RESEARCH PAPER

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Investigation of amyloid-β peptide production and clearance pathways in different stages of Alzheimer's disease

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Alzheimer's disease (AD) is an age-related, progressive decline in cognitive ability. Accumulation and deposition of amyloid-β (Aβ) is still the best-known cause of AD that worsens over time. It is unclear whether the increase in Aβ production or the inefficiency of the degradation system causes the accumulation of β-fibrils during AD development. This research investigated Aβ-producing and clearance pathways in different stages of AD. For this purpose, patients were categorized into four experimental groups: patients with mild cognitive impairment, patients with moderate cognitive decline, patients with very severe cognitive decline, and healthy patients as control. Levels of Aβ-40, soluble amyloid precursor protein beta (sAPPβ), matrix metalloproteinase-9 (MMP-9), matrix metalloproteinase-3 (MMP-3), neprilysin (NEP), angiotensin-converting enzyme (ACE), and insulin-degrading enzyme (IDE) were determined by ELISA kits and immunoblotting in serum samples. According to the results, the levels of Aβ-40 and sAPPβ increased in AD patients from an early stage, and levels were maintained in progressive AD stages. MMP-9 also increased in the early stage, but its content decreased with disease development. MMP-3 was significantly higher in the three stages of AD compared to the control patients. However, IDE, NEP, and ACE enzymes as clearing systems decreased in all studied AD samples, with their reductions more remarkable in the middle and late stages. The results showed that multiple Aβ-degrading enzymes such as NEP and IDE in AD patients decline as AD progresses, while Aβ-40 and sAPPβ increased from the early stage of the disease. Therefore, it could be concluded that detection of the dementia phase is a critical step for therapeutic strategies.

Key words: Alzheimer's disease, amyloid-β clearance, insulin-degrading enzyme, neprilysin, angiotensin-converting enzyme, matrix metalloproteinase enzymes

INTRODUCTION

Alzheimer's disease (AD) is a multifactorial progressive disease, besides the classical neuropathological features (including not only amyloid beta (Aβ) deposition but also neuronal loss and neurofibrillary tangles), AD comprises other traits such as neuroinflammation, oxidative stress, and insulin resistance (Sadigh-Eteghad et al., 2015). As reported, the cumulative action of β and gamma-secretase enzymes digest the amyloid precursor protein (APP) into insoluble Aβ peptides, which cluster together to form Aβ plaques that deposit inside and outside of neural cells (Hur, 2022). Therefore, strong evidence suggests that Aβ is still the best-known cause of AD through its accumulation and deposition in the frontal cortex and hippocampus tissue, causing the neurodegenerative process (Selkoe & Hardy, 2016). Thus, strategies for developing AD drugs have been focused on the reduction of Aβ deposits in the brain, possibly through reduction of Aβ formation and/or increase of Aβ-degrading pathways. The clearance of Aβ from the brain is accomplished by several mechanisms, which include non-enzymatic and enzymatic pathways (Baranello

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et al., 2015). Multiple Aβ-degrading enzymes (ADE), including neprilysin (NEP) (Hafez et al., 2011), insulin-degrading enzyme (IDE) (Pivovarova et al., 2016), matrix metalloproteinase-9 (MMP-9) (Yan et al., 2006), matrix metalloproteinase-3 (MMP-3) (Kim & Hwang, 2011), glutamate carboxypeptidase II (Kim et al. 2010). and angiotensin-converting enzyme (ACE) (Hemming & Selkoe, 2005) are implicated in the clearance system that is involved in Aβ deposition and dementia progression in AD.

