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Histomorphological Changes and Cognitive Function in Fluoride and Arsenic Induced Injury to Hippocampus and the Cerebral Cortex in Wistar Rats

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Abstract: Fluoride and Arsenic are two inorganic contaminants found in drinking water, and ingestion of such drinking water may result in complicated adverse effects. This research aimed to study the effects of Fluoride and Arsenic alone and their co-exposure on the histomorphological changes in sub regions of the hippocampus and cerebral cortex and learning and memory ability in Wistar rats. One-month-old male Wistar rats 32 were randomly allocated and assigned to a Control group, Fluoride group, Arsenic group, and Fluoride + Arsenic group. The control rats were treated with potable water. The remaining rats were treated with 120 ppm of Fluoride and 70 ppm of Arsenic water. Hebb William's Maze and T-Mazes have been used as learning and memory tests. Learning and memory ability declined in Arsenic and Fluoride + Arsenic-treated rats compared to Fluoride and Control rats. The assessment of histomorphological changes in the hippocampus and cerebral cortex by Cresyl violet stain. In the control group, neurons exhibited a clear nucleus and cytoplasm, In Arsenic and Fluoride + Arsenic-treated rats showed irregularly arranged cells and evidence of Karyopyknosis compared to Fluoride and Control rats. The hippocampus and cerebral cortex showed many vacuoles. The pyramidal cells were irregular in shape and had darkly stained nuclei, Granular cells were shrunken and deeply stained in Arsenic and Fluoride + Arsenic-treated rats compared to Fluoride and Control rats. Combined Fluoride and Arsenic exposure for long duration has damaging effects on the brain as resulted in diminished learning and memory ability and histomorphological changes compared to the effect of Fluoride and Control groups.

Keywords: Fluoride, Arsenic, hippocampus, cerebral cortex.

氟化物和砷致威斯达大鼠海马和大脑皮层损伤的组织形态学变化和认知功能

摘要：氟化物和砷是饮用水中发现的两种无机污染物，摄入此类饮用水可能会导致复杂的不良影响。本研究旨在研究氟化物和砷单独及其共同暴露对威斯达大鼠海马和大脑皮层亚区域组织形态学变化和学习记忆能力的影响。1月龄雄性威斯达大鼠32只随机分为对照组、氟化物组、砷化物组和氟化物+砷化物组。对照大鼠用饮用水处理。其余的大鼠用120

ppm的氟化物和70
ppm的砷水处理。赫布·威廉的迷宫和吨型迷宫已被用作学习和记忆测试。与氟化物和对照大鼠相比，砷和氟化物+砷处理的大鼠的学习和记忆能力下降。通过甲酚紫染色评估海马和大脑皮层的组织形态学变化。在对照组中，神经元显示出清晰的细胞核和细胞质，在砷和氟化物+砷处理的大鼠中，与氟化物和对照大鼠相比，显示出不规则排列的细胞和核固缩的证据。海马和大脑皮层显示出许多空泡。与氟化物和对照大鼠相比，砷和氟化物+砷处理的大鼠的锥体细胞形状不规则且细胞核染成深色，颗粒细胞缩小且染色深。与氟化物和对照组的影响相比，长期接触氟化物和砷会对大脑产生破坏性影响，导致学习和记忆能力下降以及组织形态学变化.

关键词：氟化物、砷、海马体、大脑皮层。

1. Introduction

Fluoride (Fl) and Arsenic (As) are two inorganic contaminants found in drinking water, and their consumption can lead to various complications such as dental fluorosis, bone disease, osteoarthritis, numbness in limbs, skin pigmentation and so on [1]. In trace amounts, Fl is required for the mineralization of bone, teeth, and the formation of dental enamel. However, at higher doses (>1.5mg/L), it may cause skeletal and dental fluorosis [2]. Kolar is one of Karnataka's 16 fluorosis-endemic districts. The concentration of Fl in Kolar ground water ranges between 2.8 and 4.3 mg/L, which are above the permissible limits. Dental fluorosis was found in 31.05% of people [3]. The toxicity of FI to the developing brain, which results in lower intelligence quotient (IQ) and impairment of learning and memory, is a contentious issue. It could also be the cause of Autism, Alzheimer's, and Parkinson's diseases [4].

