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Flubendiamide and lead exposure alters glial fibrillary acidic protein and aspartic acid concentrations in cerebrospinal fluid in buffalo calves

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Abstract

Changes in glial fibrillary acidic protein (GFAP) and aspartic acid (AA) concentrations in CSF following flubendiamide and lead exposure was studied. Male buffalo calves received daily oral dose of flubendiamide alone (n=4) @ 0.024mg/ kg/ day or lead acetate alone (n=4) @ 9.2 mg/ kg/ day or a combination of these two (n=4) for 90 days. Blood and CSF samples were collected and analyzed. GFAP in CSF did not alter in animals receiving flubendiamide alone. However, a significant increase in GFAP concentration in CSF was observed in animals receiving lead acetate. GFAP concentration showed a positive correlation (r = +0.545) with blood lead concentration in lead exposed animals, but no significant correlation was evident in animals exposed to both lead and flubendiamide. On the basis of results, it can be concluded that Aspartic acid concentration in CSF did not change significantly in lead treated animals, but increased significantly in flubendiamide treated animals. No significant alteration in AA level in CSF was observed in combined flubendiamide and lead treated animals. Flubendiamide exposure is associated with increase in AA level in CSF. Flubendiamide may have interactive effects with lead as evident from increase in GFAP and AA level in CSF after their combined exposure. This is the first report documenting alterations in GFAP and AA concentrations in CSF after flubendiamide exposure in mammals.

Keywords: CSF, Heavy metal, Pesticide, Toxicity

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1. Introduction

Adoption of intensive agriculture farming practices across the world has resulted in high insecticides and heavy metals residues in the food chain. This may be because over 98% of sprayed insecticides and 95% herbicides reach a destination other than their target species, air, water and soil[1]. Man and animals are often exposed to a cocktail of pollutants comprising different insecticide and heavy metals, posing a serious health hazard. One pollutant may have additive or synergistic effects or may antagonize the toxic effects of others[2]. Therefore, it is prudent to investigate the toxicity potential of a single pollutant alone as well as in combinations.

Flubendiamide belongs to a new class of insecticides known as phthalic acid diamides. It activates insect ryanodine receptors that are present on neurons, muscle and possibly other tissues of insects, leading to massive intracellular release of calcium ions. It has a potent and broad spectrum activity against the insects of order Lepidoptera and has great potential application in controlling these pests as a part of the insecticide resistance management[3]. The mammalian toxicity of flubendiamide has not been widely investigated so far. It does not activate mammalian ryanodine receptors, hence considered safe for non-target species. However, in a preliminary study, we observed marginal alterations in erythrocytic indices in buffalo calves exposed to fluobendiamide[4].

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Lead is considered as one of the most hazardous heavy metal pollutant that affects all biological systems. Lead toxicity has been reported from many countries as a cause of endemic chronic plumbism or saturnism in man and animals[5]. It poses a serious health hazard in developing countries due to strict environmental and occupational regulations[6]. Reports of high lead concentration in feed, fodder, mineral mixture and drinking water available for animal consumption indicated existence of Pb toxicity problem in livestock[7]. Moreover, its public health significance cannot be overlooked because high lead residues in milk have been reported in animals reared in the vicinity of industrial units[8].

The mechanism of lead induced neurotoxicity is complex and still not fully understood. The direct neurotoxic actions of lead include increased apoptosis, excitotoxicity affecting neurotransmitter storage and release, alterations in neurotransmitter receptors, mitochondrial damage and damage to cerebrovascular endothelial cells, astroglia and oligodendroglia[9]. Calcium dependent proteins and neurotransmitter receptors are major targets in lead toxicity[10].

