Memory-Enhancing Effects of Folic Acid Against Fluoride-Induced Cognitive Deficits in Adult Male Rats

EBTIHAL A. ABD EL-AZIZ, M.D.*; AZZA S. ABDELHAFFEZ, M.D.* and ASMAA M. AHMED, M.D.**

The Departments of Medical Physiology* and Pathology**, Faculty of Medicine, Assiut University

Abstract

Background: The present study interrogated the potential protective role of folic acid (FA) against fluoride induced neurocognitive dysfunction in adult male rats.

Methods: Forty rats were included, divided into four groups: Control, sodium fluoride (NaF) group which received NaF for 8 weeks, therapeutic group which received NaF for 4 weeks then stopped and was given FA for another 4 weeks and prophylactic group which received NaF and FA concomitantly for 8 weeks.

Results: Exposure to fluoride resulting in remarkable impairment of the memory capacity as observed by Morris water maze (MWM) test and the novel object recognition test (NORT). Augmentation of the hippocampal oxidative stress (increment of malondialdehyde (MDA) and the decrement of reduced glutathione (GSH) levels) and the enhancement of hippocampal apoptosis as evidenced by increased of tumor suppressor protein 53 (p53) and reduction of survivin expressions were also observed. Furthermore, hippocampal tissue inhibitor of metalloproteinase-1 (TIMP-1) expression and the levels of monoamine neurotransmitters (serotonin (5-HT), norepinephrine (NE) and dopamine (DOP)) were reduced. FA treatment significantly abrogated these effects. The histopathological analysis corroborated the biochemical studies with apparent hippocampal neurodegeneration and apoptotic changes with fluoride exposure and marked alleviation of these findings with FA treatment. However, the mitigation influences of FA administration in the prophylactic group were more obvious than the therapeutic group.

Conclusion: The present work signifies the valuable wholesome influences of FA in protection as well as an incoming therapeutic agent against cognitive deficits.

Key Words: Neurocognitive dysfunction – Fluoride – Folic acid – Oxidative stress – Apoptosis – Tissue inhibitor of metalloproteinase-1 (TIMP-1) – Monoamine neurotransmitters.

Introduction

THE acquisition of memory is a procedure including molecular and cellular events that occur early after a training session and last from several hours to a few days in specific brain regions, like as the hippocampus [1]. Dysfunction in intellectual performance and learning are symptoms characterize cognitive dysfunction [2].

Fluorides are exceedingly used in wire insulation, pipe linings, rodenticides, refrigerants, plastic polymers, isotopes preparation, phosphate fertilizers, ceramics and brick industries [3]. Human being is incurred to fluoride via food, drinking water, toothpastes, fluoride additives, and professional administration of fluoride gel [4]. Fluorosis occurred due to exposure to water and other foods containing fluoride for long term [5]. Fluoride exposure has injurious effects on the brain as reflected in decline learning and memory [6]. Chronic fluoride exposure starts oxidative stress which is a causative factor for cell deterioration and apoptosis [7].

P53 is a transcription apoptotic protein responding to plentiful extrinsic and intrinsic stress stimuli, involving DNA damage, oncogene activation, and hypoxia [8]. Survivin was known to be the strongest inhibitor of apoptosis determined to date and it is involved in cell division regulation [9,10]. Reduction of survivin stimulates the activity of caspase-3, 9 and enhances apoptosis [11].

Matrix metalloproteinases (MMPs) are family of enzymes consist of more than 20 members, all of which require Zn$^{+2}$ for their enzymatic activities [12]. It was conceivable that MMPs adjust the levels and the functionality of extracellular matrix components and cell surface signaling receptors [13]. The proteolytic activity of MMPs are regulated by tissue inhibitors of metalloproteinases (TIMPs) which are endogenous inhibitors that play a significant role in normal growth and development of CNS [14]. Imbalance between MMPs/anti-MMPs with reduction of TIMP-1 has been reported in a
model of cerebral ischemia and reperfusion [15]. Moreover, it was reported that MMPs inhibitors may be beneficial neuroprotective therapeutic agents [15]. TIMP-1 actions are not limited to MMPs inhibition, it can act as a signaling molecule influencing various biological processes including cell growth, apoptosis, differentiation and angiogenesis too [16].