ACE is a zinc (Zn^{2+}) metalloprotease containing two homologous regions, termed the N- and C-domains, each of which is proteolytically active (Wei et al., 1991). A potential relationship between ACE and AD was first suggested by human genetic studies, which reported that an insertion (I)/deletion (D) polymorphism within intron 16 of the ACE gene is associated with AD (Kehoe et al., 1999). Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-containing and calcium (Ca^{2+})-requiring endo-proteases capable of degrading extracellular matrix (Page-McCaw et al., 2007). They are released from astrocytes, neurons, and microglia, as well as leukocytes and macrophages, and their target compounds include collagen, gelatin, fibronectin, laminin, elastin, and extracellular deposits of Aβ (Beuche et al., 2000). MMP expression has been investigated in postmortem brain tissue from AD patients, and results showed that MMP-9 is localized in hippocampal neurons, and is capable of degrading Aβ (Backstrom et al., 1996). While MMP-3 is a multifunctional metalloprotease, its polymorphisms in the coding region have been linked to an increased risk of AD (Helbecque et al., 2007). The MMP-3 content of cerebrospinal fluid (CSF) and plasma has been demonstrated to be altered in AD and to correlate with cognitive impairment and core AD biomarkers (Hanzel et al., 2014). IDE is another metalloprotease enzyme responsible for insulin degradation and has been shown to play a key role in Aβ peptide degradation, both *in vitro* and *in vivo*, but its role as an amyloid-degrading enzyme remains controversial (Farris et al., 2003; Leissring, 2021). Genetic linkage studies have connected AD and plasma Aβ levels to chromosome 10q, which harbors the IDE gene (Bertram et al., 2000). NEP is a type II integral membrane protein, known as zinc metallopeptidase, that is capable of degrading monomeric and oligomeric forms of Aβ (Wang et al., 2010). It has been previously shown that NEP-deficient mice have significantly elevated brain Aβ levels, and treatment with NEP inhibitors can cause a rapid increase of Aβ levels in the brain as well as memory impairment (Bertram et al., 2000).

AD is a progressive disease that can be studied in four separate stages during the development of dementia and memory loss accompanied by increased Aβ plaque accumulation (Kumar et al., 2024). However, it is not clear whether the accumulation of Aβ plaques during the conversion of the healthy brain to the AD brain is due to increased Aβ production and/or poor Aβ clearance system. The proposed research aims to investigate Aβ production and clearance pathways in different stages of AD (early, middle, and late) (Kumar et al., 2024). For this purpose, systemic concentrations of Amyloid beta-40 (Aβ-40) and soluble APP beta protein (sAPPβ) were evaluated as plaque-producing elements, and MMP-9, MMP-3, NEP, ACE, and IDE were measured as Aβ clearance systems in patients suffering from different stages of AD. Based on our knowledge, studies evaluating Aβ clearance pathways by stage of dementia are rare, but these kinds of studies could elucidate the molecular mechanism of disease progression and help in target selection for AD treatment.

METHODS

Experimental design

This study is a case-control experiment on AD patients referred to Baghdad Medical City, Baghdad, Iraq, between September 2021 and September 2022. The stage of the disease and the pathophysiological symptoms of the disease were confirmed by a neurologist based on brain scans, such as computed tomography (CT), magnetic resonance imaging (MRI), or positron emission tomography (PET), and biochemical and behavioral analysis. The group of control patients (n=25) was free of neurologic illness and did not suffer from diabetes or memory loss, and people with a family history of dementia were excluded from the study. The patients in the early phase group had the fewest participants (n=4), as detection was more difficult due to the lack of severe cognitive problems or memory loss. Mild cognitive impairment (MCI) in this group was detected based on hand movement analysis (Kumar et al., 2024). The middle phase group consisted of patients who suffer from moderate cognitive decline (MCD) and manifest hallmark signs of dementia, including difficulties with language and reduced problem-solving skills and thinking ability (n=25). The late-phase group consisted of patients in very severe cognitive decline (VSCD) who suffered from very severe cognitive impairment and a loss of physical abilities (n=25). Late-onset dementia symptoms include obvious memory loss, incontinence, and an inability to move without help. The experimental design is shown in Fig. 1.