As a metalloid element, it is found in water, air and soil and is also released into the environment by industrial processes such as metal smelting, fossil fuel consumption, insecticide, and pesticide manufacturing and so on.[5] Arsenic in ground water exists primarily as oxy-anions in two oxidation states: arsenite and arsenate. Arsenite is more toxic than arsenate. According to the WHO, more than 200 million people worldwide may be chronically exposed to As in drinking water at concentrations greater than the WHO safety standard of 10g/L [6].

UNICEF and the Government of Karnataka conducted studies that revealed several villages with well water containing more As than the WHO accepted value (>10μg/L). One of these studies hypothesized that local gold mining was a factor in As contamination [7]. Traces of As in ground water might be due to the presence of sulfide bearing ore such as arsenopyrite. As is released during the oxidation of sulfide [8]. High concentrations of As exposure can affect children's intelligence and growth [9].

The wide use of Fl-containing toothpastes and mouth washes together with high As in groundwater causes co-exposure to Fl and As. Fl and As can cross the blood-brain barrier and the placental barrier, and accumulate in the brains of rats exposed to high levels of Fl and As [10]. Chronic exposure to Fl and As in drinking water has been linked to lower intelligence and impairment in cognitive and neurobehavioral function [11].

The hippocampus is an important part of the brain for learning and memory. The hippocampus is divided into four regions: CA1, CA2, CA3, and CA4, with the CA3 region controlling spatial memory. Fluoride damages the hippocampus and interferes with its physiological functions [12]. The hippocampus is involved in learning and memory, and accumulation of As and its metabolites in animal models modulates the long-term potentiating threshold for expression [13].

Individual toxicities of Fl and As have been shown in previous studies to impair a child's intelligence and neurobehavioral function, including learning and memory [11]-[13]. However, little is known about the combined effects of Fl and As on histomorphological changes and learning and memory impairments.

The current study aimed to investigate the effects of Fl and As alone and in combination Fl+As on histomorphological changes in sub regions of Hippocampus and Cerebral cortex and Learning and Memory ability in Wistar rats.

2. Materials and Methods

2.1. Animal Model

INVIVO Biosciences in Bengaluru provided 32 male Wistar albino rats weighing 80-100 g. All the rats were kept in the Central Animal House facility at Sri Devaraj Urs Academy of Higher Education & Research under pathogen-free conditions. The rats were housed individually in polypropylene ventilated cages with paddy husk bedding and kept at a temperature of 25 ± 3° C with a 12 h/12 h light/dark cycle. The mice were fed a standard pellet diet and given unlimited access to water (*ad libitum*).

The Institutional Animal Ethics Committee approved the animal experimental protocol (No. IAEC/PHARMA/SDUMC/2017-18/03) and experiments were carried out in accordance with the guidelines of the Committee for Control and Supervision of experiments in Animals (CPCSEA), Ministry of Environment and Forests (Government of India), New Delhi, India.

2.2. Inclusion Criteria

Male rats in good health were included.

2.3. Exclusion Criteria

Female and unhealthy rats were excluded.

2.4. Chemicals

Nice chemicals, Kochi, supplied sodium fluoride (NaF-98% purity) and Sodium Arsenite (NaAsO2-98.5% purity). Purified water was used to dissolve the chemicals. One hundred and twenty ppm of Fluoride 120 ppm and 70 ppm of arsenic water solutions were freshly prepared every day.

2.5. Experimental Design

After one week of acclimatization, the rats were randomly assigned to a control group (C), Fluoride group (FI), Arsenic group (As) and Fluoride + Arsenic group (FI +As) using the block randomization method. Each group contained eight rats and was individually housed in cages.

Group I (Control): Rats were given unlimited access to water (*ad libitum*) for 180 days.

Group II (Fluoride): Rats were given 120 ppm of sodium fluoride in their drinking water (*ad libitum*) for 180 days.

Group III (Arsenic): Rats were given 70 ppm of sodium arsenite in their drinking water (*ad libitum*) for 180 days.

Group IV (Fluoride + Arsenic): Rats were given 120 ppm of sodium fluoride and 70 ppm of sodium arsenite in their drinking water for 180 days.

2.6. Growth and Development

Every 7 days, the body weights of all rats were recorded and the volume of water and feed consumed was measured daily.