Folic acid (FA), a water-soluble B vitamin, is naturally present in a variety of foods [17]. Folic acid is not only related to hematopoietic system, but also has an influence in the nervous system growth and development. It plays a pivotal role on the proliferation and differentiation of neural stem cells [18]. It is one of the powerful antioxidant because has a free radical scavenging property [19]. It enhances the cell survival rate and inhibits apoptosis [20].

This study was designed to elucidate the conceivable salubrious role of folic acid against fluoride-induced cognitive dysfunction. Also, it aimed to explore the possible molecular mechanisms and related nervous pathways that might contribute to the salutary effects of folic acid treatment.

**Material and Methods**

**Experimental animals:**

Fourty adult male albino rats with average body weight (175±25g). They were obtained from Assiut University Animal House (Assiut, Egypt) during 2016. The animals were housed in stainless steel good aerated cages at temperature 23 ±3°C, fed on standard rodent pellet diet and tap water were given ad libitum. These animals were maintained at constant daily light/dark periods for 12 hours. Animal care and use followed the guidelines of the Animal Committee of the Faculty of Medicine of Assiut University and were in accordance with procedures outlined in the National Institutes of Health Guidelines.

**Chemicals:**

FA and NaF were purchased from Sigma (St. Louis, MO, USA). All the other chemicals and solvents used in the study were of analytical grade and were obtained either from Sigma Company or commercial suppliers, unless otherwise mentioned.

**Experimental design:**

After 1 week of acclimatization, rats assigned randomly to four groups and each group comprised of 10 as follows:

*Control group*: Rats were given distilled water. *NaF group*: Rats were given NaF at a dose of 4mg/kg [21] for 8 weeks.

*Therapeutic group*: Rats treated with NaF of the previous dose for 4 weeks then stopped and followed by FA at a dose 1.5mg/kg [22] for another 4 weeks.

*Prophylactic group*: Rats were treated with NaF and FA of the previous doses for 8 weeks.

FA and NaF were dissolved in distilled water. Each animal received its treatment daily in the morning by oral gavage. Behavioral tests were done in the last week of the experiment.

**Behavioral assessments:**

*Morris water maze (MWM) test:*

A modification of the spatial version described by Morris [23] was used. This behavioral test was developed to evaluate spatial memory acquisition and retention, and is employed to assess the function of the hippocampus in the development of this type of memory. The MWM consisted of a circular dark tank (180 cm diameter and 60cm depth) filled with 24±1°C opaque water (made white with powdered milk). It was divided into four quadrants with a small hidden platform (12cm in diameter) fixed in a perma-nent position located 1-2cm under the water’s surface. The experiment was started by placing the rat on a start platform in the tank from 4 different randomly chosen start positions with the head facing the wall of the tank. The rat swims around until it finds the other platform to stand on. The researcher measures how long it takes for a rat to find hidden platform. Each experiment lasted until the rat finds the invisible platform at fixed positions in the water tank or for a maximum duration of 120 seconds. The escape latency, the time to reach the platform, was recorded for each animal. If a rat could not find the platform within 120 seconds, it was guid-ed to the platform and also allowed to stay there for 30 seconds; the latency was scored as 120 seconds. Acquisition trials were per-formed five times daily for five consecutive days. On the sixth day, the probe test was conducted as in the acquisition trial. However, the platform was removed in probe test. Animals were allowed to swim freely for one minute, the time spent in the target quadrant and the numbers of crossing the plat-form were recorded as indica-tors of memory retention.

*Novel object recognition test (NOR):*

NORT is a simple test depending on a rodent’s innate exploratory behavior. It is a sensitive and reliable memory assessment tool that is able to
detect behavioral and cognitive effects. It gives information on working, short-term or long-term memory depending on the elapsed testing phase. It consists of three phases: Habituation, familiarization, and test phase. In the habituation phase, each rat was allowed to freely explore the stainless steel open box (65 x 45 x 65cm) in the absence of objects for 3 min. Then the rat was removed from the box and placed in its holding cage. During the familiarization phase, two identical sample objects (familiar red round objects) were placed in the open-box and each rat was allowed to freely explore the two identical objects for 3min. To avoid bias, rat was released in the center of the open-box with its back to the objects. Test was done after retention intervals of 90 minutes (short-term memory) and twenty four hours (long-term memory). During the test phase, each rat was returned to the open-box for 3min with two objects, one was familiar (red round object) and the other was novel (novel yellow pyramid). Novel and familiar objects must have different colors, shape and size; this allows rats to recognize them as novelty. Normal rats usually remember the familiar object and therefore spend more time exploring the novel object during the test phase. Discrimination index (DI) was calculated as the difference in exploration time for novel and familiar (TF) objects divided by the total time of exploration of the novel and familiar objects \[ DI = \frac{(TN - TF)}{(TN + TF)} \] [24].

