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

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Abstract

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

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

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

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

Keywords: Alzheimer's, Dysphagia, Amyloid, Tongue, Pharyngeal muscles.

Significance statement: this research studied a possible cause of dysphagia in Alzheimer's patients, which could be a step in improving their quality of life.

1. Introduction

Alzheimer's 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 (Aβ) 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 caring and research can affect economies, as the global cost in 2018 was estimated to reach one trillion US-Dollars (Patterson, 2018).

Dysphagia is defined as an impairment in swallowing that can be due to the sensory, motor, behavioral causes or a combination of them (Özsürekci 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, malnutrition and weight loss, aspiration pneumonia, aspiration pneumonitis, and mortality (Fernández-Ruiz et al., 2021; Nagamine, Takayama, & Matsumoto, 2020). No recent publications can accurately point to the prevalence of dysphagia among AD patients (Mira, Gonçalves, & Rodrigues, 2022).

Dysphagia typically often occurs at later stages of AD; however, subclinical dysphagia can be detected in mild or moderate stages of the disease, which is often under-diagnosed or overlooked (Simoes, Oliva Filho, & Hebling, 2020). AD-associated dysphagia had been explained

by the affection of cortical control of swallowing (Humbert et al., 2010) as well as weakness and atrophy of skeletal muscles causing sarcopenic dysphagia (Özsürekci et al., 2020), which means affection of the pharyngeal muscles as a part of the whole-body skeletal muscles.

The cortical deposition of Aβ plaques and NFTs had 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, immune-histochemical local changes in pharyngeal muscles in AD . Previous studies have found indications for deposition of neuropathological proteins outside the central nervous system in other degenerative disorders like Parkinson's disease. Moreover, deposition of Aβ protein has been detected in specific skeletal muscles. If local deposition of Aβ protein in pharyngeal muscles in AD is proved, it will contribute to better understanding of the pathogenesis of dysphagia in AD patients and may impact its management (Kuo et al., 2000). Thus, the aim of this study was to investigate structural changes including biochemical, histopathological, and immune-histochemical aspects of pharyngeal and tongue muscles in AlCl₃/D-gal-induced AD in rats.

2. Materials and methods

2.1. 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) (approval number: Vet CU28/04/2021/267). Fourteen adult male *Wistar* rats (150-170 grams, 12 week) were housed in a private animal house in 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.

2.2. Experimental design

Rats were randomly blindly allocated into 2 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 (AlCl3) 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). AlCl3 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∘C for biochemical analysis or kept in 10% formalin saline for histological and immunopathological tests.

2.3. 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 of the experiment. Body weight change percentage was calculated from the following formula (body weight at the end of the experiment (g)- body weight at the

beginning of the investigation* 100). The aim of measuring body weight is to monitor any decrease in the feed intake indeirectly.

2.4. Validation of AD in rats

2.4.1 Behavioral validation

2.4.1.1.Open field test

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

2.4.1.2. 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 depend on the natural tendency of rats to alternate between three different arms.

2.4.1.3. Novel object recognition (NOR) test

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

$$\frac{New\ object\ exploration\ time - familiar\ object\ exploration\ time}{New\ object\ exploration\ time + familiar\ object\ exploration\ time}.$$

While the recognition ratio which is defined as the capacity of the rat to determine the same object on different occasions, was calculated using the equation: RI = $\frac{\text{New object exploration time} + \text{familiar object exploration time}}{\text{New object exploration time}}$. (Costa et al., 2008; Lueptow, 2017).

2.4.2.Biochemical validation

2.4.2.1.Preparation of tissue homogenates

Hippocampal and pharyngeal tissue homogenates, at a concentration of 20% (w/v), were meticulously prepared in ice-cooled phosphate-buffered saline, employing a homogenizer. Subsequently, these homogenates underwent a centrifugation step, 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 the quantification of different biomarkers and mediators as described previously byEl-Shoura et al. (2023)

2.4.2.2.Measurement of tissue malondialdehyde (MDA) concentration

Malondialdehyde Colorimetric/Fluorometric Assay Kit (K739-100) was purchased from BioVision, USA. It was prepared as described by Halliwell and Chirico (1993).

