

Research Paper



Structural Changes in Pharyngeal and Tongue Muscles as a Potential Contributor to Dysphagia in Alzheimer Disease Rat Model

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ABSTRACT

Introduction: Alzheimer disease (AD) is a progressive neurodegenerative disease that accounts for 60% of dementia cases worldwide. Despite the lack of concrete information about the prevalence of dysphagia among AD patients, it still significantly impairs their quality of life (QoL). That outcome necessitates more investigations to understand the pathophysiology of this condition and how to manage it. In this study, we examined if AD-associated changes in pharyngeal and tongue muscles could explain dysphagia.

Methods: Fourteen adult male rats were allocated into 2 groups: Group I (control) received distilled water orally, group II (AD) received aluminum chloride (AlCl₃) (200 mg/kg, per os) and D-galactose (60 mg/kg, subcutaneous) daily for 45 days. Biochemical parameters were conducted, including amyloid beta-peptide (Aβ), histopathological investigation of the hippocampus, tongue, and pharynx, and immune-histochemical expression of brain glial fibrillar acidic protein (GFAP).

Results: Our AD model showed marked cognitive impairment, hippocampal oxidative stress, and increased brain Aβ expression (P=0.0003) compared to controls. Dysphagia was confirmed by loss of body weight (P=0.0077) and decreased eating and drinking patterns by 25%-35% in AD versus the control group. Histopathological, immune-histochemical, and biochemical evidence, including increased levels of pharyngeal Aβ (P=0.0017), were detected in AD rats' tongue and pharyngeal muscles.

Conclusion: Dysphagia in AD can result not only centrally but also due to local involvement of the tongue and pharynx. Further translational studies linking dysphagia to AD pathology will be needed.

Keywords:

Alzheimer disease (AD),
 Dysphagia, Amyloid, Tongue,
 Pharyngeal muscles

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Highlights

- Male rats with Alzheimer disease (AD) showed cognitive impairment and hippocampal oxidative stress.
- Dysphagia was confirmed by loss of body weight and decreased eating and drinking patterns in male rats with AD.
- Histopathological, immune-histochemical, and biochemical evidence of pathological changes were detected in AD rats' tongue and pharyngeal muscles.
- Dysphagia in AD can result from central and local involvement of the tongue and pharynx

Plain Language Summary

AD causes changes in the brain that can lead to many complaints in patients, including dysphagia (difficulty swallowing), which can affect the patient's quality of life. In this study, Alzheimer's disease was induced in a group of male rats, and brain and behavioral changes were confirmed. The difficulty of swallowing was also confirmed, and the study of tongue and pharynx muscles proved that there are local changes in muscles associated with brain changes.

1. Introduction

Alzheimer disease (AD) is a progressive neurodegenerative disease usually characterized by behavioral and mental alterations, loss of recent memories, cognitive deficits, and the inability to live independently (Waldemar et al., 2007). The most severely affected brain regions are the neocortex and hippocampus, which classically show the main pathological hallmarks of AD, including abnormal accumulation of extracellular amyloid beta-peptide (A β) plaques as well as intracellular aggregates of tau-containing neurofibrillary tangles (NFTs) or phosphorylated tau (DeTure & Dickson, 2019; Frisoni et al., 1999). It accounts for 60% of causes of dementia worldwide, with a higher prevalence in females by 1.9 times (Cao et al., 2020). Moreover, the high cost of care and research can affect economies, as the global cost 2018 was estimated to reach \$ 1 trillion (Patterson, 2018).

Dysphagia is an impairment in swallowing due to sensory, motor, or behavioral causes or a combination of them (Özsürekcı et al., 2020). Neurogenic dysphagia occurs in patients with neurological diseases of different etiologies, such as degenerative, dementia, myopathy, traumatic, and vascular etiologies (Panebianco et al., 2020). It is considered one of the geriatric syndromes associated with impaired quality of life (QoL), malnutrition and weight loss, aspiration pneumonia, aspiration pneumonitis, and mortality (Fernández-Ruiz et al., 2021; Nagamine et al., 2020). No recent publications can accurately point to the prevalence of dysphagia among AD patients (Mira et al., 2022).

Dysphagia typically occurs at later stages of AD; however, subclinical dysphagia can be detected in mild or moderate stages of the disease, which is often underdiagnosed or overlooked (Simoes et al., 2020). AD-associated dysphagia has been explained by the involvement of cortical control of swallowing (Humbert et al., 2010) as well as weakness and atrophy of skeletal muscles causing sarcopenic dysphagia (Özsürekcı et al., 2020), which means the involvement of the pharyngeal muscles as a part of the whole-body skeletal muscles.

The cortical deposition of A β plaques and NFTs has been proved in AD (Kloskowska et al., 2010), which are the pathological hallmarks of the disease. Although dysphagia is a common comorbidity in AD, no studies have investigated if there are biochemical, histopathological, and immune-histochemical local changes in pharyngeal muscles in AD. Previous studies have found indications for the deposition of neuropathological proteins outside the central nervous system in other degenerative disorders like Parkinson disease. Moreover, deposition of A β protein has been detected in specific skeletal muscles. If local deposition of A β protein in pharyngeal muscles in AD is proven, we better understand the pathogenesis of dysphagia in AD patients and may improve its management (Kuo et al., 2000). Thus, this study investigated structural changes in rats, including biochemical, histopathological, and immune-histochemical aspects of pharyngeal and tongue muscles in AlCl₃/D-gal-induced AD.