**Sample collection:**

At the end of behavioral tests, the animals sacrificed. Following sacrifice, the rats were perfused intracar-dially with isotonic saline and whole brains were carefully removed and dissected. Each brain sample was mid-sagittally divided into two halves. The first half fixed in 10% formalin buffer for histopathological and immunohistochemical study. In the second half, the hippocampus was micro-dissected, washed in isotonic saline, dried, weighed then homogenized with ice cold 0.1M phosphate buffer (pH 7.4) in a volume 10 times the weight of the tissue. The homogenate was centrifuged at 10,000g for 15min (4°C) and aliquots of supernatant were separated and stored at –80 for further biochemical analysis.

**Biochemical analysis:**

1- **Assessment of tissue levels of Oxidative stress markers:**

Lipid peroxidation, as evidenced by the formation of malondialdehyde (MDA), was assayed by the method described by Mihara and Uchiyama [25]. Tissue levels of reduced glutathione (GSH) were measured using Ellman assay method [26].

2- **Estimation of the brain monoamines neurotransmitters concentrations by high-performance liquid chromatography (HPLC) method:**

Serotonin (5-HT), norepinephrine (NE) and dopamine (DOP) levels were determined using by high performance liquid chromatography (HPLC) with fluorescence detection (Agilent Technologies 1200 series). The hippocampal tissue was homogenized (1/10, w/v) in 0.2 N HClO4 containing 1 mM EDTA and 0.1 % cystine. The homogenate was centrifuged at 15000 rpm for 10min at 4°C. Then 100 µl supernatants were directly injected to the Zorbax 300 SB-C18 column. (4.6mm x 150mm, 5 µm) under the following conditions: The mobile phase consisted of acetate buffer (pH 3.5, 12mM acetic acid, 0.26mM Na2Edta)100, flow rate 1 ml/min, the injection volume was 20 µl, the temperature was 35°C and the fluorescence excitation and emission wave lengths were set at λex 279nm and λem 320nm, respectively. The resulting chromatogram identifying each neurotransmitter position and area under curve for each sample was compared to that of the standard curve made by Eurochorm HPLC Software, version 1.6.

**Molecular analysis:**

1- **Detection of survivin, TIMP-1 and P53 by Western blotting:**

Fifty micrograms from each protein homogenate were denatured by boiling for 5min in 2% SDS and 5% 2-mercaptoethanol and loaded in each lane [27]. Sodium dodecyl sulfate–poly acrylamide gel electrophoresis (SDS–PAGE) was done at 100 V for 2h using 12% gels. The electro-transfer was done using T-77 ECL semidry transfer unit (Amersham Biosciences) for 2h. The membrane was blocked in TBS buffer that contains 0.05% Tween and 5% non-fat milk for 1h. The primary antibodies used were rabbit anti-survivin polyclonal antibody (Bioss Inc., Massachusetts, USA), rabbit anti-TIMP-1 polyclonal antibody (Biospes Co., Ltd., Chongqing, China) and mouse monoclonal anti-p53 antibody (Novus Biologicals, LLC, Littleton, CO, USA) at dilutions of 1:1500 and 1:1200 respectively. After being washed three times with TBST buffer, each membrane was incubated for one hour at room temperature with an alkaline phosphatase-conjugated goat anti-mouse secondary antibody (Novus Biologicals, LLC, Littleton, CO, USA) at a dilution of 1:1500 and 1:1200 and 1: 1500 respectively. After being washed three times with TBST buffer, each membrane bound antibodies were detected with a commercially available BCIP/NBT substrate detection Kit (Genemed Biotechnologies, Inc., CA, USA). Equivalent protein loading for each lane was confirmed by stripping and re-blotting each
membrane against mouse monoclonal anti-β actin antibody (Santa Cruz Biotechnology, Inc., CA, USA). The analysis was repeated to assure reproducibility of results.

**Histopathology and immunohistochemistry:**

The hippocampal samples were paraffin-embedded and 5-µm thick sections were prepared and stained with haematoxylin and eosin (H/E) for histopathological study.

