Original Article

Protective effect of chronic caffeine intake on gene expression of brain derived neurotrophic factor signaling and the immunoreactivity of glial fibrillary acidic protein and Ki-67 in Alzheimer’s disease

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Abstract: Alzheimer’s disease (AD) is a neurodegenerative disorder with progressive degeneration of the hippocampal and cortical neurons. This study was designed to demonstrate the protective effect of caffeine on gene expression of brain derived neurotrophic factor (BDNF) and its receptor neural receptor protein-tyrosine kinase-β (TrkB) as well as glial fibrillary acidic protein (GFAP) and Ki-67 immunoreactivity in Aluminum chloride (AlCl₃) induced animal model of AD. Fifty adult rats included in this study were classified into 5 group (10 rats each); negative and positive control groups (I&II), AD model group (III), group treated with caffeine from the start of AD induction (IV) and group treated with caffeine two weeks before AD induction (V). Hippocampal tissue BDNF and its receptor (TrkB) gene expression by real time RT-PCR in addition to immunohistochemical study of GFAP and Ki67 immunoreactivity were performed for all rats in the study. The results of this study revealed that caffeine has protective effect through improving the histological and immunohistochemical findings induced by AlCl₃ as well as BDNF and its receptor gene expression. It could be concluded from the current study, that chronic caffeine consumption in a dose of 1.5 mg/kg body weight daily has a potentially good protective effect against AD

Keywords: Caffeine, BNDF signaling, glial fibrillary acidic protein, Ki-67, Alzheimer’s disease

Introduction

Alzheimer’s disease (AD) is a neurodegenerative disorder with progressive degeneration of the hippocampal and cortical neurons, and thus impairment of memory and cognitive ability. The pathological hallmarks of AD are spheroidal accumulations of b-amyloid protein (senile plaques) and degenerating neuronal processes as well as neurofibrillary tangles (paired helical filaments and other proteins) [1, 2] Many biochemical cellular changes induce neuronal programmed cell death. Among these cellular changes in AD are metabolic disturbances, disruption of Ca²⁺ homeostasis, oxidative stress, inflammation, and accumulation of unfolded/mis-folded proteins [3].

Brain derived neurotrophic factor (BDNF), a member of the neurotrophin family, is essential for growth, survival and neuronal cell differentiation. Moreover, it is involved in learning and memory by binding to its main functional receptor (neural receptor protein-tyrosine kinase-β; TrkB), present in the hippocampus, cortex and basal forebrain. The BDNF signaling, through binding to TrkB, is involved in the pathophysiology of AD [4, 5].

Astrocytes are critical for the survival of neurons in the central nervous system (CNS) by playing a role in the energy metabolism, maintenance of the blood-brain barrier, vascular reactivity, regulation of extracellular glutamate levels and finally protection from reactive oxygen species. These cells react to the neuronal damage, resulting from physical or chemical insults, by over expression of the glial fibrillary acidic protein (GFAP), an intermediate cytoskeletal filament protein specific for astrocytes [6]. Ki-67
is a reliable marker of cell proliferation expressed during all active phases of the cell cycle [G(1), S, G(2)] but is absent in resting cells [G(0)] [7]. During interphase, Ki-67 antigen can be exclusively detected in the nucleus, whereas in mitosis most of the protein is relocated to the chromosomal surface. Previously, Smith and Lippa [8] reported that Ki-67 may be involved in the pathogenesis of neurofibrillary degeneration in AD, other neurodegenerative disorders, normal aging, and neuronal neoplasms as ganglioglioma.

Santos et al. [9] have demonstrated that caffeine intake, one of the most widely consumed psychoactive substances, may reduce the risk of AD and cognitive impairment in elderly patients. Other studies have indicated that caffeine intake (1.5 mg/day) may reverse cognitive impairment and decrease brain amyloid beta (Aβ) levels in aged AD mice [10]. However, epidemiologic studies in humans came to be inconclusive, with some suggesting a reduced risk of dementia with higher coffee intake in midlife, and others pointing to a lack of association [11].

So, the present study was designed to clarify the role of chronic habitual use of caffeine intake as a protective agent in AD and to investigate its effect on gene expression of BDNF and TrkB as well as GFAP and Ki-67 immunoreactivity in aluminum chloride induced animal model of AD.

Materials and methods

Animals

A total of 50 adult male albino Sprague-Dawley rats (200-250 gm) were used in this study.

Used drugs

Aluminum chloride (AlCl₃, MW = 133.34) was purchased from Sigma-Aldrich Co. (Munich, Germany) and Caffeine was purchased from Pratap chemical industry Pvt Ltd (India).

Induction of Alzheimer's disease (AD) in rats

Induction of AD in rats was carried out by administering AlCl₃ orally at a dose of 17 mg/kg body weight daily for 4 successive weeks, according to the procedure described by Karam et al. [12].