2. Materials and Methods

Study animals

Animals were housed according to the regulations approved by the Veterinary Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, [Cairo University \(VET- IACUC\)](#). Fourteen adult male Wistar rats (150-170 g, 12 weeks) were housed in private plastic cages with softwood chips for bedding and were fed on a balanced commercial diet and water ad libitum. Animals were acclimatized for two weeks before proceeding with the experiment.

Experimental design

Rats were randomly blindly allocated into two groups, each of 7 rats. Group I (control) received distilled water orally and subcutaneously daily for 45 days, while group II (AD) orally received $AlCl_3$ (Sigma-Aldrich Co., USA) (200 mg/kg) and subcutaneously received D-galactose (HEGENG Co., China) (60 mg/kg) daily for 45 days ([Chiroma et al., 2018](#); [Ezzat et al., 2022](#)). Aluminum chloride ($AlCl_3$) and D-galactose (D-gal) were used as AD inducers due to their neurotoxicity, including morphological alterations, cognitive impairment, and altered brain neurochemistry in rodents due to its pro-oxidant nature as reported in previous studies ([Liaquat et al., 2019](#); [Rebai, & Djebli, 2008](#)). $AlCl_3$ is considered a neurotoxin, and D-gal is used to model subacute aging. Consequently, their combination can create a non-transgenic AD animal model ([Xiao et al., 2011](#)).

After the scheduled 45 days, behavioral tests were carried out for 5 days. Then, blood samples were taken for sera separation. Animals were decapitated, and the brains, tongue, and pharynx of each rat were dissected out, washed, and frozen at $-20\text{ }^\circ\text{C}$ for biochemical analysis or kept in 10% formalin saline for histological and immunopathological tests.

Measurement of rats' body weight

The rats' body weight in the two groups was measured at the beginning of the experiment, then twice a week until the end. Body weight change percentage was calculated from the [Equation 1](#):

$$1. \text{ Body Weight at the End of the Experiment (g) - body Weight at the Beginning of the Investigation} \times 100$$

The aim of measuring body weight is to monitor any indirect decrease in feed intake.

Validation of AD in rats

Behavioral validation

Open field test

The locomotion and exploratory behavior were evaluated using an open-field test, performed as mentioned by [Khalil et al. \(2021\)](#). The measured parameters were the number of crossing squares and the rearing frequencies.

Y-maze test

The Y-maze test was used to evaluate short-term memory and motor activity. The test was conducted according to [Khalil et al. \(2020\)](#) and [Khalil et al. \(2021\)](#), and the measured parameters were the number of arm entries and the spontaneous alternation percentage (SAP%). The spontaneous alternation percentage depends on the rats' natural tendency to alternate between three different arms.

Novel object recognition (NOR) test

The NOR test was used to evaluate hippocampal-dependent memory impairment ([Lueptow, 2017](#)) and was conducted as mentioned in previous research ([Khalil et al., 2021](#)). The calculated parameters were the total exploration time, the discrimination ratio (DR), and the recognition index (RI). DR was calculated using the [Equation 2](#):

$$2. \text{ DR} = \frac{\text{New Object Exploration Time} - \text{Familiar Object Exploration Time}}{\text{New Object Exploration Time} + \text{Familiar Object Exploration Time}}$$

The recognition ratio, the rat's capacity to determine the same object on different occasions, was calculated using the [Equation 3](#):

$$3. \text{ RI} = \frac{\text{New Object Exploration Time} + \text{Familiar Object Exploration Time}}{\text{New Object Exploration Time}}$$

([Costa et al., 2008](#); [Lueptow, 2017](#)).

Biochemical validation

Preparation of tissue homogenates

At a concentration of 20% (w/v), hippocampal and pharyngeal tissue homogenates were meticulously prepared in ice-cooled phosphate-buffered saline, employing a homogenizer. Subsequently, these homogenates under-

went centrifugation, lasting 15 minutes at 5000×g while being maintained at a temperature of 4 °C. The resultant tissue homogenates were then carefully portioned into aliquots and preserved at -80 °C, pending further analysis for quantifying different biomarkers and mediators as described previously by [El-Shoura et al. \(2023\)](#).

Measurement of tissue malondialdehyde (MDA) concentration

Malondialdehyde (MDA) colorimetric/fluorometric assay kit (K739-100) was purchased from BioVision, USA. It was prepared as described by [Halliwell and Chirico \(1993\)](#).

Measurement of tissue total antioxidant capacity (TAC)

Rat TAC1/Substance P ELISA Kit (LS-F14457) was purchased from LSBio, Inc., USA. It was examined in hippocampal and pharyngeal homogenates as described by [Koracevic et al. \(2001\)](#).