For immunohistologic staining, 4-µm thick sections were prepared from the formalin fixed paraffin-embedded paraffin blocks. For immunohistologic staining, sections were deparaffinized and rehydrated. Endogenous peroxidase activity was blocked using 3% hydrogen peroxide treatment. For antigen retrieval, sections were heated in 10mM citrate buffer (pH 6.0) with microwave at 80 °C for 12min. Primary antibodies (Rat monoclonal Anti-TIMP1 antibody (ab86482) isotype IgG, dilution 1:50, abcam) was used to cover sections on each slide, and were incubated overnight at room temperature. Secondary staining kits were used according to the manufacturer’s instructions (Thermo scientific corporation Fremont, CA, USA).

**Positive and negative controls:** Breast carcinoma tissue was used as positive control [28].

**Statistical analysis:**

For statistical evaluation, the software package Prism (graph pad version 3.0) was used. Data were presented as means ± standard error of the mean (SE). Indicators of the acquisition trial of MWM were analyzed by two-way ANOVA, followed by paired student’s Newman-Keuls "t" test. The discriminative index, results of probe trial in MWM model and biochemical parameters were analyzed using one way ANOVA followed by post hoc Student’s Newman-Keuls "t" test. p-value of less than 0.05 was considered significant.

**Results**

Behavioral tests:

**A- MWM test:**

The escape latency of NaF group was significantly longer than that of the control group on day 2 to 4, the time spent in the target quadrant was significantly shorter than control group and the number of crossing the platform was lesser than the control group. In both therapeutic and prophylactic groups, the escape latencies were significantly improved over days as compared to NaF group but still significantly lower than the control group in the therapeutic group. In the day of probe test, after removal of the hidden platform, the time spent in the target quadrant of therapeutic and prophylactic groups was significantly longer with significant greater in the number of crossing the platform as compared to NaF group. Comparing to the control group, the number of crossing the platform and the time spent in the target quadrant of therapeutic group were significantly fewer. However, in the acquisition trial, the escape latency of the therapeutic group was significantly increased as compared to the prophylactic group. Also, the number of crossing the platform and the time spent in the target quadrant were significantly decreased in the therapeutic group as compared to prophylactic group. Normalization of number of crossing the platform and time spent in the target quadrant in the prophylactic group was observed.

The results of MWM test demonstrated significant alleviation of fluoride induced spatial memory impairment with FA treatment and this alleviation was significantly more prominent in the prophylactic group more than the therapeutic group.

**B- Novel object recognition test:**

The DI of fluoride exposed rats was significantly decreased for short-term memory and long-term memory as compared to the other groups. FA treatment resulted in remarkable improvement of DI in both prophylactic and therapeutic groups as compared to NaF group. The mitigation effect of FA treatment was significantly noticeable in prophylactic group as compared to therapeutic group for long-term memory. No significant difference in DI of long-term memory in prophylactic group as compared to control group (Fig. 2).

**Biochemical results:**

**Effect of FA on lipid peroxidation and GSH activities of the hippocampal homogenate in fluoride exposed rats:**

The changes in the lipid peroxidation and GSH in the hippocampus were presented in Table (1). Compared with the control group, MDA level in the hippocampus of the NaF group was significantly higher and GSH level was markedly diminish, whereas the levels in the prophylactic and therapeutic groups were significantly reduced for the MDA and strongly enhanced to the GSH levels when compared to NaF group but the changes for both didn’t reach the levels of the control group. In prophylactic group, the level was significantly decreased for the MDA and markedly increased for the GSH as compared to the therapeutic group.
Effect of FA on monoamine neurotransmitters of the hippocampal homogenate in fluoride exposed rats:

5-HT, NE and DOP levels were significantly reduced in NaF exposed group in comparison to the other groups. FA fortification resulted in significant increment of the three monoamines in the hippocampal homogenate in therapeutic and prophylactic groups as compared to fluoride treated group. Observed significant increase in the monoamines levels in prophylactic group as compared to therapeutic group. The monoamines levels of the prophylactic groups were not significantly different from those of the control group (Table 2).

Effect of FA on P53 expression of the hippocampal homogenate in fluoride exposed rats:

As evidenced in Fig. (3), treatment with NaF markedly enhance the expression manner of P53. Their expressions in the prophylactic and therapeutic groups were significantly decreased when compared to NaF group but the reduction didn’t reach the expression of the healthy control group. In prophylactic group, the expression was significantly decreased as compared to the therapeutic group.