Experimental protocol

The experimental protocol of the study was approved by the ethical committee of Medical Faculty of Mansoura University. Animals were used in accordance with the Animal Welfare Act and Guide for Care Use of MERC (Mansoura Experimental Research Center) prepared by Mansoura University. In this study 50 adult male rats weighing (200-250 gm) each were housed in a quiet non-stressful environment for one week before study. They were fed ad libitum and allowed free access to water during the experimental period. Animals were divided into five equal groups (10 rats each):

Group I: Each animal received 1 ml normal saline 0.9% by gavage for 4 successive weeks and served as the negative control group.

Group II: Each animal received 1.5 mg/day of caffeine [5] by gavage for 4 successive weeks and served as the positive control group.

Group III: Each animal received 17 mg/kg body weight of aluminum chloride once daily [12] by gavage for 4 successive weeks.

Group IV: Each animal received a combination of caffeine (1.5 mg/day) with AlCl₃ (17 mg/kg body weight) for 4 successive weeks.

Group V: Rats were given caffeine for 2 successive weeks, followed by a combination of caffeine with AlCl₃ for 4 successive weeks.

Sampling

At the end of each experiment, all animals were sacrificed by cervical dislocation. Craniotomy was performed to dissect out the intact brains for histological, immunohistochemical and molecular study. Brains were removed and sagittally divided into right and left hemispheres. Total RNA was extracted from 25-30 mg hippocampus tissue from one hemisphere immediately after shock freeze in liquid nitrogen and real time RT-PCR for BDNF and its couple receptor TrkB was performed [13].

Histological study

For histological study, the brain specimens (the other hemisphere) were fixed in Bouin's solution. After fixation, specimens were dehydrated
in an ascending series of alcohol, cleared in two changes of xylene and embedded in molten paraffin. Sections of 5 microns thickness were cut using rotary microtome and mounted on clean slides. For histological examination, sections were stained with hematoxylin and eosin (H&E) [14].

**Immunohistochemical (IHC) study**

The other paraffin-embedded sections were immunohistochemically stained with Anti-Ki-67 and with GFAP.

a)→Brain sections were immunohistochemically stained with proliferating cell nuclear antigen (Mouse Monoclonal Anti-Ki-67, Clone GM010, from Genemed Biotechnology Inc., 458 Carlton Ct. South San Francisco, CA 94080, USA), at dilution 1:50 or 1:100. Tonsil was used as a positive control.

b)→Brain sections were immunohistochemically stained with GFAP mouse monoclonal antibody (Mouse Monoclonal anti-human clone6F2 code No. M 0761, from DakoCytomation. Produktionsvej 42 DK-2600 Glostrup Denmark) at dilution 1:50 or 1:100. Brain was used as a positive control.

Sections were taken on positive slides and immunostained using avidin-biotin technique. Sections were deparaffinized in xylene, rehydrated and pretreated with 0.01% hydrogen peroxide (H₂O₂) for blocking endogenous peroxidase activity and unmasking of the antigenic site was carried out by transmitting sections into 0.01 M citrate buffer (ph 6) for 10 minutes in ethanol for 10 minutes. Microwave-assisted antigen retrieval was then performed for 20 minutes. Sections were incubated overnight at 40C with the diluted primary antibody at dilution 1/500 & 1/100 monoclonal mouse antibodies for GFAP and Ki-67. Sections were incubated with the avidin-biotin complex (ABC) reagent for 60 minutes then incubated in peroxidase substrate solution for 6-10 minutes. Finally, haematoxylin was used as a counter stain, dehydration in absolute alcohol, clearing and mounting were done. Immunoreactivity was visualized as dark brown cytoplasmic staining for GFAP while for Ki-67 it was visualized in the nucleus [15]. For the negative control slide, the specific 1 ry antibody was replaced by phosphate buffer saline.

**Quantitative analysis**

*Astrocyte counting in CA1 and CA3 regions:*

Slides were digitized using Olympus® digital camera installed on Olympus® microscope with 1/2 X photo adaptor, using 40 X objective. The result images were analyzed on Intel® Core i3® based computer using VideoTest Morphology® software (Russia) with a specific built-in routine for immunohistostaining analysis and stain quantification. The system measured the area percentage of GFAP positive expression.

*Ki67-positive cells counting in the dentate gyrus*

The numbers of Ki-67 positive cells in samples from all groups were determined using an image analysis system equipped with a computer-based charge-coupled device (CCD) camera (Optimas 6.5; Media Cybernetics, USA). Ki67-positive cells in the dentate gyrus of each section were counted using Optimas 6.5 software (Media Cybernetics). Cell counts for all the sections from every rat were averaged and are presented as a percentage.

*Total RNA extraction from the hippocampal tissue samples:*

According to the manufacturer’s instructions, total RNA was extracted from 25-30 mg hippocampal tissue samples. The tissue samples were snap frozen in liquid nitrogen and then used immediately for RNA extraction using the TriFast TM reagent (PeqLab. Biotechnologie GmbH, Carl-Thiersch St. 2B 91052 Erlongen, Germany, Cat. No. 30-2010). Any remained DNA was removed by digestion with DNase I (Sigma). The concentration and purity of the isolated RNA was determined using NanoDrop™ 2000 Spectrophotometer (Thermo Scientific., USA). Purity of the extracted RNA was also, confirmed using 2% formaldehyde agarose gel electrophoresis with ethidium bromide staining, to find the 2 sharply purified bands that represent 28S and 18S ribosomal RNA.