2.7. Behavioral Tests

At the end of the experimental period, all the groups (C, Fl, As and Fl + As) of rats were subjected to behavioral tests. T-maze and Hebb Williams maze tests were used to assess learning and memory.

2.7.1. T- Maze Test

The goal of this test was to evaluate the rat's spatial learning ability. This examination included both spontaneous alternation and rewarded alternation tests [1].

2.7.2. Hebb-Williams Maze

The Hebb-Williams maze is an exteroceptive behavioral model that uses incentives to assess spatial working memory and long-term memory in rodents. The Hebb-Williams maze operates on the principle that "the faster the rat navigates the maze, the better its spatial memory". The Hebb-Williams maze has fewer stresses on the animals than the water version of the maze [16].

After the maze tests were completed, rats were sedated with an intraperitoneal injection of Ketamine: xylazine (60:6 mg/kg, i.p.) [17]. The sacrificed rats were then decapitated and the brain tissues from each group were collected, washed with normal saline and fixed in 10% neutral buffered formalin. After fixation, the brain tissue samples were processed, embedded in paraffin blocks, 5 μm tissue sections were prepared using microtome and stained with Cresyl violet and examined under a light microscope for histomorphological changes.

2.8. Light Microscopic Examination

The stained slides were examined with a light microscope at 10X and 40X magnifications, the CA1, CA3 regions of the hippocampus, DG and Cerebral cortex were identified.

2.9. Statistical Analysis

The data were expressed as mean ± standard deviation (SD) and statistical significance of group differences was determined using one-way Analysis of Variance (ANOVA), followed by Bonferroni's post hoc test. IBM SPSS statistics version 22 was used for all statistical analyses. To determine statistical significance, a *p*-value of less than or equal to 0.05 was used.