Effect of FA on survivin and TIMP-1 expressions of the hippocampal homogenate in fluoride exposed rats:

Exposure to NaF resulted in significant decrement of survivin and TIMP-1 expressions as compared to control rats. However, there were remarkable increment of survivin and TIMP-1 expressions in the prophylactic and therapeutic groups as compared to NaF group, although the enhancement didn’t reach the expressions of the control group. In prophylactic group, the expressions were significantly increased as compared to the therapeutic group (Figs. 4,5).

Histopathological results:

The histopathological features of hippocampal tissues of rats in the four groups included in this study were described in Fig. (6). In NaF group, the cells were distorted and disorganized with remarkable degenerative changes. Also, shrunken neurons with darkly stained nuclei and vacuolated cytoplasm were observed (Fig. 6B). These pathological changes were markedly lesser in both therapeutic and prophylactic groups (Fig. 6C,D). This improvement was more apparent in prophylactic group which was nearly similar to that of the control group.

Immunostaining results:

Evaluation of the Immunostaining results: The proper positive and negative controls were positive and negative, respectively indicating the validity of our staining. Cells were considered positive when cytoplasmic staining [28] was observed. For the determination of the staining levels, immunoreaction against TIMP-1 was quantified by scoring between 0 to 3, i.e. 0, no immunoreactivity; 1, light immuno-reactivity; 2, moderate immunoreactivity and 3, strong immunoreactivity [29].

Staining for TIMP1 was found in the cytoplasm of the neurons. Strong cytoplasmic expression was found in normal control group. The expression was markedly decreased in NaF group which revealed weak cytoplasmic expression. The intensity was increased in both therapeutic and prophylactic groups IV. Moderate intensity was found in most neurons in prophylactic group and focally in therapeutic group (Figs. 7,8).

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NaF</th>
<th>Therapeutic</th>
<th>Prophylactic</th>
</tr>
</thead>
<tbody>
<tr>
<td>MDA (µmol/mg protein)</td>
<td>9.88±0.88</td>
<td>15.70±0.96*</td>
<td>13.37±1.32*,#</td>
<td>11.00±1.20*,#.•</td>
</tr>
<tr>
<td>GSH (µmol/mg protein)</td>
<td>12.70±1.07</td>
<td>6.42±0.45*</td>
<td>8.59±0.69*,#</td>
<td>10.78±0.30*,#.•</td>
</tr>
</tbody>
</table>

Data were displayed as mean ± SEM of ten rats.

Table (1): Mean Levels of MDA and GSH in the tissue homogenate of the hippocampus of the studied groups.

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>NaF</th>
<th>Therapeutic</th>
<th>Prophylactic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Serotonin</td>
<td>3.48±0.19</td>
<td>1.94±0.14*</td>
<td>2.41±0.15*,#</td>
<td>2.95±0.18#,•</td>
</tr>
<tr>
<td>Norepinephrine</td>
<td>15.67±0.48</td>
<td>9.38±0.36*</td>
<td>13.32±0.36*,#</td>
<td>14.5±0.4#,•</td>
</tr>
<tr>
<td>Dopamine</td>
<td>7.07±0.5</td>
<td>2.18±0.31*</td>
<td>3.92±0.71 *,#</td>
<td>5.94±0.55#,•</td>
</tr>
</tbody>
</table>

Data were displayed as mean ± SEM of ten rats.

Table (2): Mean monoamine neurotransmitters levels in the hippocampal homogenate of the studied groups.
Fig. (1): Effect of folic acid on the cognitive function obtained by MWM test in fluoride treated rats of the studied groups. The escape latency of the acquisition trial (A), the number of crossing the platform (B) of the probe test and the time spent in target quadrant (C).

Values are expressed as mean ± SEM; n=10 per group.

* p<0.05 vs. control group.

# p<0.05 vs. NaF group.

* p< 0.05 vs. therapeutic group.

Fig. (2): Effect of folic acid on cognitive function obtained by NORT discrimination indexes (DI) in fluoride exposed rats for short-term memory (STM) (A) and long-term memory (LTM) (B) of the studied groups.

Values are expressed as mean ± SEM; n=10 per group.

*p<0.05 vs. control group.  
#p<0.05 vs. NaF group.  
* p< 0.05 vs. therapeutic group.
Fig. (3): Representative Western blotting analysis of p53 in hippocampal homogenate of the studied groups. P-actin was used in parallel as an internal control.

