent experiments in triplicate. ***P<0.001 indicates significant difference compared to V or as indicated.

**H.** Cortical and hippocampal tissues from vehicle (V), individual metal, binary mixture and tertiary mixture-treated 10X-P90 rats were assayed for IL-1α and IL-1β. Representative immunoblot and densitometry showing IL-1α and IL-1β in cortex (LHS) and hippocampus (RHS) relative to β-actin. Data represent means ± SE of five pups from five litters. ***P<0.001, **P<0.01 and *P<0.05 indicate significant difference compared to V or as indicated, and cP<0.001 indicates significant difference compared to all.

**Figure 5: 10X-metal mixture impairs learning memory performance in developing rats**

**A.** Increase in number of errors (%) during learning, and fall in memory retained (% saving memory) at 24hr, 48hr and at 7th day post-learning measured against learning session in Y-Maze test of 10X-P90 compared to vehicle (V-P90) rats. Data represent means ± SE of twenty pups from five litters. ***P<0.001, **P<0.01 and *P<0.05 compared to 10X-P90 in learning session.

**B.** Transfer latency time in passive avoidance test measured during acquisition trial and 1st, 2nd and 3rd retention trials for 10X-P90 compared to vehicle (V-P90) rats. Data represent means ± SE of twenty pups from five litters. ***P<0.001 indicates significant difference compared to acquisition of vehicle, or as indicated.

**Figure 6: Proposed schematic As+Cd+Pb-induced AD-like pathology in young rats**

As, Cd and Pb-mixture induce oxidative stress that induces IL-1 via microglial and astrocytic activation in young rat frontal cortex and hippocampus. Up-regulated IL-1 promotes synergistic translation of APP through the 146-nt-APP mRNA 5’UTR that bears an IL-1 responsive element in +51-+94 nt. The APP undergoes enhanced processing by up-regulated
BACE, PS1 and PS2 to generate $\text{A}\beta_{1-42}$ and $\text{A}\beta_{1-40}$ in synergism. Induction of A$\beta$ peptides culminates in cognitive impairments and thereby manifests premature AD-like pathology.
**Table 1: Metal treatment given to pregnant, lactating and post-weaning rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>Treatment</th>
<th>Concentrations</th>
</tr>
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<tbody>
<tr>
<td>Group 1</td>
<td>Vehicle</td>
<td>Water</td>
</tr>
<tr>
<td>Group 2</td>
<td>1X (As+Cd+Pb)</td>
<td>NaAsO$_2$: 0.380ppm + CdCl$_2$: 0.098ppm + Pb(C$_2$H$_3$O$_2$)$_2$: 0.220 ppm</td>
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<tr>
<td>Group 3</td>
<td>10X (As+Cd+Pb)</td>
<td>NaAsO$_2$: 3.80ppm + CdCl$_2$: 0.98ppm + Pb(C$_2$H$_3$O$_2$)$_2$: 2.220 ppm</td>
</tr>
<tr>
<td>Group 4</td>
<td>As+Cd</td>
<td>NaAsO$_2$: 3.80ppm + CdCl$_2$: 0.98ppm</td>
</tr>
<tr>
<td>Group 5</td>
<td>As+Pb</td>
<td>NaAsO$_2$: 3.80ppm + Pb(C$_2$H$_3$O$_2$)$_2$: 2.220 ppm</td>
</tr>
<tr>
<td>Group 6</td>
<td>Cd+Pb</td>
<td>CdCl$_2$: 0.98ppm + Pb(C$_2$H$_3$O$_2$)$_2$: 2.220 ppm</td>
</tr>
<tr>
<td>Group 7</td>
<td>As-individual treatment</td>
<td>NaAsO$_2$: 3.80 ppm</td>
</tr>
<tr>
<td>Group 8</td>
<td>Cd-individual treatment</td>
<td>CdCl$_2$: 0.98 ppm</td>
</tr>
<tr>
<td>Group 9</td>
<td>Pb-individual treatment</td>
<td>Pb(C$_2$H$_3$O$_2$)$_2$: 2.220 ppm</td>
</tr>
</tbody>
</table>
Table 2: Metal treatment in neurons and astrocytes