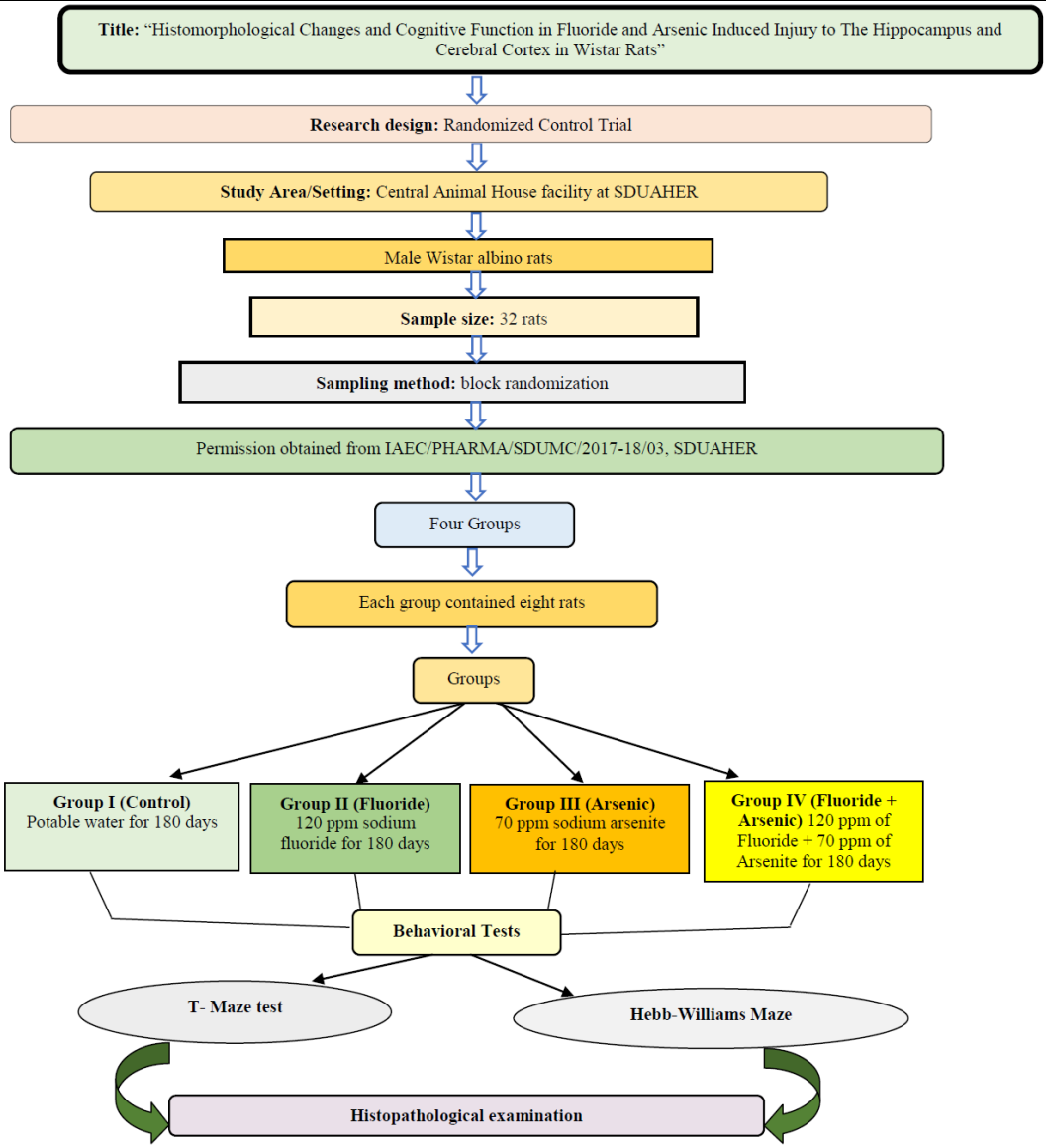

Fig. 1 Research methodology: schematic representation of research methodology

3. Results

3.1. Growth and Development

All rats were examined daily for general appearance and physical condition, which included moving activities, appetite, and alertness as well as the appearance of hair, nose, eyes, and limbs. The general appearance of rats exposed to Fluoride, Arsenic or Fluoride + Arsenic for six months did not differ from the control group. There was no mortality in any groups of rats.

3.2. Water and Food Consumption

Every week, rats' average daily water and food consumption was recorded. At the end of the study, consumption of food and water was significantly lower in groups Fl, As and F+As compared to control (Table 1).

Table 1 Volume of water intake (ml/rat/day) and feed consumed (g/rat/day)

Groups	Number	Daily water consumption (ml)	Daily food consumption (g)
Control	8	44.85 ± 3.844	27.915 ± 0.9649
Fl	8	32.50 ± 3.942*	26.123 ± 0.5309*
As	8	29.27 ± 1.614*	24.831 ± 0.9927*
Fl+As	8	28.15 ± 1.515*	24.458 ± 1.2100*

Notes: Data represent mean ± SD; * $P < 0.05$ compared to the control group

From the eighth week to the end of the experiment, weekly body weight gains in the As and Fl +As groups were significantly (P<0.05) lower than in the control and Fluoride groups. The body weight gain of rats in the FI group did not differ significantly from that of the control group (Fig. 2).

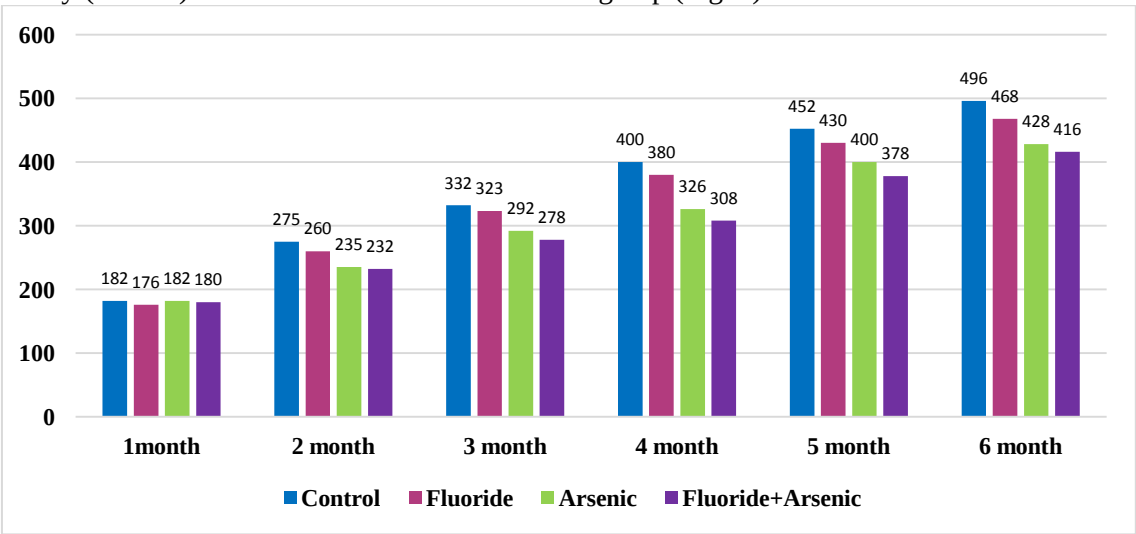

Fig. 2 Body weight of the rats (g/rat/month) exposed to Fl, As, Fl+As and control rats