Fig. (4): Representative Western blotting analysis of survivin in hippocampal homogenates of studied groups. P-actin was used in parallel as an internal control.

Fig. (5): Representative Western blotting analysis of TIMP-1 in hippocampal homogenate of studied groups. P-actin was used in parallel as an internal control.
Fig. (6): Histopathological changes in hippocampal tissue from rats (400 x): (A) Control group revealed unremarkable pathological changes. (B) NaF group revealed significant degenerative changes in the form of shrunken neurons with darkly stained nuclei and vacuolated cytoplasm. (C) Therapeutic group revealed neurodegenerative changes but to a lesser extent with less cytoplasmic vacuolation than NaF group. (D) Prophylactic group revealed histologic picture which was nearly similar to the normal control group.

Fig. (7): Immunohistochemical expression of TIMP-1: (A) Strong cytoplasmic TIMP-1 expression in Control group. (B) Weak TIMP-1 cytoplasmic expression in neurons of NaF group. (C&D): Moderate TIMP-1 cytoplasmic expression in Therapeutic and Prophylactic groups respectively.

Fig. (8): Mean TIMP-1 expression of the studied groups. Values are expressed as mean ± SEM; n=10 per group. *p<0.05 vs. control group. #p<0.05 vs. NaF group. $p<0.05$ vs. therapeutic group.
Discussion

The present study elucidated the putative neuroprotective role of FA treatment in NaF induced behavioral, biochemical, histological and abnormalities in rats. In the existing literature, fluoride administration elicited learning and memory impairment, increased oxidative stress and hippocampal apoptotic neuronal degeneration in rats. Impaired spatial learning and memory were obvious as observed by magnifying the escape latency time, reduction of the time spent in the target quadrant and enhance the number of platform crossings during probe testing in the MWM task. Spatial learning sounds to depend upon the coordinated action of different brain regions constituting a functionally integrated neural network, such as hippocampus and cortical brain regions [30]. Non-spatial memory can be assessed through NORT. NORT paradigm relies the rat's innate explorative behavior. In the current investigation, NORT used to assess the short-term as well as long-term episodic memory deficits. Fluoride exposure produced a significant impairment in spatial and non spatial memory, which was in accord with previous observations [31,32]. FA fortification mended the performances of the behavioral tests of the treated rats. Several studies have related folate supplementation with enhancing memory capacity [22,33,34].

The molecular mechanism underlying the pathogenesis of CNS damage induced by chronic fluorosis remains elusive. In the current issue, fluoride exposure reinforce the oxidative stress as it is clear from enhancement of MDA, a biomarker of lipid peroxidation, and decline the glutathione level. These results proposed that the balance between the oxidative system and anti-oxidant defense mechanisms in the rats were inoperative during fluoride treatment. It has been noticed that NaF administration associated with accretion of oxidative stress with heightened levels of lipid peroxidation and attrition of the antioxidant defense mechanism [35]. The brain and neural cells contain high amount(s) of polyunsaturated fatty acids, rich iron content, and low levels of antioxidant enzymes for that, they are extremely liable, to oxidative damage [36]. Fluoride was thought to be an initiator of oxidative stress by binding to the free radicals thereby can devastate significant antioxidant enzymes leading to cell damage and apoptosis [37]. Moreover, it affects the brain by inhibiting important enzymes associated with energy production, transfer, membrane structure, transport and synaptic transmission [38].

This study was confirmed the effectiveness of FA in combating free radical interposed oxidative insult produced by sodium fluoride (NaF). It is apparent, from the reduction of MDA and the increment of glutathione levels. These results were in accord with the studies of Singh et al. [19] and Barichello et al. [39] who imputed these changes to the free radical scavenging properties and possible antioxidant activity of FA.

The data of this study demonstrated remarkable augmentation of P53 expression in hippocampus of NaF treated rats. The current investigation was in conformity with Gutiérrez-Salinas et al. [40] who found an association between fluoride exposure and up regulation of the expression of p53 protein. In many cultured cell types [41] and in experimental animal models [42] fluoride was proved to be a powerful inducer of cell death. Also, it would be expected that the disruption of mitochondrial outer membrane and release of cytochrome c into cytosol, which activates intrinsic apoptotic pathway, extrinsic pathway, death receptor were involved in fluoride-induced apoptosis [8]. Eventually, apoptosis may be has a role in enhancing neuronal and cognitive losses [43].