Glial fibrillary acidic proteins (GFAP) are synthesized in astroglial cells in all parts of the brain. In cerebrospinal fluid (CSF), its level increases during structural damage in the brain. Aspartate is an excitatory neurotransmitter which is toxic above its physiological concentration[11]. Levels of GFAP and asparate in cerebrospinal fluid increases during inflammatory and degenerative conditions of the brain[12]. However, perusal of the available literature revealed no report available on changes in GFAP and aspartic acid concentrations in CSF following exposure to flubendiamide and lead, either alone or in combination. Since both flubendiamide and lead toxicity is associated with disruption in calcium ion homeostasis at cellular level, it was hypothesized that their simultaneous exposure may alter the toxic effects of each other. The present study aimed to investigate the effects of flubendiamide, lead and their combined exposure on GFAP and aspartic acid concentrations in CSF.

2. Materials and Methods

Experimental protocol- Sixteen healthy, 8 to 12 months old, male water buffalo (*Bubalus bubalis*) calves with body weight between 120-180 kg were used as experimental animals. They were provided balanced ration, dewormed and acclimatized for two weeks in the experimental animal house of the department. The ethical guidelines of CPCSEA, New Delhi on the proper care and use of animals, as approved by the Institutional Animal Ethics Committee, were followed throughout the experiment. The animals were divided into four equal groups. Group I animals did not receive any treatment and served as healthy control. Group II animals received flubendiamide (Fame, Bayer Cropscience Limited, Sabarkanta, Gujarat, India) at 0.024 mg/ kg/ day orally for a period of 90 days. Group III animals were given lead acetate at 9.2 mg/ kg/ day orally, while group IV received both flubendiamide (at 0.024 mg/ kg/ day) and lead acetate (at 9.2 mg / kg/ day) orally for 90 days. Blood and CSF samples were collected on 0, 30, 60, 90 days of treatment and on day 30 post-treatment. Blood samples were collected by jugular venipuncture. CSF samples were collected by puncturing lumbo-sacral cistern after anaesthetizing the local skin with 2% Lidocaine as per the procedure recommended by Smith and George (2009)[30]. The CSF samples were centrifuged at 2300 rpm for 10 min just after collection and the supernatant obtained was stored at -80^{0}C till analysis.

Laboratory analysis - Blood samples were wet digested as per the standard procedure after adding double acid mixture consisting of 3 parts nitric acid (70%) and 1 part perchloric acid (70%). Lead concentration in digested sample was estimated using Atomic Absorption Spectrophotometer (AAS, Analyst 700, Perkin Elmer, USA) equipped with a Deuterium background corrector and a graphite furnace. A wavelength of 283.3 nm and a spectral slit width of 0.7 nm were used.

The quantitative determination of Glial Fibrillary Acidic Protein (GFAP) concentration in CSF was done by microplate competitive enzyme immunoassay using standard kits of GenX-Bio. Aspartic acid (AA) concentration in CSF was determined using High Performance Liquid Chromatography (HPLC, Perkin Elmer, USA)[13]. HPLC system consisted of a single pump (Perkin Elmer, 200) and autosampler injector with 200 micro-liter loop, fluorescence detector (Perkin Elmer, 200 series), and total chrom software® (version 6.1) for analysis. The excitation and emission wavelengths were 330 and 445 nm, respectively. Browniee Analytical Amino Column (Perkin Elmer®, Particle size 5µ, and 4.6 X 150 mm; Catalog no-N9303504, USA) severed as a stationary phase. The limit of quantification was 0.625 µ mol/ l. The

precision of the analytical method was determined by evaluating intra-day and inter-day variation. Intra-day variation was determined by assaying three replicates of known concentration on three different occasions, at least 24 hours apart. Precision of the method was expressed as percent of co-efficient of variation (CV)/ RSD (Relative Standard Deviation). The inter-day and intra-day variations were found to be 8.2 % and 9.15 % respectively.

Statistical analysis - The data obtained were presented as mean ± SE and evaluated using Duncan Multiple Range Test. The statistical analysis was carried out using Statistical Product and Service Solutions (SPSS), version 16.0 (SPSS Inc., China) statistical software. $P< 0.05$ was considered statistically significant.