3.3. Learning and Memory Tests

3.3.1. T-Maze Test

In the T- maze test, rats given Fluoride, Arsenic, or Fluoride +Arsenic showed significant impairment in spatial learning and memory in the form of more percentage bias, fewer alternations and a lower percentage of correct responses compared to the control group of rats (Table 2).

Table 2 Learning and memory ability by T–Maze test

Groups	N	Spontaneous alternation test		Reward alternation test
		Number of alternations	% Bias	% of Correct Responses
C	8	15.88 ± 0.835	52.50 ± 2.976	76.00 ± 4.140
Fl	8	13.25 ± 0.707*	61.75 ± 3.732*	66.75 ± 3.412*
As	8	11.13 ± 0.835*	66.63 ± 4.173*	58.50 ± 3.338*
Fl+As	8	9.75 ± 1.035*	68.88 ± 3.944*	54.50 ± 3.338*

Notes: Data represent mean ± SD; C – control; Fl – Fluoride; As - Arsenic; Fl + As – Fluoride + Arsenic; * P < 0.05 compared to the control group

3.3.2. Learning and Memory Ability by Hebb Williams Maze

The Hebb Williams maze was used to assess learning and memory ability. The animal's time in seconds to reach the Reward Chamber (TRC) from the start box was recorded. The animals were trained for three days (three trials per day) before the readings were taken on the fourth day. A significant decrease in the TRC value indicated improved memory in animals, whereas a high TRC value indicated poor memory in animals. When compared to the control group of rats, Fl, As and Fl+As treated rats had significantly higher TRC values. When compared to the control group, learning and memory ability in the Fl, As and Fl+As treated rats decreased (Table 3).

Table 3 Learning and memory ability by Hebb-William's maze

Groups	N	TRC (Sec)
Control	8	35.38 ± 8.2
Fluoride	8	104.13 ± 5.0*
Arsenic	8	102.63 ± 7.2*
Fluoride + Arsenic	8	114.25 ± 5.3*

Notes: Data represent mean ± SD; * P < 0.05 compared to the control group

3.4. Histopathological Examination

Cresyl violet staining was used to assess histomorphological changes in the hippocampus, dentate gyrus and cerebral cortex subfields. The cellular architecture of the rat hippocampus and cerebral cortex.

3.4.1 Qualitative Analysis

A light microscope at 40X magnification was used to evaluate the general morphology of brain sections from each animal. Images of the brain regions were captured using a microscope-mounted digital camera. These images were then examined for morphological changes

such as cell shrinkage and cell size in the hippocampus and cerebral cortex's CA1, CA3, and DG sub-regions.

Cresyl violet staining revealed that no histological changes were seen in the control group's CA1, CA3, and DG sub-regions of the hippocampus. CA3 is the region with large and less densely packed cells, CA1 is the region with densely packed medium sized cells, and Dentate gyrus is the region with densely packed granular cells. Cells were arranged orderly and intact, with nuclei stained clear and dark blue, whereas in Fl, As and Fl+As treated groups showed more degenerative neurons and Pyramidal cells were shrunken and darkly stained with a small nucleus (nuclear pyknosis), showing vacuolar changes and a decrease in the viable neurons (Figures 2, 3 & 4).