*Quantitative real-time PCR*

According to the manufacturer’s instructions, Reverse Transcription (RT) of the extracted RNA was performed using Maxima First Strand cDNA Synthesis Kit for RT-qPCR provided by ThermoScientific, U.S.A, cat No. #K1641. A control reaction, with 2 μl of purified RNA, was
run without reverse transcription to ensure absence of any DNA contamination in the extracted RNA samples. The synthesized cDNA is stored at -20°C until use for real time RT-PCR.

Gene-specific real time RT-PCR primers (purchased from Oligo™ Macrogen): Oligonucleotide primers (specific for the coding region of exon 5) were designed to amplify rat BDNF. Sequences of BDNF primers [16] were 5'-CAGGGGACAGACAAAG-3' (forward); and 5'-CTTCTCCCTTTATGTC-3' (reverse) (PCR product: 167 bp); TrkB-specific primers were 5’-CTGCCAGCCTCCTCACCACAT-3’ (forward); and 5’-GCAAGAACCTTATCGGAAAGC-3’ (reverse) (TrkB-product: 315 bp) [17], while for β-actin, used as internal control, 5’-CTC- TATGCTCTGCTCGAAGC-3’ (forward) and 5’-CCATCTTGTGCTCGAAGTCT-3’ (reverse) (PCR product: 260 bp) [18].

PCR reactions for optimization of primer annealing temperature were carried out using DreamTaq™ Green PCR Master Mix (2X) (Thermo Scientific, USA, cat No. 1081). Also, optimization of the primer concentration is performed, for use in real time RT-PCR reactions, to determine the primer concentration giving the lowest CT (threshold cycle) and minimizing nonspecific amplification. Briefly, amplifications (25 μL reaction) in duplicates, containing 12.5 μL 2X SYBR green Master mix, 2 μL cDNA and a variable concentration of forward and reverse primers, were run with thermal cycling parameters as an initial step of holding at 95°C for 10 min followed by 40 cycles of denaturation (hold at 95°C, 30 sec) and annealing (at 60°C, 45 sec). Then, RT-PCR products were analyzed on 2% agarose gel electrophoresis.

Quantitative real-time PCR analysis: The Real time PCR reactions were performed using real time PCR equipment (Applied Biosystem 7500, USA) with 96-well plates, and SybrGreen reagent [SYBR® Green PCR Master Mix (Applied Biosystem, USA, cat. No. 4344463)].

Amplification were performed, in duplicates, in a 25 μL total reaction containing 12.5 μL Power Sybr® Green PCR Master Mix reaction buffer (Applied Biosystems), forward and reverse gene specific primers (10 pmol) and 2 μL cDNA. The cycling parameters included 40 cycles (each of hold at 95°C for 30 sec, 60°C for 45 sec) after one initial step of 95°C for 10 min, to activate AmpliTaq Gold polymerase. The PCR reactions were monitored by measuring the increase in fluorescence caused by binding of SYBR Green Dye to the double stranded DNA and CT values (cycle threshold) were determined. To confirm the specificity of the PCR products, melting curve analysis and agarose gel electrophoresis 2% were performed. In each experiment, negative control reaction with no template was run.

The relative quantification of the BDNF and TrkB genes in different hippocampal tissue samples was performed by the comparative method. β actin was used as an endogenous control gene to standardize the cDNA amount added to the reaction. Each analysis requires 4 reactions (2 for analysis of the target gene (BDNF or TrkB) and 2 for analysis of the internal standard gene, β actin). The ΔCT for each sample is calculated and linearized using $2^{-\Delta CT}$. Finally ΔΔCT between experimental and control samples were calculated and linearized using $2^{-\Delta \Delta CT}$ formula for overall change. Thus, the amount of target gene (BDNF or TrkB) in the experimental groups, normalized to the endogenous reference gene (β actin) and relative to control group, is calculated by an arithmetic formula.

Statistical analysis

Data were tabulated, coded then analyzed using the computer program SPSS (Statistical package for social science) version 17.0. Descriptive statistics were calculated in the form of Mean and Standard deviation (± SD). In the statistical comparison between the different groups, the significance of difference was tested using ANOVA (analysis of variance) to compare between more than two groups of numerical (parametric) data followed by post-hoc for multiple comparisons. Pearson correlation coefficient test was used correlating different parameters. A P value <0.05 was considered statistically significant in all analyses.

Results

Histological results

Group I: Microscopic examination of H&E. stained sections of all specimens in this group (negative control group) was similar and revealed the normal histological structure specific for the hippocampus. These were the hip-
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The hippocampus proper, dentate gyrus and subiculum. The hippocampus proper was formed of the Cornu Ammonis (CA) as CA1, CA2, CA3 & CA4 regions, and is continued as subiculum (S). Dentate gyrus (DG) is seen surrounding CA4 by its upper & lower limbs. Notice the presence of molecular layer (M) inside concavity of CA and DG.