2.4.2.3. 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).

2.4.2.4. 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).

2.4.2.5. Measurement of tissue amyloid beta-peptide (Aβ)

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

2.5. Histological examination

The samples were fixed, dehydrated, cleaned, and then embedded in paraffin wax. Rotatory microtome sections of 3–4 µm in thickness were prepared and stained with hematoxylin and eosin (H&E) stain as mentioned by Bancroft and Gamble (2008).

2.5.1. 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).

2.5.2. Evaluation of immunohistochemical observations (Area percent)

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

2.6. Statistical analysis

Statistical analysis was performed using GraphPad prism v6. Behavioral and biochemical data were expressed as mean +/-SEM. Unpaired student t-test was used to compare between means. Histograms were drawn using graph pad prism v6.

3. Results

3.1. 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 a decrease in the number of crossing squares (p= 0.0001) and rearing frequency (p= 0.0001) in comparison to control rats. With regards to 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 exhibited a significant reduction in the total exploration time (p= 0.03). However, the decline in the DR, and RI was not significant (p= 0.17;0.13, respectively) (Fig.1).
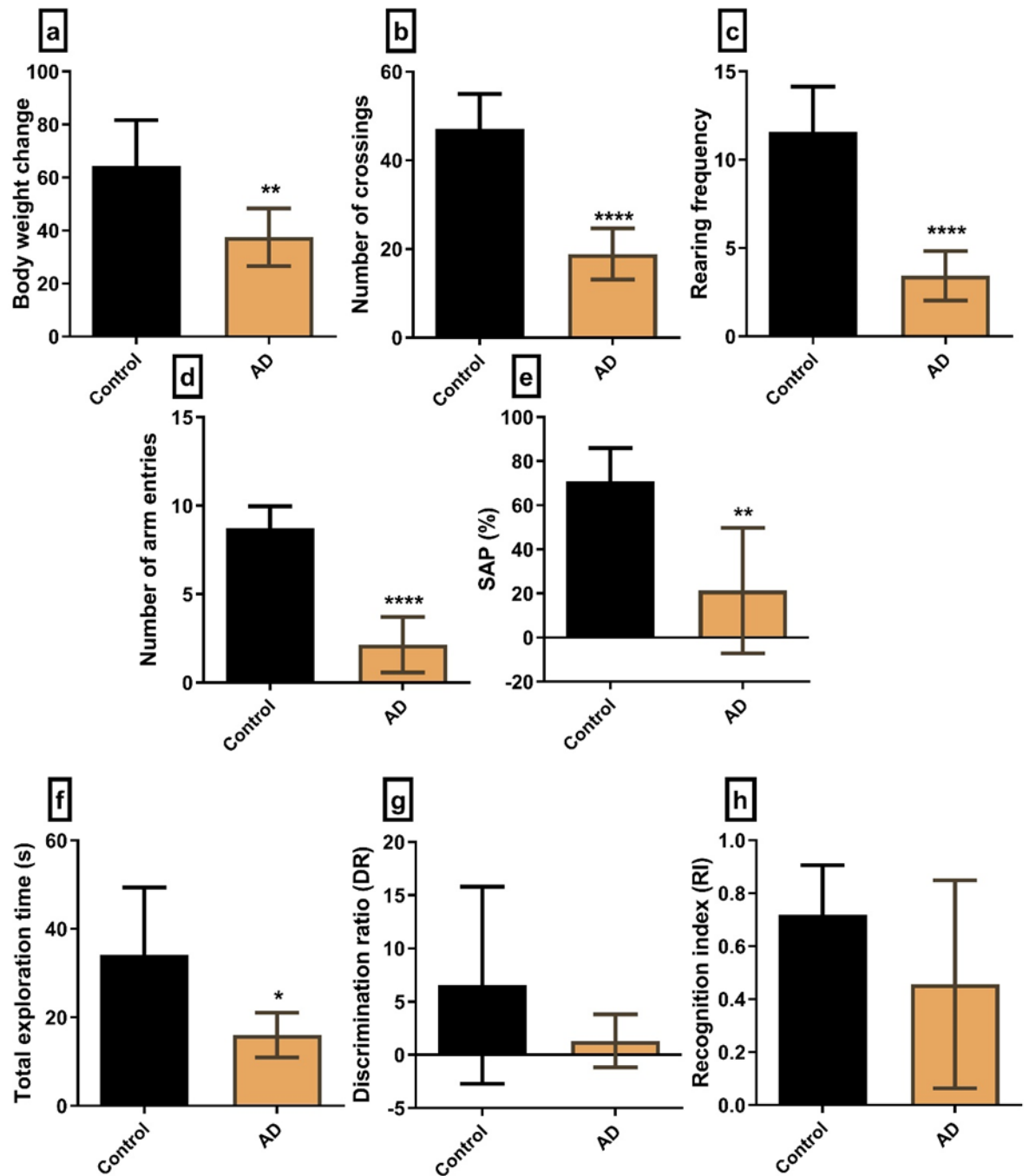
Fig.1 Effect of AlCl3/D-gal intoxication on (a) body weight changes; (b) number of crossings; (c) rearing frequency; (d) number of arm entries; (e) spontaneous alternation percentage (SAP%); (f) total exploration time; (g&h) discrimination ratio; and recognition index on both control & AD rats. **** Significantly different from the control group