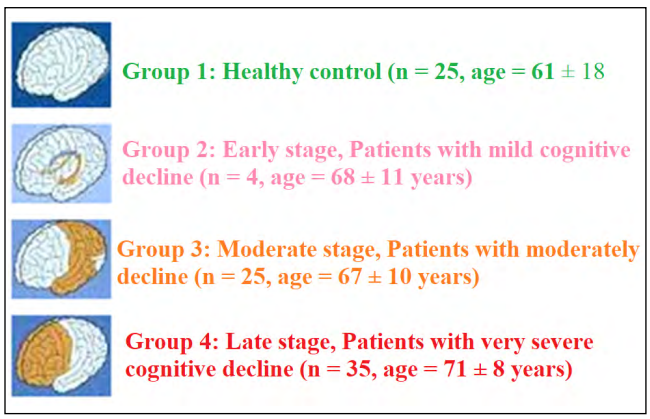

Fig. 1. This study was designed to investigate Aβ production and clearance system in four experimental groups.

Patients with liver deficiency, kidney disorders, thyroid disorder, acute coronary syndromes, different types of cancers, and diabetes were excluded from the study so as not to confound the obtained results. Following informed consent from patients or their legal guardian, blood samples were taken after fasting overnight and immediately spun down, and the plasma was aliquotted and frozen at -20°C until analysis. All patients included in this study were free of infections for at least 4 weeks before investigation. At the time of the blood draw, all patients had a normal body temperature, and C-reactive protein and erythrocyte sedimentation rates were within the normal range.

Measurement of proteins and peptides in blood samples

Commercial human ELISA kits were used for quantitative detection of Aβ-40 (Abcam, ab193692, UK), sAPPβ (Abcam, ab252904, UK), MMP-9 (Abcam, ab246539, UK), and ACE (Abcam, ab235649, UK) in blood serum samples according to the company manual.

The western blotting method was used to evaluate the proteins that the ELISA kit is not available for. Serum samples were diluted in 0.4 mL buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% sodium deoxycholate, 1% Triton X-100, 0.1% SDS, supplemented with protease inhibitors). The possible insoluble particles were separated by centrifugation at 14,000g and 4°C for 10 min. Proteins were loaded and separated using NuPage 4% to 12% Bis-Tris gels, followed by transfer onto Invitrolon polyvinylidene fluoride (PVDF) membranes. The membranes were then incubated at room temperature for 60 min in a blocking solution consisting of 5% wt/vol nonfat dry milk prepared in TBST (Tris-buffered saline (TBS), 10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20). Next, the membranes were incubated with diluted primary antibody in either 5% wt/vol BSA, 1×TBST, or 0.1% Tween 20 overnight at 4°C with gentle agitation. Anti-human MMP-9 antibody (ab73734) in 0.5 µg/ml concentration, anti-human MMP-3 antibody (sc-21732) in 1 µg/ml concentration, anti-human IDE (ab32216) in 0.5 µg/ml concentration and anti-human NEP (AF1182-SP) in 1 µg/ml concentration were used as specific primary antibodies. After washing with TBST, the blots were incubated for 1 h at room temperature with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody at a dilution of 1:5000 in blocking buffer. The HRP-labeled protein bands were detected using the chemiluminescent detection system. As a gel loading control, the membrane was also probed with an antibody against β-actin. The density of each band was quantified by using ImageJ 1.46r; Java 1.6.0_20 software after at least five repeats, and data were expressed as mean±standard deviation (SD).

Statistical test

SPSS for Windows, version 16 (SPSS Inc., Chicago, IL, USA) was used in all statistical procedures. Numerical data were expressed as mean±SD or a proportion of the sample size. All data were checked for normality by the Kolmogorov–Smirnov test (K–S test). Statistical assessment was done by using the analysis of variance (ANOVA) to determine statistical differences between results related to four experimental groups. A p-value less than 0.05 was considered significant.