Examination of CA1 & CA3 regions showed that it was formed of three layers; molecular, pyramidal and polymorphic layers. The main cellular layer was the pyramidal layer which was formed of 4-5 compact layers of small pyramidal neurons with vesicular nuclei in CA1 (Figure 2A) and many large pyramidal neurons also with vesicular nuclei in CA3 (Figure 2B). Both the molecular and polymorphic layers were relatively cell-free layers. They contained sparse nuclei of neuroglial cells as astrocytes in addition to blood vessels on a pink neuropil background consisted of neuronal and glial cell processes (Figure 2A and 2B).

The dentate gyrus consisted of molecular, granular and polymorphic layers. The granular layer constituted the principal layer. It had the greatest cell density and was formed of dense columns of granule cells that appeared rounded with vesicular nuclei. It was noticed that small dark cells variable in shape and size were present in the sub-granular zone. The molecular layer which is the outermost one was a relatively cell-free layer. It contained few neuroglial cells as astrocytes in addition to blood vessels. The polymorphic layer contained various types of cells including pyramidal cells and astrocytes in addition to blood vessels (Figure 2C). Compared with the control specimens, the animals of group II, which received 1.5 mg/day of caffeine orally by gavage for 4 weeks revealed the same general picture of the hippocampi.

Group III: Animals received aluminum chloride for 4 successive weeks showed distinct histological changes. The pyramidal layers in CA1 (Figure 3A) and CA3 (Figure 3B) showed marked shrinkage in size of small and large pyramidal cells respectively with some cell loss. The pyramidal cells lost their triangular shape, showed darkened nuclei and were surrounded with pericellular haloes. As regard the granular layer it showed an apparent increase in the number of the granule cells with decreasing in their diameter. There was marked shrinkage in size of granular cells with some cell loss and marked vacuolation. Moreover, there was a marked decrease and sometimes absence of the dark cells which were previously observed in the sub-granular zone in the control sections (Figure 3C). Molecular & polymorphic layers revealed enlarged and excess astrocytes and widened blood capillaries (Figure 3A-C).

Group IV: Examination of hippocampus sections of this group (caffeine with AlCl₃ for 4 successive weeks) revealed less prominent histopathological changes when compared with aluminum chloride group. Preservation of most pyramidal cells and markedly decreased apoptosis of cells were observed in CA1 (Figure 4A) and CA3 (Figure 4B) regions. Less vacuolations and fewer apoptotic cells persisted in the granular layer (Figure 4C) with persistence of enlarged astrocytes and widened capillaries in many fields (Figure 4A-C).

Group V: Examination of hippocampus sections of this group (caffeine for 2 successive weeks,
followed by a combination of caffeine with AlCl₃ for 4 successive weeks) showed return of hippocampus tissue towards normal morphology.
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as evidenced by preservation of small pyramidal cells (Figure 5A) large pyramidal cells

(Figure 5B) and granular cells (Figure 5C). Molecular & polymorphic layers contained nor-
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Immunohistochemical stains and Morphometric analysis

GFAP Immunostaining: Immunohistochemical staining for GFAP showed its normal distribution in the control group as a mild positive reaction in the immuno-reactive astrocytes and glial fibers. The immuno-reactive astrocytes appeared as star-shaped cells and the fibers were thin and had a regular course (Figure 6A). Group II revealed the same results as group I. In group III, there was an apparent increase in the amount and intensity of GFAP immuno-reactive astrocytes together with decreased intensity of staining of glial fibers were noticed in group IV (caffeine with AlCl₃ for 4 successive weeks) (Figure 6C). Weak GFAP immuno-reaction nearly similar to the control was seen in the star-shaped astrocytes and the thin regular glial fibers in group V (caffeine for 2 successive weeks, followed by a combination of caffeine with AlCl₃ for 4 successive weeks) (Figure 6D).

In Table 1; GFAP positive areas (%) are significantly higher in group III (AD disease model) than that of group I (negative control) as well as group II (positive control). It starts to be lower in group IV (AD + Caffeine) but still significantly higher than that of both control groups (I&II). In group V, GFAP positive areas (%) showed the lowest expression in experimental AD groups; it showed significant decrease than that of group...
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**Table 1.** Ki67 and GFAP immunoreactivity as well as BDNF and its receptor gene expression in the different studied groups