Measurement of tissue brain-derived neurotrophic factor (BDNF)

Rat BDNF ELISA Kit (SEA011Ra) was purchased from Cloud-Clone Corp., USA. It was measured in hippocampal and pharyngeal homogenates, as described by [Klein et al. \(2011\)](#).

Measurement of tissue A β

Rat A β 1-42 ELISA Kit (LS-F23254) was purchased from LSBio, Inc., USA. Soluble and insoluble/formic acid-soluble A β were quantified in hippocampal and pharyngeal homogenates, as mentioned by [Liu et al. \(2011\)](#).

Histological examination

The samples were fixed, dehydrated, cleaned, and embedded in paraffin wax. Rotatory microtome sections of 3–4 μ m in thickness were prepared and stained with hematoxylin and eosin (H&E), as [Bancroft and Gamble \(2008\)](#) mentioned.

Immunohistochemical examination

Glial fibrillar acidic protein (GFAP) was used to detect astrocyte protein in the hippocampus. The method was done according to [Stoltenburg-Didinger et al. \(1996\)](#).

Evaluation of immunohistochemical observations (area percentage)

The sections were stained and assessed by the Leica Quin 500 analyzer computer system (Leica Microsystems, Switzerland). The image analyzer was calibrated automatically to convert the pixels into actual micrometer units, then statistically analyzed for each specimen.

Statistical analysis

Statistical analysis was performed using GraphPad Prism software, version 6. Behavioral and biochemical data were expressed as Mean \pm SEM. An unpaired student t-test was used to compare the means. Histograms were drawn using GraphPad Prism.

3. Results

Body weight change, locomotor activity, and cognitive function

AD rats displayed a significant decrease ($P=0.0077$) in their body weight compared to control rats. Also, they exhibited a marked reduction in their locomotor activity, as evidenced by decreased crossing squares ($P=0.0001$) and rearing frequency ($P=0.0001$) compared to control rats. Regarding cognitive functions, AD rats showed a marked cognitive dysfunction both in the Y maze and the NOR test and displayed a significant decrease in the number of arm entries ($P=0.0001$) and SAP% ($P=0.001$). Furthermore, they significantly reduced the total exploration time ($P=0.03$). However, the decline in the DR and RI was not significant ($P=0.17$ and $P=0.13$, respectively) ([Figure 1](#)).