RESULTS

Systemic level of Aβ and its precursor changed during AD progression

According to the results presented in Fig. 2A, the level of Aβ-40 increased remarkably in AD patients compared to the control patients. Circulating levels of Aβ-40 were estimated to be 112.67±26.31 pg/ml in control individuals *versus* 295.52±68.71 ng/ml in the early phase, although this difference was not significant, possibly due to the small size of the group. Aβ-40 concentrations in blood samples of middle (381.19±137.19 ng/ml) and late (405.07±104.99 ng/ml) AD, were significantly higher than the control ($P<0.0001$), however, there was no significant difference in Aβ-40 level in different stages of AD disease ($P>0.05$).

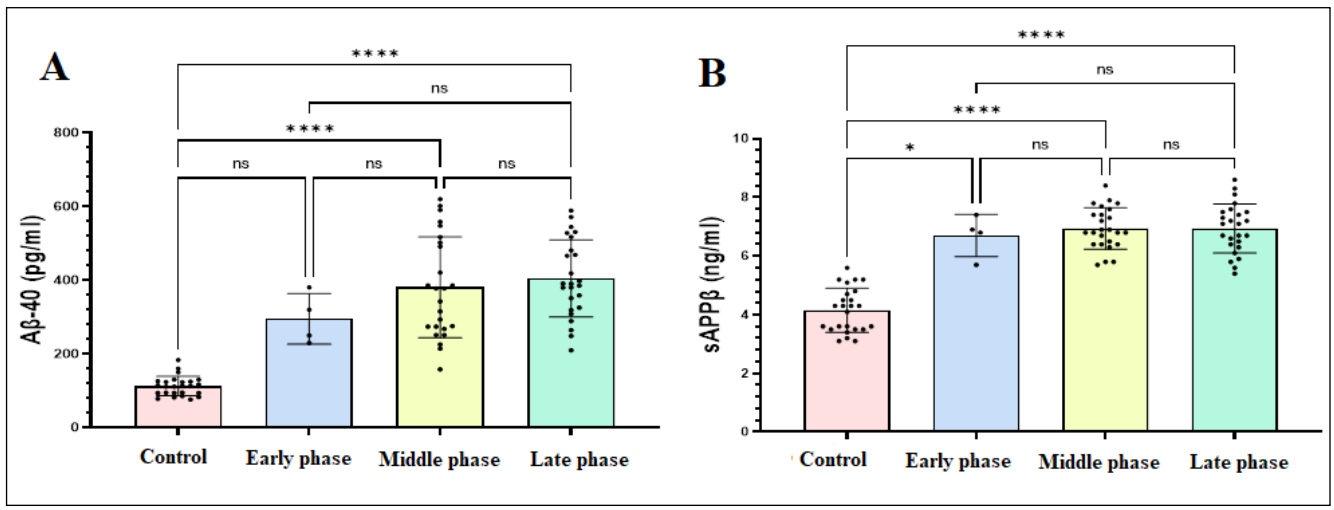

Fig. 2. Comparison of Aβ production in different phase of AD. (A) Aβ-40 level in blood samples of AD patients and control revealed that Aβ-40 content increased in all of the AD phases rather than control. (B) Evaluation of sAPPβ in experimental groups confirmed the blood level of this precursor protein increased in three studied phase of disease and sAPPβ level is the same in early, middle and late phases. All data were expressed as mean ± SD. Asterisk symbols show the significant difference between experimental groups (****, P<0.0001; ns, non-significant).

Our results also showed that the level of sAPPβ was significantly higher in AD patients compared to control patients. The amounts of sAPPβ measured were 4.15±0.75 ng/ml in control patients *versus* 6.70±0.72 ng/ml, 6.94±0.70 ng/ml, and 6.94±0.83 ng/ml in early, middle and late AD, respectively, confirming the significant increase of precursor protein in the studied stages of AD (P<0.05; P<0.0001) *versus* control patients. Conversely, there was no significant difference in sAPPβ levels in different stages of AD disease.