<table>
<thead>
<tr>
<th>Group</th>
<th>Ki67-positive cells (%) Mean ± SD</th>
<th>GFAP positive areas (%) Mean ± SD</th>
<th>BDNF gene expression ($2^{-\Delta\Delta CT}$) Mean ± SD</th>
<th>TrkB gene expression ($2^{-\Delta\Delta CT}$) Mean ± SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (I)</td>
<td>95.26 ± 10.55</td>
<td>9.79 ± 2.77</td>
<td>1.00 ± 0.10</td>
<td>1.00 ± 0.10</td>
</tr>
<tr>
<td>Control + Caffeine (II)</td>
<td>96.63 ± 12.01</td>
<td>9.09 ± 2.93</td>
<td>1.00 ± 0.11</td>
<td>1.08 ± 0.24</td>
</tr>
<tr>
<td>AD disease model (III)</td>
<td>22.28 ± 5.92</td>
<td>28.93 ± 6.18</td>
<td>0.35 ± 0.07</td>
<td>0.32 ± 0.07</td>
</tr>
<tr>
<td>AD + Caffeine (IV)</td>
<td>58.88 ± 8.36</td>
<td>19.50 ± 3.46</td>
<td>0.53 ± 0.08</td>
<td>0.55 ± 0.12</td>
</tr>
<tr>
<td>AD + Caffeine Before (V)</td>
<td>84.91 ± 10.76</td>
<td>11.95 ± 2.34</td>
<td>0.85 ± 0.13</td>
<td>0.77 ± 0.13</td>
</tr>
<tr>
<td>P</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
<td>&lt; 0.0001</td>
</tr>
</tbody>
</table>

SD: standard deviation; P: Probability Test used: ANOVA followed by post-hoc Tukey for multiple comparisons; a: significance relative to Control Group; b: significance relative to Control + caffeine Group; c: significance relative to AD disease model Group; d: significance relative to AD + Caffeine Group.

**Figure 7.** Photomicrographs of Ki67 immune-stained sections in the rat hippocampus. (A) Group I showing Ki67-positive nuclei in the subgranular zone of the dentate gyrus (arrow). (B) Group III showing an apparent decrease in the number of Ki67 immunoreactive nuclei (C) Group IV showing an apparent increase in the number of Ki67-positive nuclei compared to group III (D) Group V showing Ki67-positive nuclei in the subgranular zone of the dentate gyrus (arrow) nearly similar to the control group.

III and IV with no significant difference than that of group I&II.

**Ki-67 immunostaining**

Ki67-positive nuclei were detected in the subgranular zone of the dentate gyrus in the control group (Figure 7A). Group II revealed the same results as group I. The number of Ki67 immunoreactive nuclei was significantly decreased in group III as compared to control groups (group I&II) (Figure 7B; Table 1). An apparent increase in the number of Ki67-positive nuclei was observed in group IV (Figure 7C) when compared to group III (AD model) but still significantly lower than that of the control groups (I&II) as shown in Table 1. In group V the...
number of Ki67-positive nuclei was nearly similar to the control group (Figure 7D). Moreover, it shows significant increase than that of group III and group IV (Table 1).

The correlation between the percentage of GFAP and Ki67 immunoreactive positive cells is presented in Figure 8 in the form of significant negative correlation ($r = -0.871 \& P < 0.0001$).

Real time RT-PCR study of BDNF and its receptors TrkB genes expression

BDNF and TrkB genes expression by real time RT-PCR study showed statistically significant decrease in group III when compared to control groups (I&II) which started to increase in group IV but still significantly lower than that of the control groups. Group V became significantly higher than that of group III and IV with BDNF gene expression has no significant difference when compared to group I (negative control group) (Table 1).

In Figure 8 hippocampal BDNF gene expression shows significant positive correlation with the percentage of Ki67 immunoreactive positive cells ($r = 0.869 \& P < 0.0001$). While, it shows significant negative correlation with the percentage of GFAP immunoreactive positive cells ($r = -0.834 \& P < 0.0001$).

Discussion

In the current study, aluminum chloride ($\text{AlCl}_3$) was used for induction of AD in a dose of 17 mg/kg body weight orally as a single daily dose through a gastric tube as described by Karam et al. [12]. The choice of aluminum chloride was based on the fact that aluminum (Al) has the potential to be neurotoxic in humans and animals. It is present in many manufactured foods and medicines and is also, added to drinking water for purification purposes [19], one of the ingredients of antacid drugs, in food additives and tooth paste [20].

Aluminum chloride is considered to be an ideal substance to be used for induction of AD model as a result of being able to cross the blood brain barrier as an L-glutamate complex and it deposits in a rat’s brain [21]. It is documented...
to induce AD as it enhances neuroinflammatory events in the brain by many proposed mechanisms. It could exacerbate amyloid beta (Aβ) deposition and plaque formation in the brain of transgenic mice [22], in addition to oxidative stress potentiated by both Aβ and aluminum that will lead to genotoxicity and DNA damage [23]. This oxidative damage may lead to the formation of amyloid plaques and hyperphosphorylated tau that polymerizes to form neurofibrillary tangles which are hallmarks of Alzheimer’s [24].

In the current study, histological examination of the hippocampus of rats in group III (AD model group) revealed that the pyramidal layers in CA1 and CA3 regions of the hippocampus showed marked shrinkage in size of small and large pyramidal cells respectively with some cell loss. Choosing hippocampus for studying the pathological findings of AD comes from the fact that the hippocampus remains one of the most vulnerable brain regions to AD, and its degeneration may directly underlie memory deficit, the earliest symptom of AD [25, 26]. These histological results are in agreement with Padurariu et al. [27] and Nirmala et al. [28]. They demonstrated in their study that the cytoplasm of neurons was shrunken, the nuclei were side-moved and dark-stained, neurofibrillary degeneration and neuron loss were observed in hippocampus of rat received AlCl₃. Moreover, Yassin et al. [29] observed that sections of rat brains receiving only AlCl₃ (17 mg/kg) for 4 weeks showed brain necrosis, spongy appearance, plaques with loss of normal structure, outlines, and nuclei of cells.