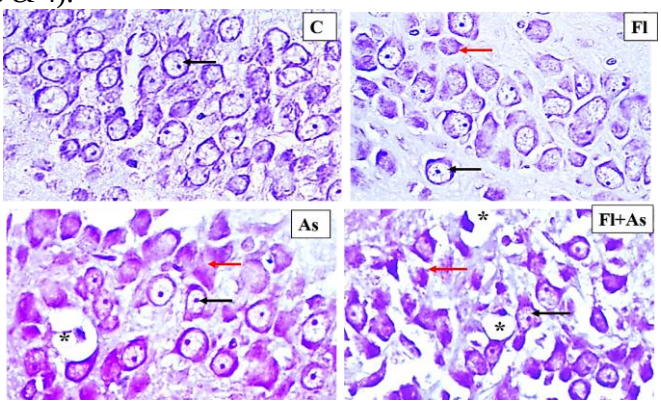

Fig. 2 Photomicrograph shows the CA3 region of Hippocampus stained by Cresyl violet stain (40X)

Notes: C - control; Fl – Fluoride; As – Arsenic; Fl + As – Fluoride + Arsenic; the black arrow mark shows normal pyramidal cells with a centrally placed nucleus; the red arrow mark shows darkly stained apoptotic cells (Karyopyknosis); (*) shows the vacuolated pyramidal cells.

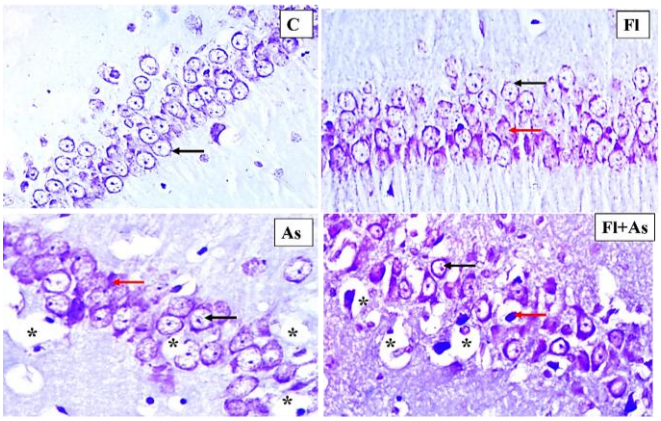

Fig. 3 Photomicrograph shows the CA1 region of Hippocampus stained by Cresyl violet stain (40X)

Notes: C - control; Fl – Fluoride; As – Arsenic; Fl + As –Fluoride + Arsenic; the black arrow mark shows normal pyramidal cells with a centrally placed nucleus; the red arrow mark shows darkly stained apoptotic cells (Karyopyknosis); (*) shows the vacuolated pyramidal cells.

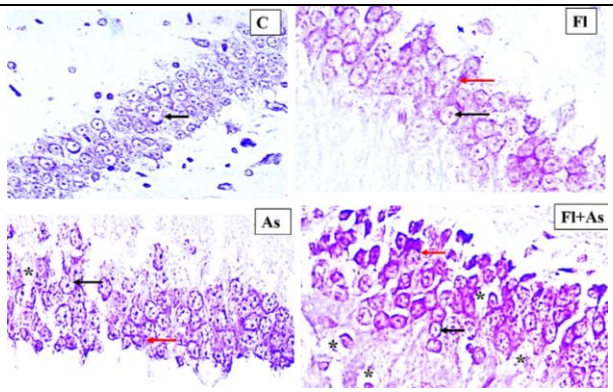

Fig. 4 Photomicrograph shows the Dentate Gyrus stained by Cresyl violet stain (40X)

Notes: C - control; Fl – Fluoride; As – Arsenic; Fl + As - Fluoride + Arsenic; the black arrow mark shows normal granule cells with a centrally placed nucleus; the red arrow mark shows darkly stained apoptotic cells (Karyopyknosis); (*) shows the vacuolated granule cells.

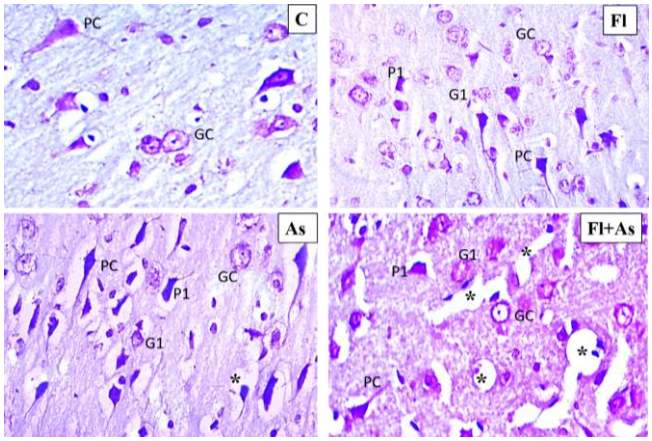

Fig. 5 Photomicrograph shows the cerebral cortex stained by Cresyl violet stain (40X)