3. Results

In flubendiamide exposed animals blood lead concentration did not alter significantly on different observation days (Table 1). The mean values varied between 0.070 ppm to 0.095 ppm, which was close to the value observed in control animals. Exposure to lead acetate in buffalo calves resulted into significant progressive increase in blood lead concentration reaching peak on day 90, when it was 414.44 % higher than day 0. On day 30 post-treatment, the concentration decreased by 37.08 % from day 90. Combined oral administration of flubendiamide and lead acetate resulted into significant increase in blood lead concentration on day 60 and 90 by 604.28 and 975.71 % respectively from day 0. On day 30 post-treatment, the concentration decreased by 34.26 % from day 90, but was still above the critical level of 0.35 µg/ ml.

Flubendiamide administration did not induce any significant change in GFAP concentration on different observation days, though the values increased on day 30, 90 and post-treatment day 30 (Table 2). GFAP concentrations on different observation days did not differ significantly from corresponding value in control. However, on day 60 and 90, values were significantly lower than group III and IV. Lead acetate exposure resulted into non-significant decrease in GFAP on day 30, but the level increased significantly by 81.55 and 225 % on day 60 and 90 respectively. On day 30 post-treatment, the level decreased significantly by 23.44 % from day 90, but the concentration was still significantly higher (148.81%) than day 0. Combined exposure to flubendiamide and lead resulted into significant increase in GFAP concentration on day 60 and 90 by 53.57 and 173.98 % respectively from day 0. On day 30 post-treatment, the level declined non-significantly by 4.28 % from day 90, but it was 162.24 % higher than day 0.

Flubendiamide exposure resulted into progressive increase in AA concentration on day 30, 60 and 90 by 17.61, 55.11 and 73.29 % respectively (Table 3). However, only day 60 and 90 levels differed significantly from day 0. The level on day 30 post-treatment decreased non-significantly from day 90, but it was 30.68% higher than day 0. Lead acetate exposure resulted into increase in AA concentration on day 30, 60 and post-treatment day 30 by a level up to 28.95 %, though the values within the group on different observation days did not differ significantly from each other. The values on different observation days were statistically comparable to corresponding values in control. Combined administration of flubendiamide and lead induced progressive but non-significant increase in AA concentration on day 30, 60 and 90 by 44.58, 61.78 and 71.34 % respectively from day 0. The level on day 60 and 90 were significantly higher than corresponding values in control animals and group III, but did not differ significantly from group II.

Table 1. Changes in blood lead concentrations (µg/ ml)

Group	Day of observation				
	0	30	60	90	30 post-treatment
I	0.075 ± 0.014^{aA}	0.082 ± 0.016^{aA}	0.092 ± 0.023^{aA}	$0.072\pm0.011^{a\,A}$	0.070 ± 0.011^{aA}
II	0.095 ± 0.015^{aA}	0.075 ± 0.006^{aA}	0.080 ± 0.008^{aA}	$0.070\pm0.010^{a\,A}$	0.072 ± 0.005^{aB}
III	0.090 ± 0.019^{aA}	$0.107\pm0.018^{a\,A}$	0.463 ± 0.081^{bB}	$0.747\pm0.037^{c\,B}$	0.470 ± 0.090^{bB}
IV	0.070 ± 0.020^{aA}	0.092 ± 0.012^{aA}	0.493 ± 0.175^{bB}	$0.753\pm0.037^{c\,B}$	0.495 ± 0.015^{bB}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead

Values bearing different superscript in small letters in a row and capital letters in a column differ significantly ($P<0.05$)

Table 2. Changes in glial fibrillary acidic protein (GFAP) concentration (µg/ ml) in CSF