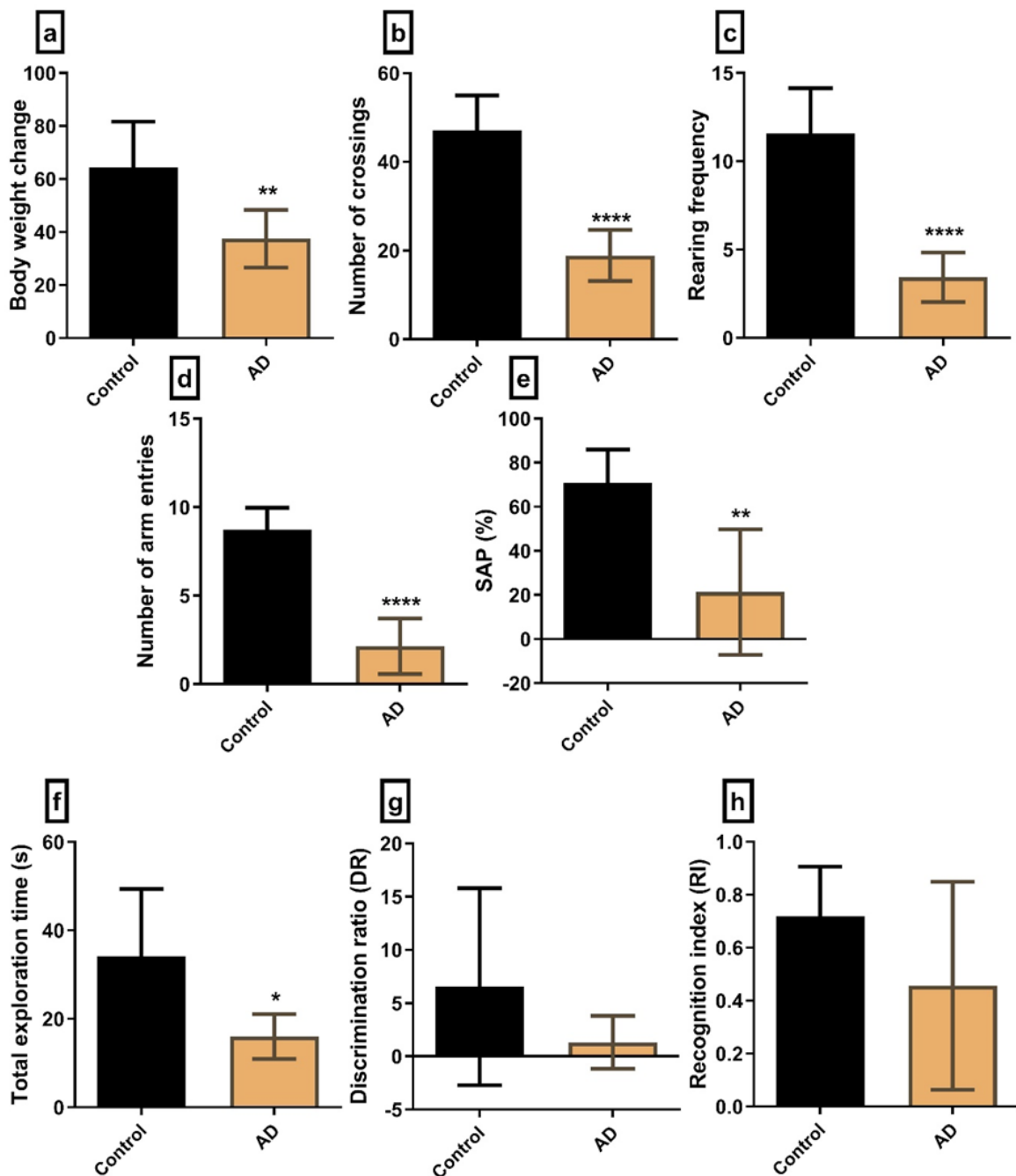
Biochemical parameters

Oxidative stress markers

The hippocampal and the pharyngeal MDA were significantly higher in the AD group compared to the controls ($P\leq 0.0001$, 0.0003 , respectively) ([Figures 2A](#), and [2B](#)). Additionally, the hippocampal and pharyngeal TAC was significantly lower in the AD group compared to the controls ($P=0.0005$ and $P=0.0002$, respectively) ([Figures 2C](#), and [2D](#)).

Brain-derived neurotrophic factor

The BDNF was significantly lower in the hippocampus and the pharyngeal muscles of the AD group compared to the control group ($P=0.0001$ and $P<0.0001$, respectively) ([Figures 3A](#), and [3B](#)).



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Figure 1. Effect of AlCl₃/D-gal intoxication

a) Body weight changes, b) Number of crossings, c) Rearing frequency, d) Number of arm entries, e) Spontaneous alternation percentage (SAP%), f) Total exploration time, g & h) DR, and RI on both control & AD rats

****Significantly different from the control group.

Aβs

The Aβ levels in the hippocampus and the pharyngeal muscles were significantly higher in the AD group compared to the control group (P=0.0003 and P=0.0017, respectively) (Figures 3C, and 3D).

Hematoxylin and eosin-stained sections

Hippocampus H&E-stained sections

The H&E-stained hippocampus sections of the control rats revealed the normal histological structure of the mo-