3.3. Biochemical parameters

3.3.1. Oxidative stress markers

The hippocampal and the pharyngeal MDS were significantly higher in AD group compared to the controls ($p= < 0.0001$, 0.0003, respectively) (Figures 2A, 2B) (Fig.2B). Additionally, the hippocampal and pharyngeal TAC was significantly lower in the AD group compared to the controls ($p=0.0005$, 0.0002, respectively) (Figures 2C, 2D)
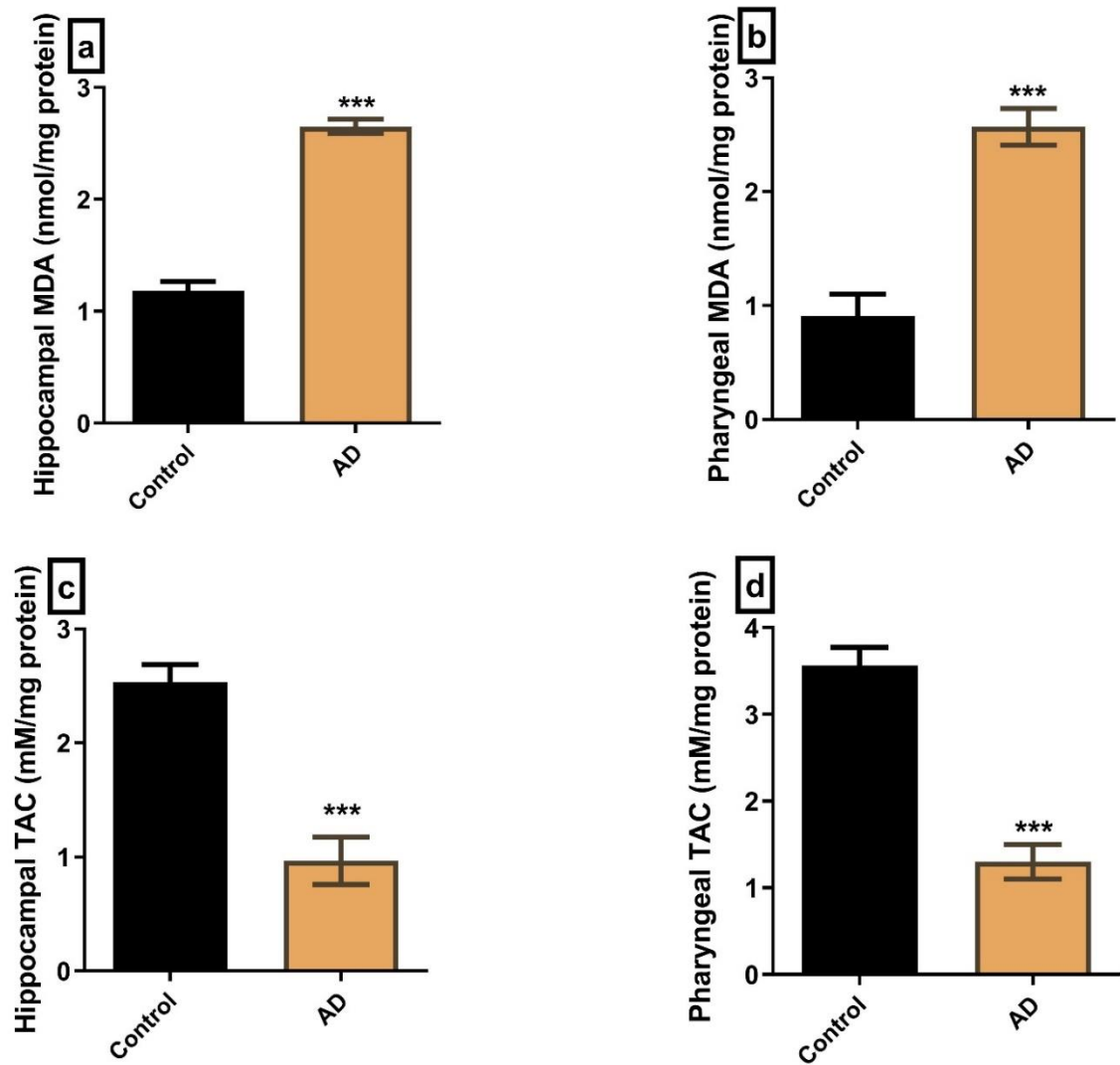
Fig.2 Effect of AlCl3/D-gal intoxication on the oxidative stress markers of the rats. MDA levels in the hippocampus (a) & pharyngeal muscles (b); TAC levels in the hippocampus (c) & pharyngeal muscles (d). *** Significantly different from the control group. MDA; malonaldehyde, TAC; total antioxidant capacity

3.3. 2. Brain-derived neurotrophic factor (BDNF)