Notes: C - control; Fl –Fluoride; As – Arsenic; Fl + As – Fluoride + Arsenic; PC - pyramidal cell; GC - granule cell, granule cells with rounded nuclei; G1 - shrunken and deeply stained granule cells; P1 - pyramidal cells are irregular in shape and have darkly stained nuclei; (*) shows the vacuolated pyramidal and granule cells.

Microscopic examination of CV-stained sections of the cerebral cortex were obtained from Fluoride, Arsenic & Fluoride + Arsenic groups of rats revealed severe multifocal histological changes in all layers of the Cerebral cortex compared to the control group. Many vacuoles of variable sizes, either single or multiple appeared in As and Fl + As groups compared to the Fl and Control group of rats. Most of the nerve cells were shrunken with loss of their processes and had pericellular halos. The pyramidal cells were more affected; they lost their processes and had deeply stained nuclei and became irregular in shape in As and Fl + As groups compared to Control and Fl groups. The neuropil among the nerve cells and neuroglia showed vacuolation. Most of the

granule cells were affected and became faintly stained, ill- defined boundaries with the loss of their nuclei and nucleoli. Both Pyramidal & Granule cells had pericellular halos and showed a vacuolated background in the As and Fl + As groups compared to Control and Fluoride groups (Fig. 5)

4. Discussion

The research findings revealed that, individual and combined exposure to Fl and As, affect the animals' physiological and anatomical responses. Animals' biological responses to Fl and As are related to dosage and other factors, according to epidemiological and experimental research.

According to [4], there were no statistical differences in body weight and water and food intake between dams and pups exposed to Fl. These results are inconsistent with this study, which may be attributable to the dose and duration of exposure and animal species.

A decrease in body weight gain in rats was observed beginning in the third week of treatment with sodium fluoride alone and continuing throughout the treatment period [18]. It was found that Fluoride experimental group's body weights and physical activity were significantly lower [19]. As reported in [20], rats body weight decreased As exposed group. Animals exposed to high concentrations of Fl for long periods of time gained less body weight than those exposed to low doses of Na-Fl for a short period of time and rats in the control group [21].

A reduction in body weight was observed in rats exposed to arsenic at various doses and times [22]. According to [23], the growth rate (g/rat/day) was not significantly different among groups. However, when compared to other groups, water intake was significantly lower in the highest dose group, whereas feed intake was significantly lower in the 0.4 and 40 ppm groups compared to the 4-ppm group. As found in [24], the weekly body weight gains in As and Fl + As groups were significantly lower than in the control and Fl groups. It was reported in [10] that rats exposed to arsenic alone or along with fluoride and arsenic had a significant decrease in body weight compared to control and fluoride-exposed rats. Our findings are comparable to these studies. The loss of body weight could be a result of the general toxic effect of the chemical, reduced food and water consumption, reduction in the repair and synthetic activities of various cells.

The effects of Fl and As alone and Fl + As co-exposure on cognitive function, learning, and memory were assessed in rats using T-maze and Hebb-Williams maze. As reported in [4], in a rodent model, exposure to Fl during early stages of rat development leads to memory impairment in young offspring. According to [25], fluoride exposure, even at low concentrations, can exacerbate learning and memory deficits. Rats exposed to sodium fluoride had a higher percentage bias and a lower number of alternations, which is considered as an indicator of learning and memory impairment [2]. Compared to the control group, the Fl-toxicated rats made more mistakes. Fl causes oxidative damage in the Hippocampus by producing excessive ROS [26]. As reported in [21], the learning and memory assessed during maze test showed reduced memory retention in rats exposed to high Fl for long periods. According to [27], the learning and memory ability decreased in rats exposed to Fl. Postnatal As exposure impaired learning and memory in rats [28]. According to [29], the behavioral tasks showed significant spatial memory impairment in males but only a marginal effect in females.