<table>
<thead>
<tr>
<th>Group</th>
<th>Neurons</th>
<th>Astrocytes</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Vehicle</td>
<td>Water (Vehicle)</td>
</tr>
<tr>
<td>2</td>
<td>*MM (As+Cd+Pb)</td>
<td>NaAsO$_2$: 5 µM + CdCl$_2$: 1 µM + Pb(C$_2$H$_3$O$_2$)$_2$: 10 µM</td>
</tr>
<tr>
<td>3</td>
<td>As-individual treatment</td>
<td>NaAsO$_2$: 15 µM (3x5 µM)</td>
</tr>
<tr>
<td>4</td>
<td>Cd-individual treatment</td>
<td>CdCl$_2$: 3 µM (3x1 µM)</td>
</tr>
<tr>
<td>5</td>
<td>Pb-individual treatment</td>
<td>Pb(C$_2$H$_3$O$_2$)$_2$: 30 µM (3x10 µM)</td>
</tr>
</tbody>
</table>

* Mixture of 1/3rd IC50 on neurons or astrocytes
Table 3: CI-Value of $A\beta_{1-42}$, $A\beta_{1-40}$, APP, BACE and PS in tertiary mixture

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>As+Cd+Pb</td>
<td>(As+Cd)+Pb</td>
</tr>
<tr>
<td>$A\beta_{1-42}$</td>
<td>0.32</td>
<td>0.49</td>
</tr>
<tr>
<td>$A\beta_{1-40}$</td>
<td>0.39</td>
<td>0.46</td>
</tr>
<tr>
<td>APP</td>
<td>0.54</td>
<td>0.61</td>
</tr>
<tr>
<td>BACE</td>
<td>0.52</td>
<td>0.62</td>
</tr>
<tr>
<td>PS1</td>
<td>0.55</td>
<td>0.62</td>
</tr>
<tr>
<td>PS2</td>
<td>0.51</td>
<td>0.63</td>
</tr>
<tr>
<td>IL-1$\alpha$</td>
<td>0.52</td>
<td>0.65</td>
</tr>
<tr>
<td>IL-1$\beta$</td>
<td>0.64</td>
<td>0.72</td>
</tr>
</tbody>
</table>
Table 4: Relative fold change of IL-1α, IL-1β, IL-1R1, TNF-α and IL-6 in 10X-treated rats

<table>
<thead>
<tr>
<th>Inflammatory Markers</th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td>Relative levels of IL-1α</td>
<td>2.4 fold ± 0.14 ***</td>
<td>2.2 fold ± 0.13 ***</td>
</tr>
<tr>
<td>Relative levels of IL-1β</td>
<td>2.0 fold ± 0.06 ***</td>
<td>1.9 fold ± 0.08 ***</td>
</tr>
<tr>
<td>Relative levels of IL1-R1</td>
<td>2.3 fold ± 0.13 ***</td>
<td>1.8 fold ± 0.17 **</td>
</tr>
<tr>
<td>Relative levels of IL-6</td>
<td>2.0 fold ± 0.16 **</td>
<td>1.7 fold ± 0.13 **</td>
</tr>
<tr>
<td>Relative levels of TNF-α</td>
<td>1.8 fold ± 0.12 **</td>
<td>1.9 fold ± 0.10 **</td>
</tr>
</tbody>
</table>

Data are expressed as means of ± SE of five postnatal rats from different litters. ***P<0.001 and **P<0.01 compared to vehicle
Table 5: Oxidative stress parameters in vehicle and 10X-treated rats

<table>
<thead>
<tr>
<th>Oxidative Stress Markers</th>
<th>Cortex</th>
<th>Hippocampus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Vehicle-P90</td>
<td>10X-P90</td>
</tr>
<tr>
<td>LPO nmoles MDA formed/min/mg protein.</td>
<td>5.11 ± 1.02</td>
<td>14.79 ± 1.08***</td>
</tr>
<tr>
<td>SOD nmol formazan formed/min/mg protein.</td>
<td>3.29 ± 0.48</td>
<td>1.68 ± 0.13***</td>
</tr>
<tr>
<td>CAT µmol H₂O₂ consumed/min/mg protein</td>
<td>7.70 ± 0.30</td>
<td>4.36 ± 0.27**</td>
</tr>
<tr>
<td>GST nmol CDNB conjugate formed/min/mg protein</td>
<td>20.04 ± 0.20</td>
<td>14.19 ± 0.59**</td>
</tr>
</tbody>
</table>

Data expressed as means ± SE of five pups from five different litters. **P<0.01 and ***P<0.001 compared to vehicle
2A

Frontal Cortex

Hippocampus

2B

Frontal Cortex

Hippocampus