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

3.3. 3. Amyloid beta-peptides

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, 0.0017, respectively) (Fig.3C, 3D).
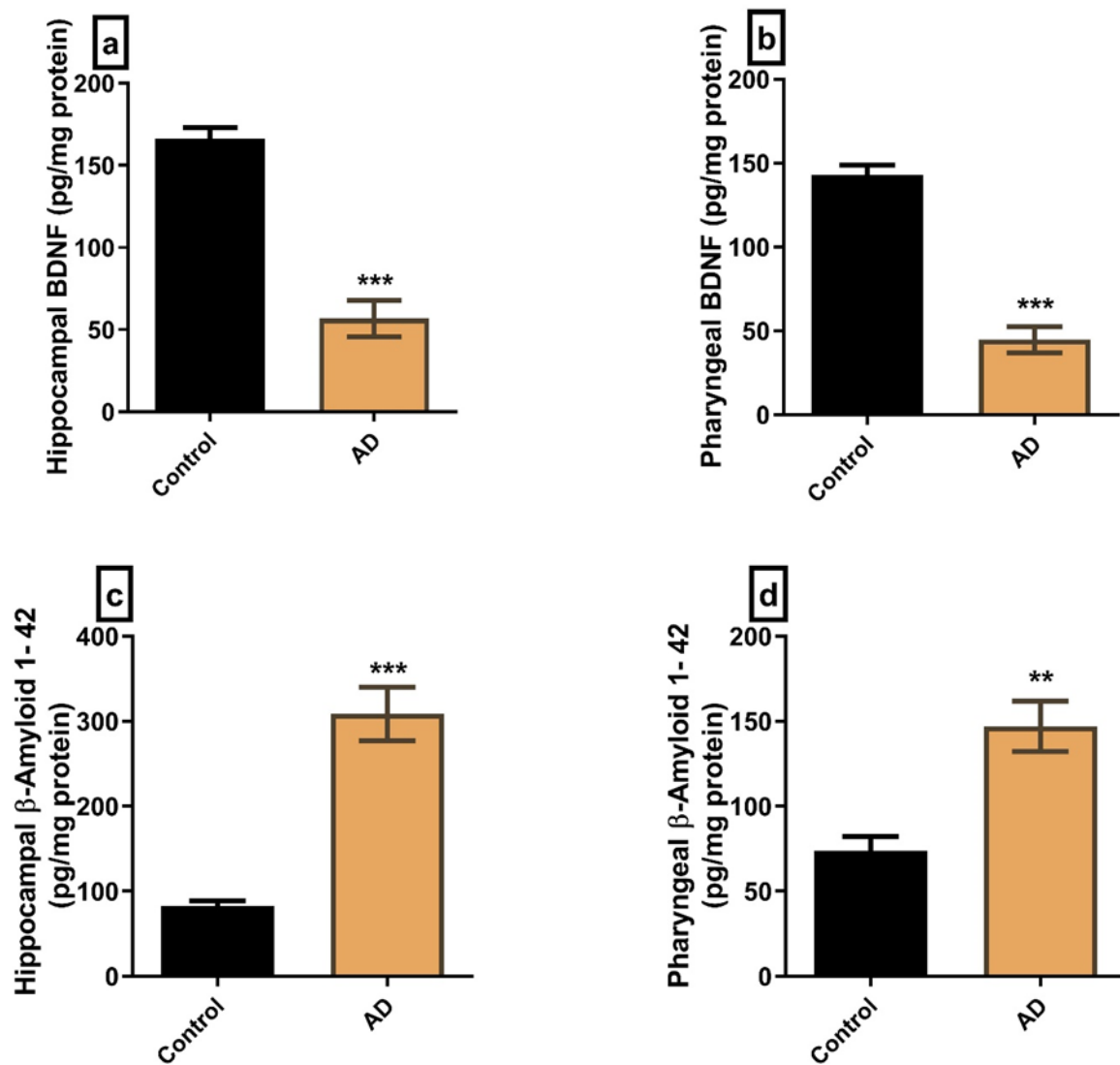
Fig.3 Effect of AlCl3/D-gal intoxication on BDNF and Aβ of the rats. BDNF levels in the hippocampus (a) & pharyngeal muscles (b); Aβ levels in the hippocampus (c) & pharyngeal muscles (d). *** Significantly different from the control group

3.4. Hematoxylin and eosin-stained sections

3.4.1. Hippocampus H&E-stained sections

The H&E-stained hippocampus sections of the control rats revealed the normal histological structure of the molecular layer, pyramidal cell layer, and polymorphic cell layer. (Fig.4A). On the other hand, hippocampus sections of AD rats showed histopathological disruption in the form of cellular disorganization of pyramidal cells that had neurofibrillary tangles confirming the induction of AD (Fig.4B).

### 3.4.2. 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 C.T. in between. Lingual myofibers appeared cylindrical, unbranched, striated, and multinucleated (Fig.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,edematous interstitial tissue, inter-/extra- myofibril hemorrhage were observed (Fig.4D).