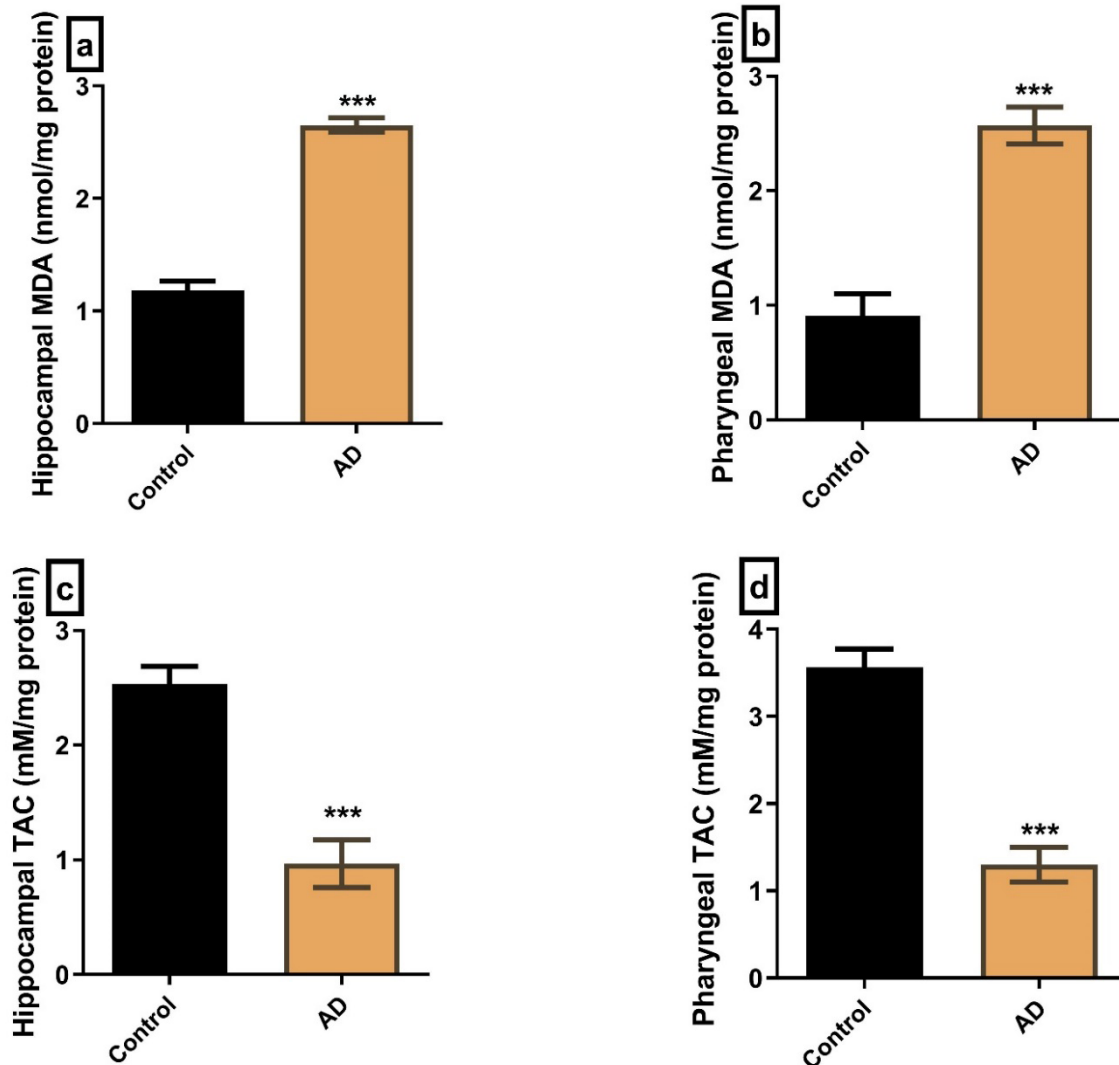


Figure 2. Effect of $AlCl_3$ /D-gal intoxication on the oxidative stress markers of the rats

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a) MDA levels in the hippocampus, b) MDA levels in the pharyngeal muscles, c) TAC levels in the hippocampus, d) TAC levels in the pharyngeal muscles

MDA: Malonaldehyde; TAC: Total antioxidant capacity.

***Significantly different from the control group.

lecular layer, pyramidal cell layer, and polymorphic cell layer (Figure 4A). On the other hand, hippocampus sections of AD rats showed histopathological disruption in the form of cellular disorganization of pyramidal cells that had NFTs confirming the induction of AD (Figure 4B).

Tongue H&E-stained sections

Tongue sections of control rats showed the normal microscopic structure of a muscular organ covered dorsally by mucous membrane and underlined by propria-

submucosa that was formed of fibro-elastic connective tissue and centrally contained skeletal muscle mass with intermuscular fibro-elastic connective tissue in between. Lingual myofibers appeared cylindrical, unbranched, striated, and multinucleated (Figure 4C). On the contrary, tongue sections of AD rats revealed multiple features of degeneration in skeletal muscles, including fragmentation of myofibers. Some myofibers appeared hyper-eosinophilic and necrotic, while others lost their striation. Additionally, rupture of some muscular fibers,