Metalloprotease expression was changed in different stages of AD

Metalloproteases are an effective member of the Aβ degrading system, and this type of enzyme could also clear other deposited proteins in biological systems. Results shown in Fig. 3A show the circulating level of MMP-9 (measured by ELISA method) was significantly higher in early and middle-stage AD patients compared to the control pa-

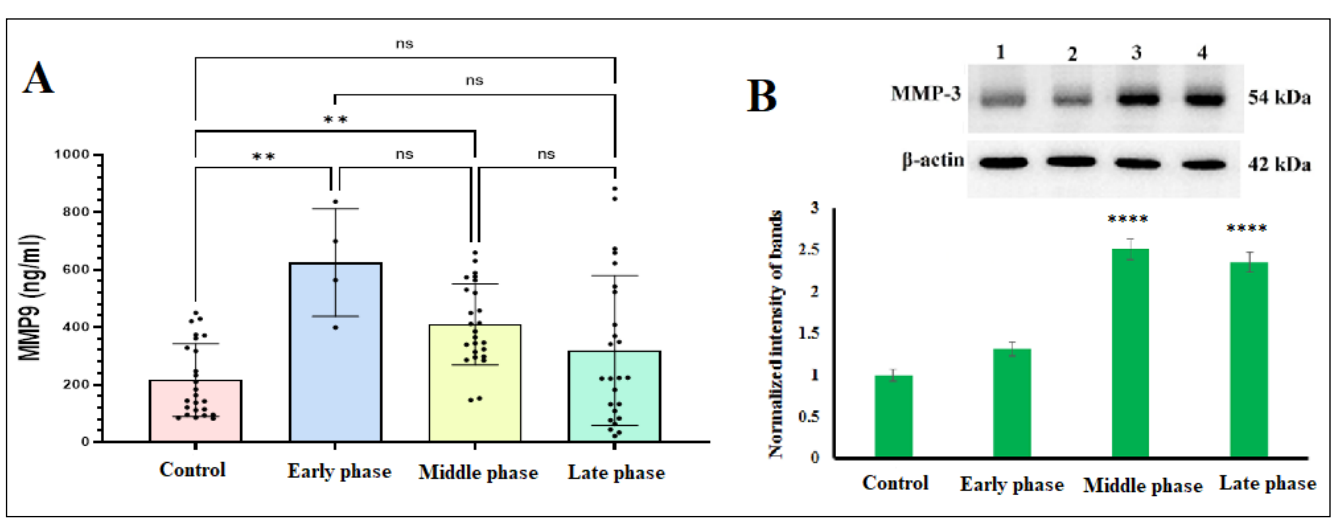

Fig. 3. The results of matrix metalloproteinase enzymes in AD patients and control participants. (A) MMP-9 level compared during AD development that results manifested increase of the enzyme content in early phase. (B) western blot analysis for MMP-3 in control (lane 1) and different phases (early, middle and late in lane 2, 3 and 4) of AD disease patients confirmed increased level of enzyme in advanced stages of AD and not in early phase. All data were expressed as mean ± SD. Asterisk symbols show the significant difference between experimental groups (****, P<0.0001; **, P<0.01); ns, non-significant).

tients (215.63±126.52 ng/ml in control patients *versus* 624.77±187.42 ng/ml and 410.01±140.95 ng/ml in early and middle AD, respectively). While there was no significant difference in the MMP-9 level of the control group and the late-stage AD patients, its concentration was determined as 215.63±126.52 ng/ml in control *versus* 319.08±260.54 ng/ml in late AD patients (P>0.05). Our analysis could not find any significant difference between MMP-9 levels in different stage AD patients, leading to the conclusion that the three studied stages of AD are similar to each other in terms of systemic MMP-9 content.