The pyramidal cells lost their triangular shape, showed darkened nuclei and were surrounded with pericellular haloes. Similar results were reported by Abd El-Rahman [30]. He demonstrated that Al administration causes the appearance of neuritic plaques with a dark center in the hippocampus and he stated that it is typical for AD. It is confirmed by Aly et al. [31] and explained it by presence of β-amyloid plaques in the cerebral cortex and the hippocampus. Slight disorganization of the pyramidal cell layer, little degeneration of pyramidal cells and slight spongiosis were reported by a finding of AD model studied by Abo El-Khair et al. [32].

As regard the granular layer it showed an apparent increase in the number of the granule cells with decreasing in their diameter. There was marked shrinkage in size of granule cells with some cell loss and marked vacuolation. This indicates a clear evidence of chronic inflammation and oxidative damage [33] and the same result was confirmed later by Abo El-Khair et al. [32] and Lynch [34]. Inflammatory changes are features of AD. Several studies have reported this observation and have shown that activated cells cluster around Aβ-containing plaques [35]. An increase in expression of inflammatory cytokines, interleukin-1β (IL-1β), IL-6 and tumor necrosis factor-α (TNF-α) has been detected in brain of AD model animal and IL-1-positive microglia present with Aβ-containing plaques, a similar event occurs in activated astrocytes [34].

During this work it was observed that the control animals showed small dark cells in the subgranular zone of the dentate gyrus. These cells were previously described by Song et al. [36] as neural stem cells that are present both in developing nervous system and in the adult nervous system of all mammals, including human. The number of these dark cells was apparently decreased after exposure to aluminum chloride. This might be due to their differentiation into granule cells, in order to compensate for their loss, caused by exposure to aluminum chloride. This might account for an apparent increase in the number of granule cells compared to the control groups. Our finding coincides with finding of Nobakht et al. [37] as they reported that there was reduction in neuronal population in hippocampus of rat model with AD. Serrano-Pozo et al. [38] mentioned that neuronal loss is the main pathological substrate of the cortex and hippocampus which is evident in sections stained with hematoxylin and eosin, it can be more readily shown with a Nissl staining or a NeuN immunohistochemistry. It is reported that the neuronal loss is a common pathway for a large number of degenerative processes in AD and can be triggered by various factors, such as β amyloid plaques, perturbed calcium regulation, glutamate, ischemia, inflammatory processes or oxidative stress [27, 39]. Moreover, neuronal loss in the hippocampus due to β-amyloid deposition might induce glucose dysregulation leading to hepatic insulin resistance which is one of the mechanisms of cognitive dysregulation in AD [40]. Molecular & polymorphic layers revealed enlarged and excess astrocytes and widened blood capillaries. This confirmed a result
increases during the progress of AD which is reflected by the increase of GFAP transcription is parallel to the 3.2-fold increase at end stage AD, this gradual transition is termed astrogliosis and characterized by an increase in the expression of their main intermediate filament (IF), glial fibrillary acidic protein (GFAP), by morphological alterations (hypertrophy) and by functional changes [44]. GFAP expression is highly associated with amyloid plaque load and to a lesser extent, with the number of neurofibrillary tangles [45]. In human study, it is reported that GFAP expression significantly increased, reaching a 1.5- to 3.2-fold increase at end stage AD, this gradual increase of GFAP transcription is parallel to the progression of AD which is reflected by the number of plaques, amyloid scores, and Braak stages [46].

The present study showed that Ki-67 positive nuclei were detected in the subgranular zone (SGZ) of the dentate gyrus in the control group. Ki-67 protein is a reliable biomarker present during all active phases of the cell cycle and is commonly used as an indicator to detect cellular proliferation [50, 51]. It is detected also, in subgranular zone of the dentate gyrus in a study of Yoo et al. [7] in agreement with our study. The subgranular zone of the hippocampal dentate gyrus constitutes one of the only two neurogenic niches of the adult brain [52]. Adult neurogenesis has been observed in all mammalian species including humans and results in the formation of new neurons in the olfactory bulb and the dentate gyrus of the hippocampus. Radial glia-like (RGL) neural stem cells (NSCs) that reside in the SGZ of the dentate gyrus, proliferate and give rise to transit-amplifying progenitors (TAP) expressing the T-box brain gene 2 (Tbr2) antigen which then give rise to doublecortin (DCX)-expressing immature neurons [53]. Neurogenesis is regulated by different physiological effects especially exposure to environmental enrichment, learning, aging and stress [36]. Several signaling molecules and pathways have been described to be essential for the maintenance, self-renewal and proliferation of NSCs and hence differentiation into mature functional neurons [54].