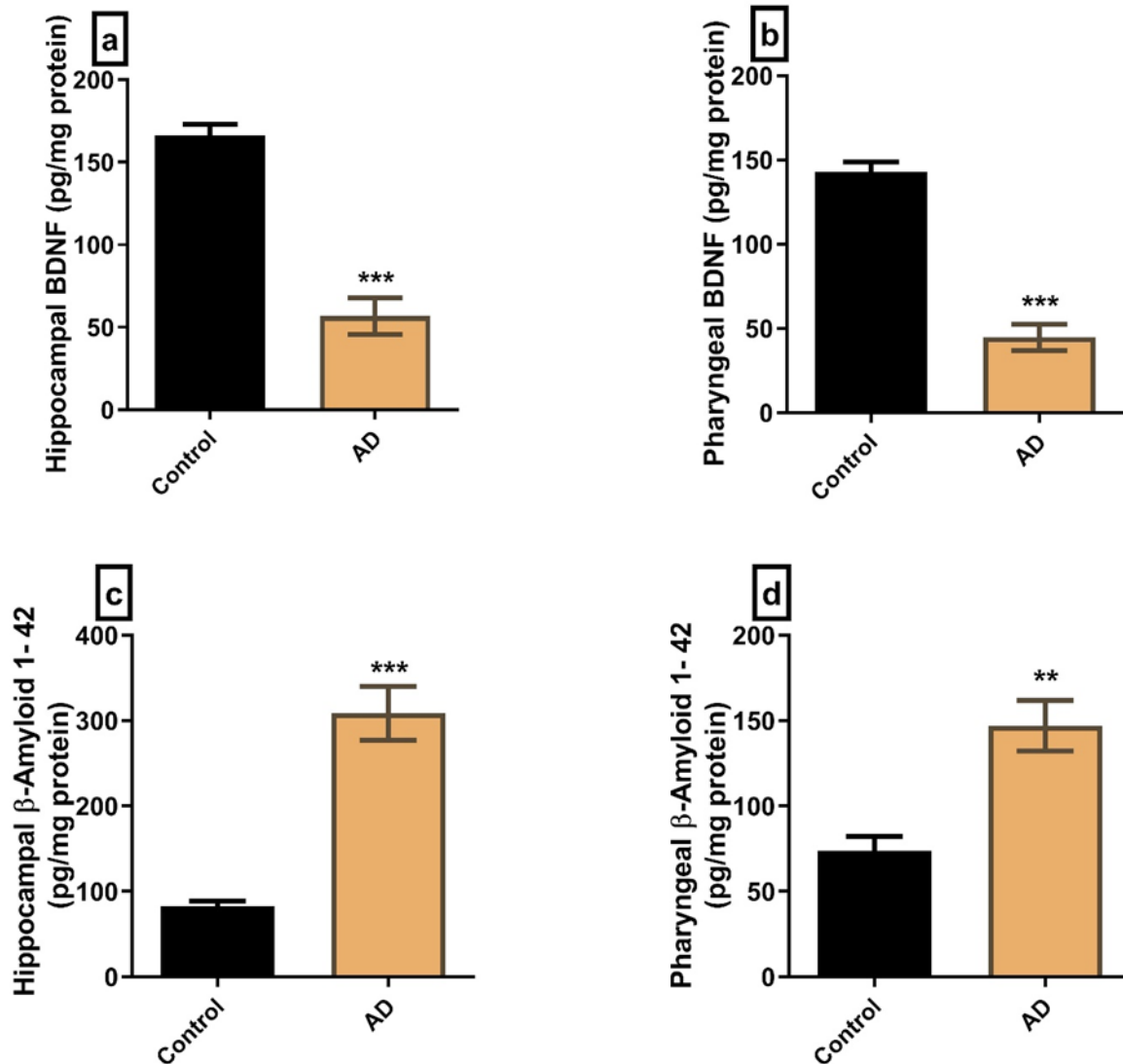


Figure 3. Effect of $AlCl_3$ /D-gal intoxication on BDNF and $A\beta$ of the rats

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a) BDNF levels in the hippocampus, b) BDNF levels in the pharyngeal muscles, c) $A\beta$ levels in the hippocampus, d) $A\beta$ levels in the pharyngeal muscles

***Significantly different from the control group.

edematous interstitial tissue, and inter-/extra- myofibril hemorrhage were observed (Figure 4D).

Pharynx H&E-stained sections

The H&E-stained oropharynx sections of control rats showed normal histological form of the lining mucous membrane that was underlined by fibro-elastic connective tissue in propria-submucosa then circularly arranged pharyngeal muscles (Figure 4E). Pharyngeal muscles were formed of cylindrical, unbranched, striated myo-

fibers that appeared multinucleated and separated from each other by intermuscular connective tissue (Figure 4F). On the other hand, oropharynx sections obtained from AD rats showed abnormal muscular architecture and fragmentation, myofiber rupture, and some myofibers appeared hyper-eosinophilic and necrotic. Hemorrhage was also observed in the myofibers (Figure 4G).

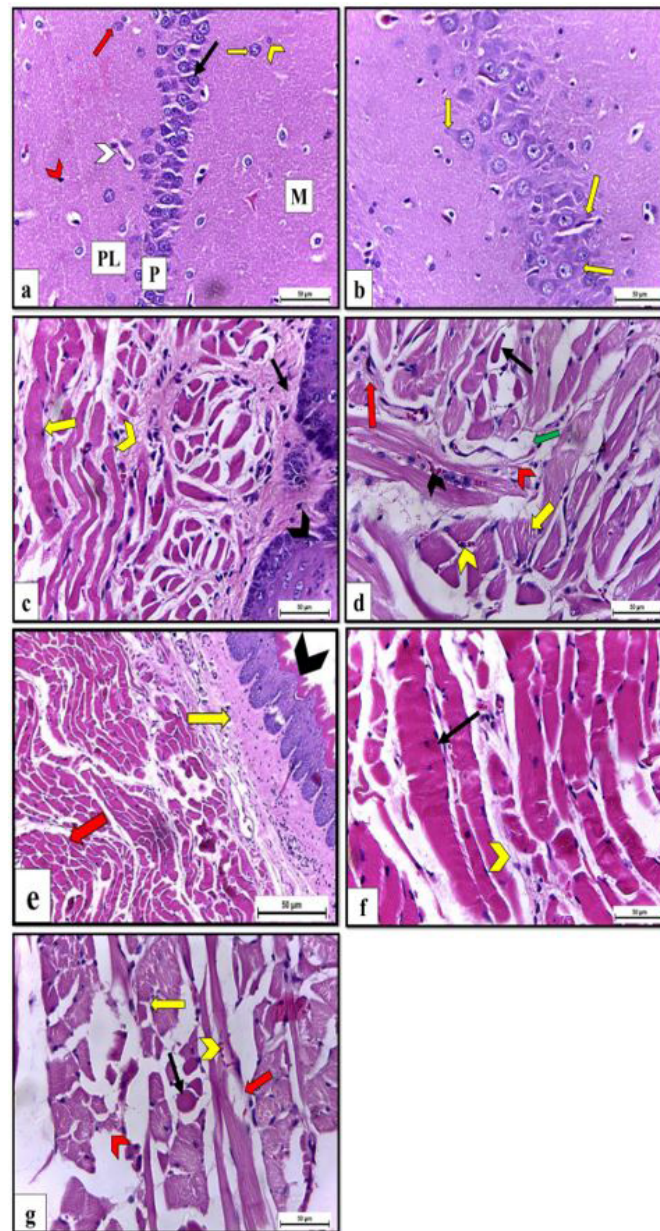


Figure 4. Hippocampus sections of albino rats (H&E stain X400)