This study also evaluated the serum concentration of the MMP-3 enzyme by using the western blotting method *via* a specific antibody. The color density of each band was quantified by using ImagJ software and compared with the control group after the normalization process (Fig. 3B). Based on the results, AD patients had higher levels of MMP-3 compared to the control patients. The difference between the early phase of AD and the control was slight, but MMP-3 concentration increased significantly in the middle and late stages (P<0.0001). Comparison of different stages of AD revealed a significant difference in MMP-3 expression between early and middle and early and late stage AD, while middle AD did not show a significant difference in comparison with late AD.

Aβ clearance system is dysregulated in various stages of AD

This study evaluated the circulating level of ACE in different stages of AD and healthy control samples. Based on the results (Fig. 4A), there was a significant difference in ACE levels between the control group and all stages of AD patients, with ACE levels lower in middle and late AD compared to the control patients. Normalized expression of ACE in control patients was estimated to be 4.30±0.63 ng/ml, while its content in the middle and late stages was 3.73±0.64 ng/ml and 3.75±0.77 ng/ml, respectively. There was no significant difference detected between control patients and early AD patients (4.30±0.63 ng/ml in controls *versus* 3.74±0.84 ng/ml in early AD) or between the middle and late stages (P>0.05).

Fig. 4B shows the western blot analysis of IDE for control and all three stages of AD. According to the results, IDE was significantly lower in AD patients compared to control patients. Reduction of IDE is gradual while developing dementia, therefore, in the late phase, the blood level of IDE was reduced more than 5-fold compared to the control.