Another finding of this study was that administration of fluoride elicited significant attenuation of survivin expression. It has been elucidated that NaF exposure exhibited decrement of apoptotic inhibitor proteins expression as it encouraged down regulation of survivin [44]. Undeniably, these results validate the success of FA administration in decreasing the expression P53 markedly with obvious increment of survivin expressions in hippocampus of NaF treated rats. Similar finding were observed by Srivastav et al. [45] who reported that FA supplementation could possibly participate in inhibiting the p53 thereby repair DNA damage. They attributed the reduction of p53 to the improvement in mitochondrial biogenesis. Of great interest, Coremans et al. [46] distinguished the function of survivin during neurogenesis, which is essential factor for the survival of developing neurons. Thus, it is worth mentioning that the impaired spatial learning, memory capacity and long-term potentiation (LTP) concordant with a deterioration of synaptic plasticity occurred as a consequence of survivin decrement [10,47].

One of the main issues of this work was the dramatic decrease of TIMP-1 gene expression in the hippocampus of NaF exposed rats and its significant increment with FA administration. It has been shown that TIMP-1 could contribute to the plasticity mechanisms involved in learning and long-term memory of hippocampus [48]. Therefore, reduced TIMP-1 observed in our study may advo-
cate its dynamic role in memory impairment. One of the possible mechanisms by which fluoride induced marked reduction of TIMP-1 was increment of oxidative stress. It has been reported that the free radicals have been known to inactivate or degrade TIMP-1 [49]. Taking into account that lowering of TIMP-1 levels may be responsible for increased activation of MMP-9, Mizoguchi et al. [12] have shown that amyloid \( \beta \) (A\( \beta \)) mediated neurotoxicity in vitro beside cognitive impairment in vivo significantly alleviated by treatment with MMP inhibitors and significantly reduced in MMP-9 homozygous knockout mice, indicating that MMP-9 expression in the hippocampus might be involved in A\( \beta \)-induced cognitive dysfunction.

In the current study, the magnitude of data clearly indicated that there was an additive advantage from treatment by FA as it significantly increased TIMP-1 expression in hippocampus. In parallel with this data, the finding obtained from Higa and its colleagues who showed up-regulation of TIMP-1 and down-regulation of MMPs with FA treatment in decidua and embryos from diabetic rats [50].

Neurotransmitter signaling was mostly linked with connection between differentiated neurons [51]. Serotonergic, dopaminergic, and noradrenergic systems participate many analogous synthetic and degradative enzymes and a reduction in all three monoamine metabolites may indicate an effect at some common metabolic point [52]. Thus, it was noteworthy that the concentrations of serotonin, dopamine and norepinephrine in the hippocampus were significantly decreased in NaF treated rats. This data was in adherence with Yu et al. [53] who clarified that fluoride affects the synthesis of monoamines and their receptors leading to changes in the neuronal function.

An accumulating evidence has demonstrated that FA markedly improve serotonin, dopamine and nor epinephrine levels in the hippocampus. These findings were in agreement with those of Miller [54] who observed that FA is necessary for the proper biosynthesis of the monoamine neurotransmitters. They added that the active metabolite of folate, 5-methyltetrahydrofolate (5-MTHF, L-methylfolate), participates in re-methylation of the amino acid metabolite homocysteine, creating methionine. S-adenosylmethionine (SAMe), the downstream metabolite of methionine, is included in numerous biochemical methyl donation reactions, including reactions forming monoamine neurotransmitters.

According to Cousens and Mayell [55] folic acid supplementation can heighten serotonin function by slowing the destruction of brain tryptophan, and that it also acts as a cofactor for enzymes that transform tryptophan into serotonin. 5-HT has been embroiled in the modulation of brain activity and function, such as sensory processing and mood [56]. Highly potent modulation of brain activity and function is accomplished by releasing 5-HT (serotonin) in targeted areas, in which several pre- and postsynaptic receptors are involved. 5-HT, mediated by the 5-HT1A receptor subtype, plays a main role in spatial learning and memory [57].

DA can modify the information processing in the hippocampus [58] and play an essential role in cognition [59]. Currently, there is great interest in the concept that hippocampal dopamine may expedite learning about relevant events by facilitating synaptic mechanisms of hippocampus dependent learning, such as place and episodic learning [60,61].