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Notes: a) Control rats (group I) revealed the normal structure of the molecular layer (M), pyramidal cell layer (P), and polymorphic layer (PL). The molecular layer contained neurons (yellow arrow) and neuroglia (yellow chevron), the pyramidal cell layer consisted of triangular-shaped neurons with vesicular spherical nuclei (black arrow), and the polymorphic layer contained neurons (red arrow), neuroglia (red chevron), and blood capillaries (white chevron). b) AD rats (group II) revealed neurofibrillary tangles of pyramidal cells (yellow arrows).

c & d) Tongue sections of albino rats H&E stain X400: c) Control rats (group I) presented the normal structure of the tongue covered by a mucous membrane (black chevron) that was underlined by fibro-elastic connective tissue (black arrow) and contained a central mass of skeletal muscles (yellow arrow) with intermuscular connective tissue (yellow chevron). d) AD rats (group II) showed degeneration in skeletal muscles such as fragmentation of myofibers (yellow arrow), some myofibers appeared hyper-eosinophilic and necrotic (black arrow), some lost their striation (yellow arrow), rupture of some muscular fibers (red chevron) and edematous interstitial tissue (green arrow) were noticed. A hemorrhage surrounded the myofibers externally (yellow chevron) and between them (black chevron).

e, f & g) Oropharynx sections of albino rats H&E stain X400: e) Control rats (group II) revealed normal lining mucous membrane (black chevron) that was underlined by fibro-elastic connective tissue (yellow arrow) and then circularly arranged pharyngeal muscles (red arrow). f) Normal cylindrical unbranched and striated multinucleated myofibers (black arrow) with intermuscular connective tissue (yellow chevron). g) AD rats (group II) showed fragmentation (yellow arrow) and rupture (red arrow) of pharyngeal myofibers, abnormal muscular architecture (red chevron), some myofibers appeared hyper-eosinophilic and necrotic (black arrow) and hemorrhage (yellow chevron).

Immunohistochemical-stained sections

Immunohistochemical examination of the hippocampus of the control rats (group I) displayed a positive immunorexpression of the fibrillary astrocyte bodies and processes to GFAP. Meanwhile, the immunoreactivity concentration was significantly increased in the AD rats (group II) (Figures 5A, and 5B). The percentage area covered by GFAP-positive immunoreactive cells within the hippocampus revealed a significant elevation in the AD rats by 44.6 fold compared with the control rats (Figure 5C).

4. Discussion

There is evidence of pathological changes outside the central nervous system in AD, as some studies reported the presence of certain species of phosphorylated tau in peripheral tissues of AD patients, such as the submandibular gland (Dugger et al., 2016) and the deposition of A β plaques in the testis and ovary of patients (Miklossy et al., 1999), with evidence of impaired sperm function in vitro (Tavares et al., 2017). In addition, phosphorylated tau has been seen in AD human participants' peripheral tissues like skin and skeletal muscle (Rodríguez-Leyva et al., 2015). Therefore, this work is meant to assess the histological abnormalities of skeletal muscles in the tongue and oropharynx in AD, in addition to the neurodegenerative impact of the disease.