In our study, the number of Ki-67 immunoreactive nuclei was significantly decreased in group III (AD model group) as compared to control groups (group I&II) which indicates decreased hippocampal neurogenesis in AD that coincides with Ihunwo et al., [26]. This finding indicates that neurogenesis is down regulated in AD.
which is reported previously by Verret et al. [55] in AD of aged rat. It has been suggested that altered hippocampal neurogenesis (HN) might be an integral part of AD progression [56]. This is confirmed in animal and human study by Gomez-Nicola et al. [57]. Also, Varela-Nallar et al. [58] demonstrated the same result and explained it by chronic hypoxia as a main mechanism of AD pathogenesis. The importance of altered hippocampal neurogenesis (HN) was summarized by Maruszak et al. [52]. They reported that HN is an integral part of AD progression. A variety of important biomolecules involved in AD pathogenesis as presenilin 1 (PS1), amyloid-β precursor protein (Aβ-PP) and its metabolites, play either a positive or negative role in adult HN [59]. In addition, many growth factors, such as brain derived neurotrophic factor (BDNF), vascular endothelial growth factor, fibroblast growth factor, and cystatin C, have been reported to be upregulated in amyloid plaques. These factors are also known to be potent modulators of neural stem cell activity [52].

In the current study, brain derived neurotrophic factor (BDNF) and its main functional receptor (neural receptor protein-tyrosine kinase-β; TrkB) genes expression showed significant decrease in group III (AD model group) when compared to control groups (I&II). The importance of studying BDNF came from its role in development and maintenance of normal neuronal circuits in the brain. It is reported that appropriate intracellular processes including transcription from BDNF gene, translation to protein, BDNF protein sorting to secretory vesicles, BDNF-containing vesicle transport, and BDNF secretion are essential to achieve normal BDNF functions as well as to activate the signaling pathways after TrkB phosphorylation [60].

BDNF acts as a neurite outgrowth and elongation factor, pro-survival factor, and synaptic regulator in the CNS through multiple mechanisms. Firstly, it promotes axon initiation through two distinct signaling pathways; it induces TrkB-dependent local elevation and stabilization of cAMP/PKA activity that are essential for axon initiation in undifferentiated neurites of hippocampal neurons [61]. Secondly, BDNF/TrkB-induced Akt phosphorylation reduces GSK-3 activation which in turn decreases production of the active form of col-lapsin response mediator protein-2 that plays a critical role in microtubule assembly during axon elongation and branching in rat hippocampal neurons [60, 62]. Thirdly, it enhances the insulin sensitivity and plays significant roles in the regulation of glucose metabolism, as impaired insulin sensitivity and signaling pathway is one of the characteristic features in AD [63].

The reduction of BDNF and its receptors TrkB genes expression in our work supports previous studies that demonstrated a decreased expression levels of BDNF protein and mRNA have been consistently reported in the hippocampus and cortex of individuals with AD [64-66]. This reduction was explained by that Aβ disturbs normal synaptic and cognitive function [67] which is associated with loss of neurotrophins, as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT3) and NT4/5, in addition to suppression of molecular transduction responsible for learning and memory as mitogen-activated protein kinases (MAPK) and cAMP response element-binding protein (CREB) [68]. The effects of reduced expression of BDNF or disruption of its signaling pathway were summarized by Poon et al. [69]. They reported that reduced BDNF signaling leads to defective hippocampal and cortical synaptic plasticity and they proved this by an experimental study that concluded that mice lack either BDNF or TrkB exhibited arborization impairment, defective synaptic sprouting, lowered synapse number and impaired hippocampal long term potentiation (LTP). In contrast to the current study, there was higher BDNF serum levels in preclinical stages of Alzheimer’s disease as well as a significant increase of BDNF concentration in hippocampus and parietal cortex and of its receptor TrkB in astrocytes and senile plaques as reported by Baglio et al. [70]. They explained this by that BDNF seems to have a protective role for brain with normal morphology in early preclinical stages of AD through its ability to induce nitric oxide production by astrocytes and promote cytokine production so, it is considered as a defensive molecule expressed early trying to suppress disease progression [70].

In the current work, studying the protective effect of caffeine in group IV (caffeine administrated with AlCl₂ for 4 successive weeks in a
dose of 1.5 mg/day) showed obvious improvement of the histological picture, increase in the number of Ki-67 positive nuclei, decrease in the amount and intensity of GFAP immunoreactive astrocytes together with decreased intensity of staining of glial fibers in addition to the increase in BDNF/B-actin and TrkB/B-actin genes expression when compared to the AD model group (group III) but still lower than that of the control groups. The histopathological changes in this study are presented by preservation of most pyramidal cells and markedly decreased apoptosis of cells were observed in CA1 and CA3 regions. Less vacuolations and fewer apoptotic cells persisted in the granular layer with persistence of enlarged astrocytes and widened capillaries in many fields.