3.4.3. 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 C.T. in propria-submucosa then circularly arranged pharyngeal muscles (Fig.4E). Pharyngeal muscles were formed of cylindrical, unbranched, striated myofibers that appeared multinucleated and separated from each other by intermuscular connective tissue (Fig. 4F). On the other hand, , oropharynx sections obtained from

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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 (Fig.4G).
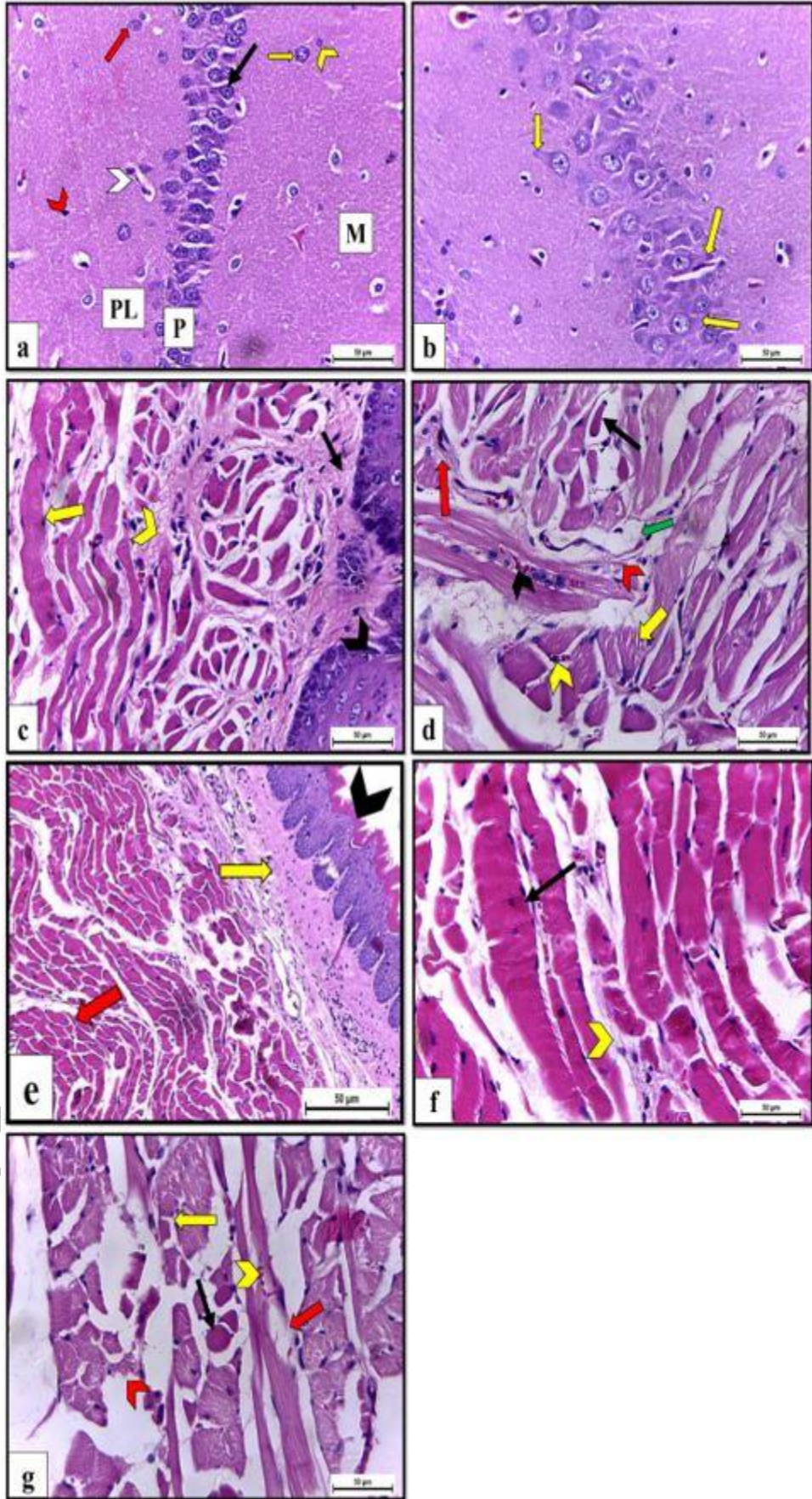
Fig.4 (a & b) Hippocampus sections of albino rats H&E stain X400. a: Control rats (Group Ⅰ) revealing the normal structure of molecular layer (M), pyramidal cell layer (P), and polymorphic layer (PL). The molecular layer contained neurons (yellow arrow) and neuroglia (yellow chevron), the pyramidal cell 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) presenting the normal structure of tongue covered by a mucous membrane (black chevron) that underlied by fibro-elastic C.T. (black arrow) and contained a central mass of skeletal muscles (yellow arrow) with intermuscular C.T. (yellow chevron). d: AD rats (Group Ⅱ) showing 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. There was hemorrhage surrounded the myofibers externally (yellow chevron) and between them (black chevron). (e: g) Oropharynx sections of albino rats H&E stain X400. e: Control rats (Group Ⅰ) revealing normal lining mucous membrane (black chevron) that was underlied by fibro-elastic C.T. (yellow arrow) then circularly arranged pharyngeal muscles (red arrow). f: normal cylindrical unbranched and striated multinucleated myofibers (black arrow) with intermuscular C.T. (yellow chevron). g: AD rats (Group Ⅱ) showing 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)