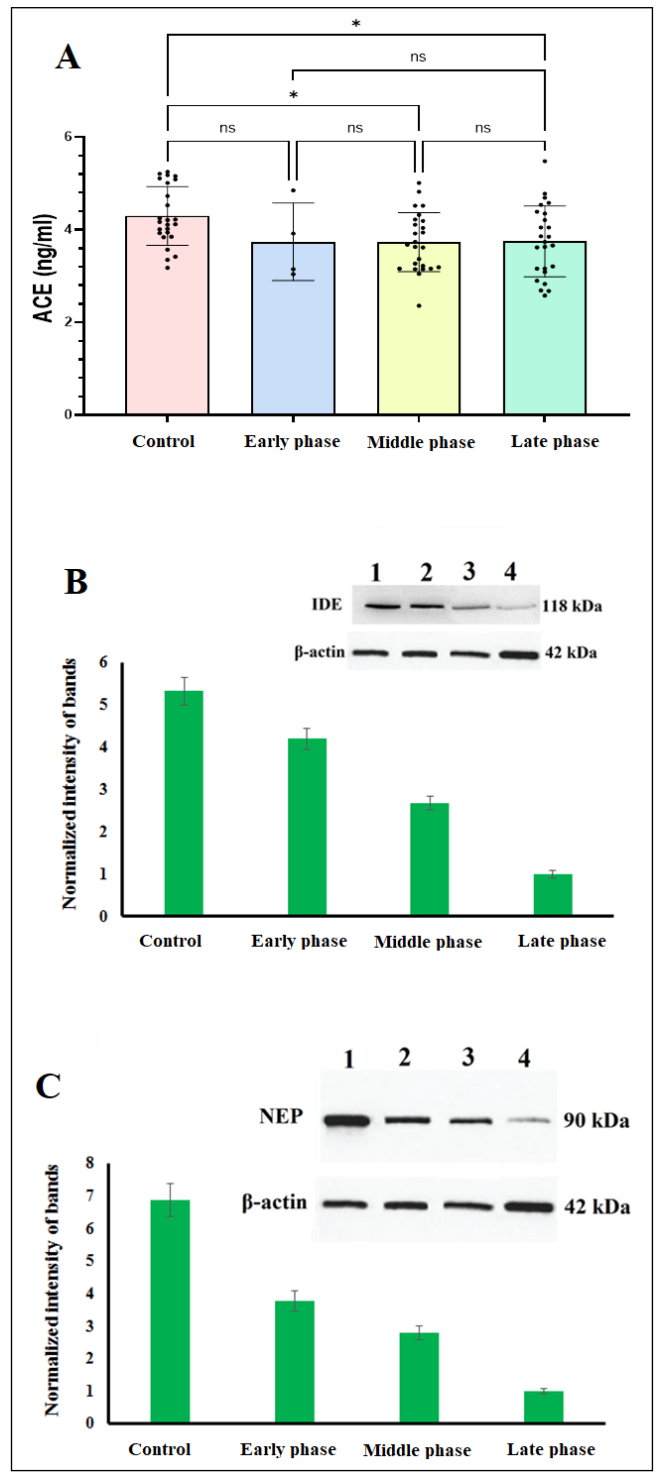

Fig. 4. The level of Aβ-degrading enzymes in AD development. (A) ACE increased significantly in both late phase of AD while IDE (B) and NEP (C) reduced in AD patients gradually by disease developing. ACE content measured by using ELISA method, while NEP and IDE evaluated *via* western blotting analysis. Immunoblotting image showed expression of protein in control in lane 1, lane 2, 3 and 4 represent protein content in early, middle and late phases of AD patients respectively. All data were expressed as mean ± SD. Asterisk symbols show the significant difference between experimental groups (*, P<0.05; ns, non-significant).

The western blot analysis for NEP is presented in Fig. 4C. Based on the western blotting results, NEP was significantly higher in control patients than in AD patients. Also, among AD patients, there were significant differences in NEP levels, with late AD having a significantly lower NEP value than the other stages and the early phase having a level significantly higher than the other two stages ($P<0.001$).

DISCUSSION

Aβ production and deposition is the main pathophysiological event in AD patients, and related animal models, and are associated with synaptic dysfunction and neural death (Selkoe & Hardy, 2016; Hur, 2022). Accordingly, prevention of Aβ processing and fibrillation, via treatment strategies or natural products, is one of the most effective therapeutic strategies (Baranello et al., 2015; Selkoe & Hardy, 2016). It is not clear whether the increase in Aβ production and processing or the inefficiency of the Aβ degradation system causes the accumulation of β-fibrils during AD development. Previous studies have confirmed that brain tissue has an effective endogenous system, including MMP-9, MMP-3, NEP, and IDE (Wei et al., 1991; Yan et al., 2006; Kim et al., 2010; Hafez et al., 2011; Pivovarova et al., 2016), to degrade extra Aβ and deposited fibrils. This research aimed to investigate Aβ production and clearance pathways in different stages of AD. Briefly, our results are in agreement with previous experiments that confirmed increased Aβ accumulation and dysregulation of Aβ degradation enzymes in AD circulation (Kehoe et al., 1999; Baranello et al., 2015; Selkoe & Hardy, 2016). According to the results, the systemic level of Aβ-40 was increased from the early stage of AD and was similar between the middle and late phases, while amounts of plaque deposition and cognitive function are not similar, suggesting the possible role of the clearance system. Hemming et al., in 2005, demonstrated that ACE expression leads to cleavage of the Aβ protein in a cellular context, and that ACE promotes the degradation of both naturally produced Aβ-40 and Aβ-42. Their results showed that ACE may modulate the progression of AD *via* degradation of Aβ. According to the results provided in this research, ACE level was reduced significantly in middle and late AD compared to control patients. Reduction of ACE content is gradual as AD develops, and we observed greater than a 5-fold reduction in VSCD stage *versus* control. IDE dysfunction in different stages of AD is similar to ACE and decreases remarkably as cognitive deficits emerge.