According to [30], As exposure can significantly reduce learning ability and accelerate memory impairment. Fluoride and arsenic may impair spatial learning and memory ability [10]. As found in [1], when rats were individually exposed to fluoride and arsenic, and when the two elements were combined, their learning and memory ability decreased. According to [9], high concentrations of As or fluoride can affect children's intelligence and growth. In this study, Fl and As exposure, either alone or in combination, impair cognitive function, including learning and memory. Our findings follow previous research demonstrating that rats co-exposed to Fl + As have significantly lower learning and memory ability than rats exposed to Fl or As alone. Treatment with Fl and As alone and in combination changed the histomorphology of the brain, where the combined effect was greater than the individual exposure. As found in [30], FI causes oxidative stress in rats' hippocampal cells in rodent models. According to [32], neuropathological examinations of cerebrum in Fl treated rats revealed that some pyramidal neurons had chromatolysis and shrank, with vacuolation surrounding them. Many granule cells were swollen in shape and size, with darkly stained nuclei and empty spaces around them. According to [33], the Fl may exacerbate the cytoarchitectural arrangement in the hippocampus by changing the structure of pyramidal neurons and neuroglia. As found in [9], brain histological structures in all experimental groups were damaged compared to the control group. The cells were shrunk and darkly stained, with a small nucleus, and their number was reduced. Furthermore, there was an increase in the cell gap. According to [19], sodium fluoride reduced the neuronal density more pronouncedly in the CA3 region of the hippocampus. A decrease was reported in the number of neurons, the presence of vacuolated cells, and degenerated nerve cell bodies in the hippocampal sub-

regions of CA1 and CA3. With neurodegenerative cells, the cerebral cortex showed "balloon-shaped" chromatolysis. Vacuolated and pyknotic neurons are found at the cerebrum's periphery in rats intoxicated with Fl [34]. Neuropathological changes in the form of necrosis, degeneration, atrophy, and pyknosis of pyramidal neurons were observed in Ammon's horn of the hippocampus of Fl treated rat [21]. A disarrangement of pyramidal cell layer of the hippocampus and a decrease in the number of pyramidal neurons in rats' brains exposed to As were discovered in [35].

In this study, we observed that the combined toxicities of Fl and As caused a more extensive neurodegeneration than Fl and As alone. In contrast, as reported in [36], the concomitant arsenic exposure and fluoride also caused cellular damage; however, it was less pronounced compared to their individual exposure. These results are inconsistent with this study, which may be attributable to the chemical dose, duration of exposure, and animal species.

5. Conclusion

The current study investigated the effects of FI and As alone and in combination of Fl + As on histomorphological changes in CA1, CA3, DG of the hippocampus and the cerebral cortex and Learning and Memory ability in Wistar rats. Our findings indicate that individual As exposure and combined Fl + As exposure have more significant effects on histomorphological changes and learning memory ability than fluoride exposure alone. Long-term intake of Fl and As combination and As alone has negative effects on the brain, resulting in decreased learning and memory ability as well as histomorphological changes by altering the structure of pyramidal neurons and granular cells in the hippocampus and cerebral cortex of rats compared to the effect of Fl alone. Oxidative stress, induced by Fl and As, may be a causative factor reducing learning and memory ability and histomorphological changes in the brain. However, a realistic human exposure simulation remains challenging, and the design extrapolation of experimental results to humans requires further studies.

5.1. Research Strengths

Our results highlight the significant impact of Fl+As Co-exposure on histomorphological changes in the brain and learning & Memory ability.

5.2. Limitations

All the complications and limitations of our study, however, would not lead to systematic errors that would challenge the main findings. However, we emphasize the need for more careful evaluation of the effects of Fluoride and Arsenic co-exposure, which may be attributable to the dose and duration of exposure and animal species.

5.3. Recommendations and Future Research

This study can form the baseline for future studies of Fluoride and Arsenic toxicity. This study recommends community campaigns and social media as the best ways to increase awareness about the toxicity of Fluoride and Arsenic consumption and follow mitigation strategies. Identify the Fluoride endemic areas and gold mining areas with well water containing high Fl and As than the WHO accepted value and create the awareness of the effects of Fluoride and Arsenic in that areas. This study can suggest to conduct learning and memory tests to determine the IQ levels of school children and to improve the IQ levels by easing methods. A standardized IQ (intelligence quotient) test, based on the classic Raven's test, can be used to determine the effects of Fl+As exposures on children's intelligence.

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