3.5. Immunohistochemical-stained sections

Immunohistochemical examination of the hippocampus of the control rats (group I) displayed a positive immunoexpression of the fibrillary astrocyte bodies and processes to GFAP. Meanwhile, the concentration of the immunoreactivity was significantly increased in the AD rats (group II) (Fig.5 A&B). The percentage area covered by GFAP-positive immunoreactive cells within the hippocampus revealed a significant elevation in the AD rats by 44.6 fold in comparison with the control rats (Fig.5C).
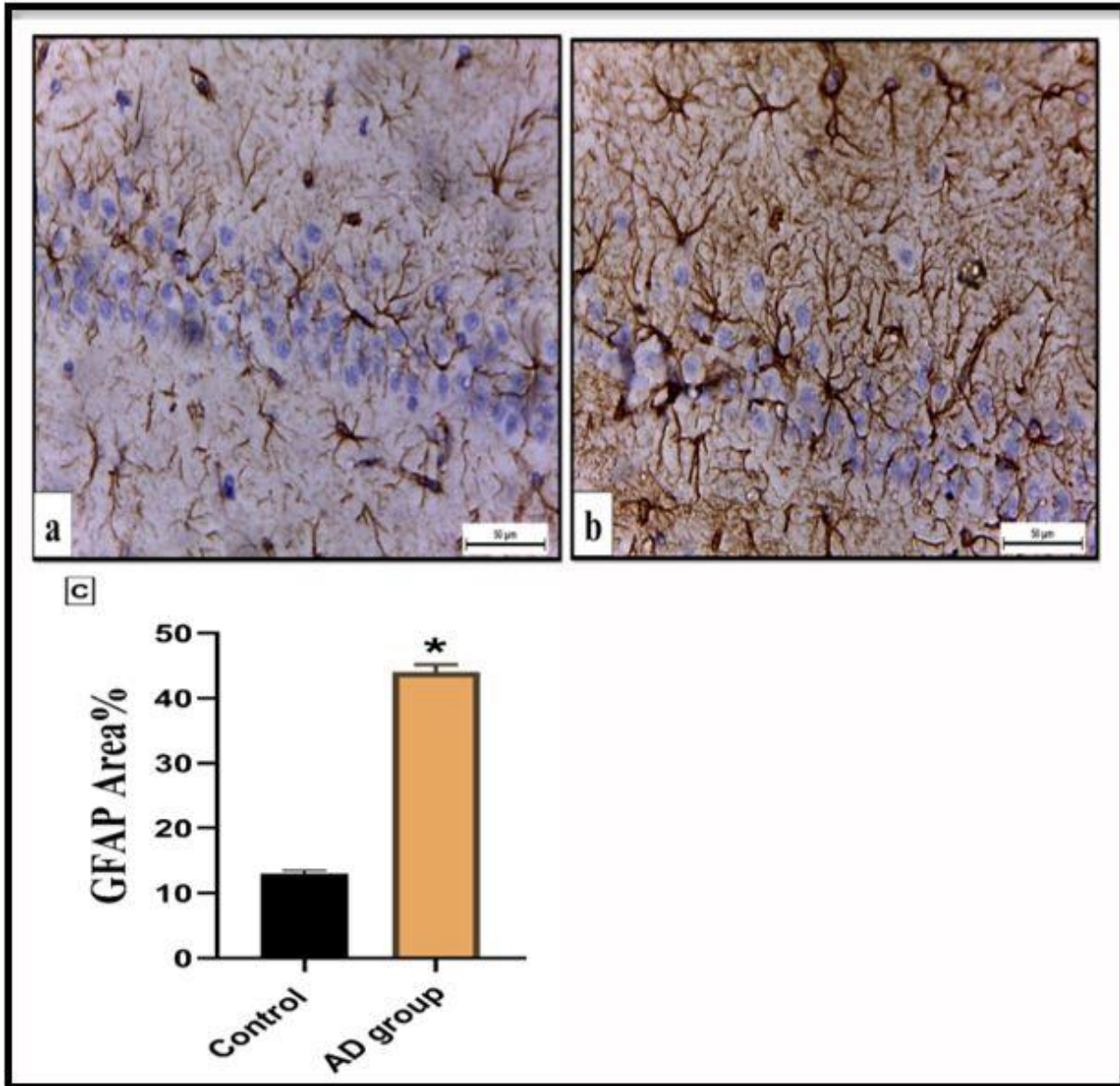
Fig.5 GFAP stained hippocampus sections (X400). a: Control rats (Group Ⅰ) revealing a positive reaction to GFAP. b: AD rats (Group Ⅱ) revealed an intensely positive reaction to GFAP. c: Bar graph represented a significant increase in the percentage area covered by GFAP-positive immunoreactive cells within the hippocampus of the AD group in comparison with the control group. Data are presented as mean values ± SE. Statistical significance level at $p \leq 0.05$. *Indicates significant difference from the control group at $p \leq 0.05$