MMP-9 has been shown to degrade fibrillar structures and compact plaques in *ex vivo* experiments and to co-localize *in vivo* with neuritic plaques, vascular amyloid deposits, and neurofibrillary tangles (Yan et al., 2006). MMP-9 is a major constituent of amyloid plaques that are observed in the cortex and hippocampus of patients affected with AD, and an elevation of MMP-9 was previously found in the AD hippocampus. MMP-9 is synthesized in neurons and is capable of degrading Aβ-40 (Backstrom et al., 1996). Fragments of the APP stimulate MMP-9 expression in cultured microglia cells and induce MMP-9 release from human monocytes (Chong et al., 2001). Additional mechanisms, such as proinflammatory cytokines, may contribute to the elevated MMP-9 levels, suggesting these cytokines and oxidative damage may be implicated in the neurodegenerative process in AD (McGeer, 1998), lymphocyte and macrophage activation, and subsequent release of MMP-9 (Gu et al., 2020). Yan et al. (2006) reported that MMP-9 can degrade Aβ fibrils and may contribute to ongoing clearance of plaques from amyloid-laden brains. Lorenzl et al. (2003) also reported that circulating levels of MMP-9 are increased in AD and may contribute to disease pathology. The results of this research show that MMP-9 levels are significantly higher in early and middle AD compared to control patients; however, there is no significant difference in MMP-9 levels in late AD *versus* control subjects. In another study, Gu et al. (2020) reported that MMP-9 levels in plasma neuronally derived extracellular vesicles (NDEV) were significantly higher in patients with AD than in healthy control patients. Evidence suggests a role of MMP-3 in the pathophysiology of AD, and our results show that MMP-3 is increased in AD patients compared to control patients. It has been suggested that Aβ toxicity may contribute to the induction of MMP-3 expression, and this enzyme has been reported to degrade secreted Aβ, suggesting that MMP-3 may contribute to reduction of Aβ deposition in AD brains (Kim et al. 2011). A recent longitudinal study demonstrated that MMP-3 levels are elevated early in AD plasma and confer a risk of AD-related cognitive decline in females, but not males (Iulita et al., 2019).

IDE has been found to degrade Aβ in neuronal and microglial cell cultures and to eliminate Aβ's neurotoxic effects. In a mouse model, Farris et al. (2003) reported that IDE deficiency resulted in a > 50% decrease in Aβ degradation in both brain membrane fractions and primary neuronal cultures. The IDE –/– mice showed increased cerebral accumulation of endogenous Aβ, a hallmark of AD (Bertram et al., 2000). Moreover, the mice had elevated levels of the intracellular signaling domain of the β-amyloid precursor protein, which was recently found to be degraded by IDE *in vitro* (Farris et al., 2003). Our results in this re-

search show that IDE and NEP are significantly lower in AD patients compared to control subjects (late AD patients have the lowest IDE and NEP levels). Reduction in the levels of both enzymes occurs gradually as dementia develops and cognitive function declines, and suggests other possible roles for these enzymes in thinking and learning processes that need further investigation. Wang et al. (2005) reported that NEP was selectively decreased in AD brains, and other research in mouse models shows that NEP expression is reduced in the hippocampus and cortex of aged mice compared to young mice (Iwata, 2002). Conversely, overexpression of NEP reduces Aβ levels in a dose-dependent manner and protects neuronal cells from Aβ toxicity *in vitro* (Iwata, 2002).

CONCLUSION

Our results report that the activity of Aβ production and clearance pathways varies in different stages of AD. Accordingly, Aβ-40 and sAPPβ increased in the early stages of disease, and their level remained high through all disease stages, while Aβ clearance enzymes manifested different behavior in correlation with dementia development. Together, we conclude that the plaque clearance system has full function in the early stage, suggesting moderate dementia in this phase is due to increased Aβ levels. Conversely, clearance pathways declined as disease developed, and reduction of multiple Aβ-degrading enzymes such as NEP and IDE in AD patients was observed, suggesting these enzymes play a more remarkable function in dementia-related disease. By considering the difference in the active molecular mechanism in each stage of disease, we can conclude that detection of the dementia phase is very important in therapeutic strategies. Our results also suggested some new therapeutic targets in AD patients.

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