The caffeine dose in our study was in agreement with many studies who used different doses of caffeine in their study and concluded that caffeine intake in a dose of 1.5 mg/day may reverse cognitive impairment and decrease brain Aβ levels in aged AD mice [5, 71] and the response to treatment was in a dose dependent manner. The mechanism by which caffeine could be a good protective agent used in treatment of AD comes from the following effects; caffeine and other adenosine receptor antagonists could prevent the accumulation of amyloid-β-peptide (Aβ) in and around cerebral blood vessels [72]. Another possible mechanism is through its stimulating effect of prosurvival cascades and inhibiting of pro-apoptotic pathways in the different brain regions [73, 74]. Caffeine and other adenosine receptor antagonists could lead to down regulation of the inflammatory response which is an obvious hallmark of AD [75]. It also, can perform its protective effect through prevention of beta amyloid (Aβ)-induced synaptotoxicity and by induction of the production of interleukin-10 (IL-10) [74].

It is reported also, that caffeine can reduce hippocampal Aβ, presenilin 1 (PSEN1), and γ- and β-secretase levels in AD transgenic mice, in addition to increased plasma level of granulocyte-colony stimulating factor (GCSF) and IL-6 [76] as well as TNF-α [77]. The anti-inflammatory and antioxidant effects of caffeine could be beneficial both for hippocampal neurogenesis and for protection against AD [52]. It is reported that caffeine is associated with reduced oxidative stress as demonstrated by reduction in ROS production, glutathione depletion, isoprostanate production, and markers of endoplasmic reticulum stress levels this would reduce Aβ accumulation [78]. Lastly, Caffeine has a role in stabilization of blood-brain barrier (BBB) integrity and has been implicated in modulating BBB functions through blocking cellular surface adenosine receptors, inhibition of cAMP phosphodiesterase (PDE) activity, and by affecting calcium release from intracellular stores [79].

In agreement with the results of the current study, it has been reported that chronic caffeine administration to mice from adulthood to old age increases hippocampus levels of BDNF and TrkB [80]. In addition, Sallaberry et al. [81] Chen et al., [82] and Mioranzza et al., [83] reported that caffeine modulates the balance between cell survival and cell death which depends upon the mature/proneurotrophin ratio, as caffeine induced increase in proBDNF, reducing neurotrophin signaling abnormalities of TrkB receptor as well as relevant morphological changes in hippocampus histology as it is known that BDNF signaling through its receptor TrkB can influence the morphology and synaptic connectivity of hippocampal neurons. Moreover, the effects of caffeine on proliferation of neuronal precursors had been investigated and it is reported that it increases hippocampal neuronal proliferation [83, 84].

The early mid-life protective effect of caffeine in the present study was studied in group V in which rats received caffeine in a dose of 15 mg/Kg body weight/day for 2 successive weeks, followed by a combination of caffeine with AlCl3 for 4 successive weeks. The results of the current study revealed marked improvement of histological picture of hippocampus which nearly returned to normal morphology, weak GFAP immuno-reaction with GFAP positive areas (%) showed the lowest expression, the number of Ki-67 positive nuclei was nearly similar to the control group. BDNF/β-actin and TrkB/β-actin genes expression became significantly higher than that of group III and IV with no significant difference when compared to group I&II (control groups). These results are in agreement with previous study that reported that chronic caffeine consumption reverses cognitive impairment and decreases brain Aβ levels in AD mice [85]. Moreover, Eskelinen and Kivipelto [86] stated that drinking of 3-5 cups of coffee per day at midlife is associated with
decreased risk of dementia/AD in later life nearly by 65%, suggesting its protective role against AD. The experimental findings of the current study come in accordance with the results of Sallaberry et al. [81] Han et al. [5] Mioranza et al. [83] Rivera-Oliver and Díaz-Ríos M [74].

Recently, in 2015, it is reported that besides the short-term effect of caffeine, epidemiological and experimental studies indicate that caffeine, the main psychoactive component of coffee and tea, when ingested chronically, has protective effects against a number of acute and chronic neurological diseases including stroke, Parkinson's disease, amyotrophic lateral sclerosis, dementia, and AD through inhibition of Aβ production in brain of rodents [87].

**Conclusion**

It could be concluded from the current study, that chronic caffeine consumption in a dose of 1.5 mg/kg body weight daily in early midlife has a potentially good protective effect against AD through modulation of the hippocampus tissue gene expression of some important molecules; BDNF and TrkB and modifying Ki-67 as well as GFAP immunoreactivity. Moreover, it could have neuroprotective effect on tissue morphology including histological preservation of the hippocampus. Further research studies are needed to prove and validate our results as well as to study other possible pathophysiological mechanisms by which caffeine can perform its protective effects on hippocampus tissue against AD and to study the potential therapeutic effect of caffeine in AD.

**Recommendations**

The daily consumption of caffeine in a dose of 1.5 mg/kg body weight daily in early midlife has a potentially good protective effect against the possibility of AD with the advance of age, so it is recommended for its habitual intake. Further investigations are needed for finding more mechanisms that explain; prove the protective effect of caffeine and study of its potential therapeutic effect.

**Limitations of the study**

This study has a limitation, It didn't evaluate the potential therapeutic effect of caffeine against AD which would be performed in further future study.

**Disclosure of conflict of interest**

None.

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