An issue is raised here hippocampal norepinephrine (NE) has, in recent decades, become well known as a neurotransmitter that is required for learning and memory [62]. It exerts its neuroprotective properties mostly via adrenergic stimulation of cAMP production and phosphorylated cAMP response element binding protein (pCREB) signaling, which in turn mediated pro-survival pathways [63]. NA defend rat primary cortical neurons against amyloid degeneration through a potential mechanism involved antioxidant protection through the stimulation of glutathione levels and energizing of antioxidant systems through the detoxification of superoxide radicals [64]. NA depletion contributed to cognitive impairment may be mediated by alterations in synapse composition and function [65].

The hippocampus plays an important role in cognitive function [33]. The present work demonstrated degenerative apoptotic changes in the hippocampal neurons of fluoride treated animals. Furthermore, Shrinkage and pyknosis of the neurons were obvious. These observations were consistent with previous findings Basha et al. [36] and Zhang et al. [66] who observed similar hippocampal pathological changes with chronic fluorosis. These morphological alterations could be due to enhanced apoptosis with fluoride administration [66]. The current work revealed improvement of morphology with FA treatment in both therapeutic and prophylactic groups. These findings were in agreement with Singh et al. [19]. These positive influences of FA supplementation could be mediated via its potent antioxidant and antiapoptotic effects.
The current study showed the ability of folic acid to be not only a protective factor but also it promotes recovery of memory impairment and cognitive deficits caused by fluoride exposure.

Considering the present results, the ameliorative effects of FA in prophylactic group were more prominent than in the therapeutic group. These results indicated that the protective role of FA against fluoride induced memory impairment was better than its therapeutic influence.

**Conclusion:**

The present study provides a valuable insight on the neuroprotective effects of FA in fluoride induced memory impairment model. FA posses powerful antioxidant and antiapoptotic with synergistic enhancement of TIMP-1 expression and monoamines neurotransmitters levels. Also, FA could be a beneficial prophylactic neuroprotective element as well as a novel adjuvant alleviative therapy in memory impairment and cognitive deficits.

**References**


أثار تعزيز الذاكرة من قبل حمض الفوليك ضد العجز الإدراكي الناتج عن الفلورايد في ذكور الجرذان البالغين

الملخص والهدف: هدفت هذه الدراسة إلى بحث الدور الوقائي المحتمل لحمض الفوليك ضد العجز الإدراكي الناتج من الفلورايد في ذكور الجرذان البالغين.

التجريبية: وقد تضمنت أربع جرذان، وقسمت إلى أربع مجموعات: المجموعة الضابطة، مجموعة فلورايد الصوديوم التي تلقى فلورايد الصوديوم لمدة 8 أسابيع، المجموعة الداعمة التي تلقى فلورايد الصوديوم لمدة 4 أسابيع ثم توقفت وأعطت حمض الفوليك لمدة 4 أسابيع. أجرت مجموعة الوقاية التي حصلت على فلورايد الصوديوم وحمض الفوليك مرتين لمدة 8 أسابيع.

النتائج: التعرض للفلورايد أدى إلى انخفاض ملحوظ في سعة الذاكرة كملاحظة من خلال اختبار متابعة موريس المائي واختبار التعرف على الشيء غير المألوفة. وزيادة في موت الخلايا المبرمج للحصين كما توضح من زيادة البروتين بين 32 ونقص البروتين سيروفين كاحد من البروتينات المثبتة لموت الخلايا المبرمج. ولاحظ أيضا زيادة الإجهاد التكسدي في منطقة الحضو (زيادة من الماندوديد). وانخفاض مستويات الجلوتاتين المنخفضة وخلوطة على ذلك. انخفاض كلا من مستويات البروتين مثبت إيزومير البروتاز البيئي، مستويات الناقل العصبي المتوازن، السيروتونين، النوربينفرين، الدوبامين. وقد أوضحت دراسة انتقال الحصين تفسيرات في موت الخلايا المبرمج مع التعرض للفلورايد. وقد أدت المعالجة بحمض الفوليك إلى تحسن في المظهر الساكنة وكلا التحسن أكثر وضوحاً في المجموعة الوقاية عن المجموعة العلاجية.

الاستنتاج: قد خلصت هذه الدراسة إلى فاعلية استخدام حمض الفوليك في علاج العجز الإدراكي بالإضافة إلى أهميته كعامل وقائي في هذا المرض.