Group	Day of observation				
	0	30	60	90	30 post-treatment
I	1.82±0.07^{aA}	1.78±0.24^{aA}	1.86±0.06^{aA}	1.75±0.13^{aA}	1.68±0.20^{aA}
II	1.72±0.22^{aA}	1.74±0.10^{aA}	1.64±0.23^{aA}	1.84±0.11^{aA}	1.99±0.05^{aA}
III	1.68±0.23^{aA}	1.57±0.24^{aA}	3.05±0.17^{bB}	5.46±0.25^{dB}	4.18±0.16^{cB}
IV	1.96±0.08^{aA}	1.73±0.21^{aA}	3.01±0.08^{bB}	5.37±0.28^{cB}	5.14±0.31^{cC}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead
Values bearing different superscript in small letters in a row and capital letters in a column differ significantly (P<0.05)

Table 3. Changes in aspartic acid concentration (µmol/ l) in CSF

Group	Day of observation				
	0	30	60	90	30 post-treatment
I	1.90±0.20^{aA}	1.75±0.44^{aA}	1.38±0.13^{aA}	1.53±0.30^{aA}	1.83±0.11^{aA}
II	1.76±0.23^{aA}	2.07±0.16^{abA}	2.73±0.25^{bcB}	3.05±0.43^{cB}	2.30±0.13^{abcA}
III	1.52±0.29^{aA}	1.96±0.53^{aA}	1.71±0.35^{aA}	1.16±0.04^{aA}	1.76±0.55^{aA}
IV	1.57±0.26^{aA}	2.27±0.59^{aA}	2.54±0.26^{aB}	2.69±0.16^{aB}	2.41±0.40^{aA}

Gr I: Control; Gr II: Flubendiamide; Gr III: Lead; Gr IV: Flubendiamide + Lead
Values bearing different superscript in small letters in a row and capital letters in a column differ significantly (P<0.05)

4. Discussion

The blood lead level of 0.35 µg/ ml is widely accepted as critical level for poisoning, but death commence when it reaches close to 1.0 µg/ ml[14]. However, the limit of blood lead concentration in occupationally exposed human adults, as recommended by Agency for Toxic Substance and Disease Registry is 60 µg/ dl[10]. Blood lead level close to this critical level, particularly in chronic Pb exposure, may not be manifested by characteristic clinical signs of lead toxicity in animals[14]. In the present investigation, most of the animals remained apparently normal up to one month with absence of clinical signs of lead toxicity. However, one calf died on day 35, after showing acute nervous signs like circling, blindness and head pressing. When blood Pb level reaches to 0.496 mg/ ml, early symptoms of lead poisoning, like mild to moderate anorexia starts and nervous signs develop; death occurs when the mean blood level reaches 0.835 mg/ ml or more[15]. Flubendiamide co-administration had no interactive effect as Pb concentration on different observation period was statistically comparable to corresponding values in animals receiving lead alone.

Lead is one of the most common causes of heavy metal poisoning in farm animals, particularly in ruminants[14]. Contamination of pasture from industrial emission and other sources such as discarded batteries, empty paint tins and machinery grease are major sources of lead exposure to domestic and wild mammals[16]. Grazing animals suffer from plumbism by ingestion of contaminated herbage and soils. The toxicity is associated with high mortality in animals in polluted environment with no or few long term premonitory signs of depression, head pressing, violent movement, blindness and salivation[17]. Lead poisoning is most frequently diagnosed in cattle being the most susceptible domestic animal species[14]. Toxic effects of lead in cattle have been widely investigated, but reports of lead toxicity in water buffaloes are meager[15]. Water buffalo is an important dairy animal with estimated population of 91 million in India. Moreover, India accounts for two-thirds of the world's entire production of buffalo milk. Water buffaloes in India are often maintained in an open environment where they are exposed to several kinds of pollutants. It is pertinent to note that few reports in the past suggested that water buffaloes are more susceptible for fluoride toxicity than cattle[18].