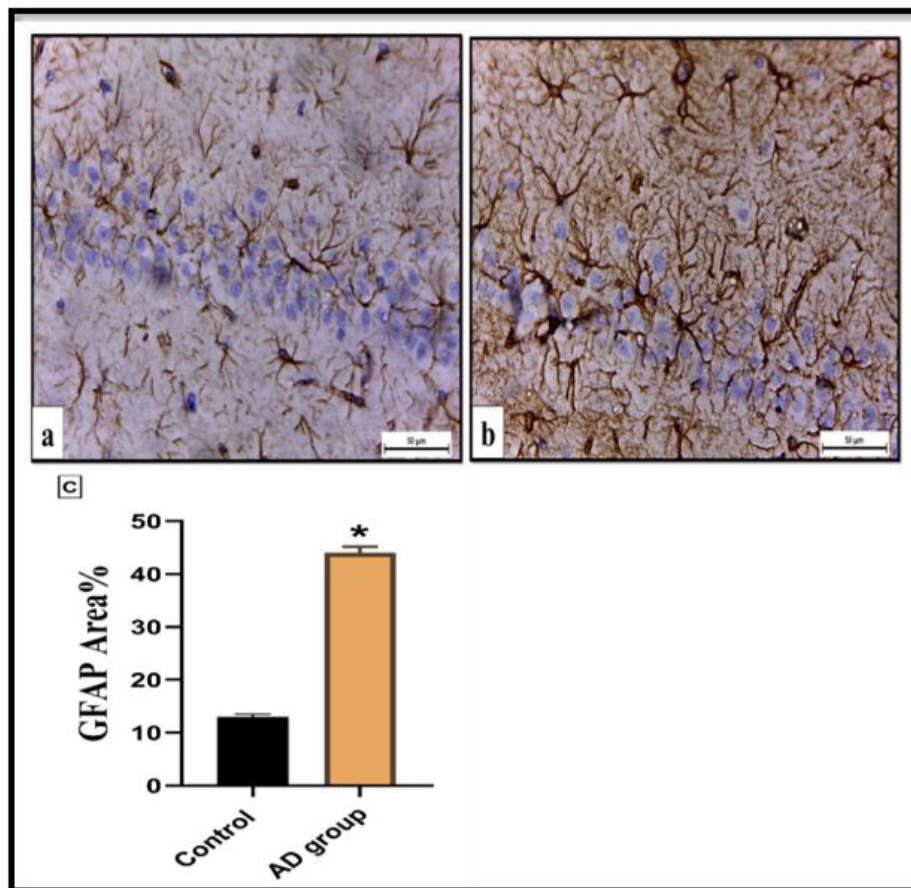


Figure 5. GFAP stained hippocampus sections (X400)

a) BDNF levels in the hippocampus, b) BDNF levels in the pharyngeal muscles, c) A β levels in the hippocampus, d) A β levels in the pharyngeal muscles

*Significant difference from the control group at $P \leq 0.05$, **Significantly different from the control group.

Notes: a) Control rats (group I) revealed a positive reaction to GFAP. b) AD rats (group II) revealed an intensely positive reaction to GFAP. c) The bar graph represented a significant increase in the percentage area covered by GFAP-positive immunoreactive cells within the hippocampus of the AD group in comparison with the control group. Data are presented as Mean \pm SE. Statistical significance level at $P \leq 0.05$.

In this study, AD was confirmed by the impairment of the working spatial and recognition memory, the locomotor disturbance, and the significant increase of hippocampal A β levels in AD rats. These results are consistent with [Khalil et al. \(2020\)](#) and [Cheignon et al. \(2018\)](#). Also, AD diagnosis was confirmed by the decreased BDNF expression in the hippocampal homogenates, the significant increase in MDA and decrease in TAC, which is in line with previous studies ([Tapia-Arancibia et al., 2008](#); [Tobore, 2019](#)), and the significant increase in hippocampal immunoreactivity to GFAP, which was explained by [Kamphuis et al. \(2014\)](#). Besides, the histopathological changes in the hippocampus established the diagnosis of AD, which agrees with the work of [DeTure & Dickson \(2019\)](#) and [Ryan et al. \(2015\)](#).

In our study, the development of dysphagia was hypothesized by the significant body weight loss observed in AD rats, as the eating and drinking patterns decreased by 25%-35% in AD versus the control group. Moreover, the histopathology of the tongue and pharynx exhibited myofibers fragmentation and rupture associated with edematous interstitial tissue representing structural affection of the muscles, which may be regarded as a potential contributor to dysphagia. Our results agree with [Ogawa et al. \(2018\)](#), who stated that sarcopenia is closely linked to AD and may be involved in the pathophysiological process of AD, as the involvement of pharyngeal muscles is a part of this generalized skeletal muscle affection. Furthermore, these local changes are associated with significant changes in local oxidative stress markers. Further research is needed to elucidate the possible relation between the pharyngeal local changes in AD and the development of dysphagia.

In our study, we show for the first time that degeneration of the tongue and pharyngeal muscles is evident in AD rats in the form of altered muscular structure, rupture and necrosis of the myofibers, and detection of oxidative stress in pharyngeal muscles. AD pharyngeal muscles even exhibited a significant increase in A β levels, which could be explained theoretically by the migration/spread of A β along cranial nerves, e. g. the vagus nerve. Otherwise, it could be formed in the muscles through metabolic alterations, leading to increased amyloid deposition. Also, the downregulation of BDNF levels in AD pharyngeal muscles could be explained by an injurious effect on the supplying cranial nerves, e. g. the vagus nerve. Further research is still needed to examine these hypotheses.

5. Conclusion

Dysphagia in AD has been discussed as originating mainly from central cerebral affection. So far, there has not been any biochemical, histopathological, or immunohistochemical evidence of AD in the tongue and pharyngeal muscles themselves. In our study, we show for the first time evidence for the presence of AD pathology in these structures, possibly one of the main contributing factors to dysphagia observed in AD. Additional investigations are needed to clarify the underlying processes that could be targeted to decrease the incidence of dysphagia and their direct linkage to clinical impairment. As a potential outlook, targeting AD pathology in these structures might, therefore, prevent dysphagia and potentially enhance the QoL in AD patients from this point. Future studies should explore the other factors contributing to dysphagia and the underlying molecular mechanisms involved in its pathogenesis. However, this is the first study to establish successfully the link between dysphagia and AD.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of the Veterinary Institutional Animal Care and Use Committee, Faculty of Veterinary Medicine, [Cairo University \(VET- IACUC\)](#) (Code: Vet CU28/04/2021/267).

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Authors' contributions

Study design, data interpretation: Ramy Abdelnaby; Data collection and analysis: Heba M. A. Khalil, Yasmine H. Ahmed, Dalia Zaafar, and Mohamed Y. Mahmoud; Writing: Alexa Häger, Ramy Abdelnaby, and Eman Mohammed Elsaeed; Final approval: All authors.

Conflict of interest

The authors declared no conflict of interest.

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