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4. Discussion

There is an 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 Aβ 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 had 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.

In this study, AD was confirmed by the impairment of the working spatial memory and the recognition memory, the locomotor disturbance, and the significant increase of hippocampal Aβ 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 are 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 and Dickson (2019) as well as Ryan, Rossor and Fox (2015).

In our study, the development of dysphagia was hypothesized by the significant bodyweight loss observed in AD rats, as the eating and drinking pattern decreased by 25-35 % in AD versus 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 affection 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 tongue and pharyngeal muscles are 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 as well. AD pharyngeal muscles even exhibited a significant increase in Aβ levels, which could be explained theoretically by migration/spread of Aβ along cranial nerves e.g. 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. vagus nerve. Further research is still needed to examine these hypotheses.

5. Conclusion

Dysphagia in AD has been discussed to mainly originate from central cerebral affection. So far, there have not been any biochemical, histopathological, or immune-histochemical evidence of AD in 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

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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 quality of life 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 publication statement: We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

Conflicts of interest/Competing interests: The authors declare they have no conflict of interest.

Authors' contributions: The study was designed by Ramy Abdelnaby. Heba M. A. Khalil, Yasmine H. Ahmed, Dalia Zaafar, and Mohamed Y. Mahmoud collected and analyzed the data. Interpretation of data was carried out by Ramy Abdelnaby. Alexa Häger, Ramy Abdelnaby and Eman Mohammed Elsaeed prepared and revised the manuscript. All the authors approved the final version of the manuscript.

Abbreviations

Aβ, amyloid beta-peptide; AlCl3, Aluminum chloride; AD, Alzheimer's disease; BDNF, brain-derived neurotrophic factor; D-gal, D-galactose; DR, discrimination ratio; GFAP, Glial fibrillar acidic protein; MDA, malondialdehyde; NFTs, neurofibrillary tangles; NOR, Novel object

recognition; RI, recognition index; SAP, spontaneous alternation percentage; TAC, total antioxidant capacity.

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