GFAP level in CSF of buffalo calves did not change following flubendiamide administration. Lead acetate administration, on the other hand, caused significant increase in GFAP concentration. GFAP is synthesized in astroglial cells in all parts of the central nervous system. GFAP is the structural subunit of the astroglial filaments which are mainly found in the fibrillary astrocytes. Since only low levels of this protein is present in serum, structural damage to the brain causes a selective leakage of this protein from the brain tissue into the cerebrospinal fluid irrespective of the blood-brain barrier dysfunction[19]. GFAP is not readily soluble, but is highly susceptible

to degradation to form water soluble products[20]. Concentration of GFAP in CSF increases as a consequence of acute encephalomyelitis, CNS vasculitis, encephalitis, meningitis, intracranial tumors, cerebrovascular damage and dementia[19]. Results of the present study were in agreement with the findings of Struzynska and coworkers[21] who observed increase in GFAP expression in rat brain after daily intraperitoneal administration of lead acetate. They attributed these changes to lead induced chronic glial activation in immature rat brain. Biphasic response with initial decrease and final increase in GFAP levels in rat brain was recorded, suggesting GFAP as a sensitive indicator for insult to astrocytes in lead-induced neurotoxicity[22]. Increase in GFAP in CSF in the present study, may therefore be assumed as end result of lead-induced activation of astroglial cells. Several studies have shown that astroglia can accumulate and store Pb and may function as a 'Pb sink'[23]. Toxic Pb insult in the early period of embryonic development may impair both glial and neuronal function[24]. The most important function of astroglial cells is maintenance of the homeostatic environment for proper functioning of neurons. In many pathological conditions including heavy metal toxicities, glial cells undergo rapid changes which have been described as reactive gliosis. One of the most important features connected with this phenomenon is the increased expression of two glial markers, GFAP and S-100b protein. Over expression of these two proteins occur in response to neuronal damage[25]. Flubendiamide appeared to have no direct toxic effect on astroglial cells, as GFAP levels did not alter after its oral exposure. GFAP level showed a positive correlation (r = +0.545) with blood lead concentration in lead alone exposed animals, but no significant correlation was evident in two other treatment groups, perhaps due to flubendiamide and lead interaction after their combined exposure. Pb exposure resulted into non-significant increase in aspartic acid concentration in CSF. Likewise, in animals treated with both lead and flubendiamide, AA concentration increased non-significantly. On the other hand, flubendiamide alone treated animals showed significant increase on day 60 and 90. We could not find any published research report documenting changes in AA concentration in CSF during lead or flubendiamide toxicity. Aspartate is an excitatory neurotransmitter which increases the likelihood of depolarization in the postsynaptic membrane. Aspartate and glycine form an excitatory/ inhibitory pair in the ventral spinal cord comparable to the excitatory/ inhibitory pair formed by glutamate and GABA in the brain. Both glutamate and aspartate are known to be neurotoxic at supraphysiologic concentration[11]. Excessive increase in extra-cellular glutamate and aspartate have been demonstrated after ischemia, hypoxia, head injuries and prolonged seizures[26]. Glutamate and aspartate levels are almost doubled in viral meningitis, acute multiple sclerosis and myelopathy[12]. They are believed to mediate excitotoxicity by acting as agonists at the NMDA receptors leading to cellular oedema and accumulation of intracellular Ca^{2+} and Na^{+} which leads to lethal and sublethal excitotoxic effects[27]. Aspartate level in CSF of patients with infantile spasms was found significantly higher suggesting role of aspartate in triggering the spasms and development of neuronal dysfunctions in these patients[28]. However, a non-significant decrease in CSF aspartate levels was observed in adult epileptic patients and the level further declined following administration of valproic acid, an antiepileptic drug[29]. Findings of the present study suggested that neurotoxicity of Pb is not associated with alteration in aspartate level in CSF. Flubendiamide exposure can cause increase in aspartate level in CSF, though its level may not reach so high to induce clinical signs of neurotoxicity. No significant alteration in AA level in CSF in combined flubendiamide and lead treated animals suggested interactive effects of lead on flubendiamide induced increase in AA level. However, further study is required to elucidate mechanism of flubendiamide associated increase in aspartate level in CSF and its clinical significance.

5. Conflict of Interest

None

6. References

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