

ORIGINAL ARTICLE

Evaluation of neural damage in Duchenne muscular dystrophy patients

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The presence of non-progressive cognitive impairment is recognized as a common feature in a substantial proportion of patients with Duchenne muscular dystrophy (DMD). Concurrently, the amyloid beta peptide ($A\beta_{42}$) protein has been associated with changes in memory and cognitive functions. Also, it has been shown that different subtypes of neural stem/progenitor cells (CD 34, CD 45, nestin) are involved in the innate repair of plasticity mechanisms by the injured brain, in which Nerve Growth Factor (NGF) acts as chemotactic agents to recruit such cells. Accordingly, the present study investigated levels of CD 34, CD 45, nestin and NGF in an attempt to investigate makers of neural regeneration in DMD. Neural damage was assayed in terms of $A\beta_{42}$. Results showed that $A\beta_{42}$ (21.9 ± 6.7 vs. 12.13 ± 4.5) was significantly increased among DMD patients compared to controls. NGF (165.8 ± 72 vs. 89.8 ± 35.9) and mononuclear cells expressing nestin (18.9 ± 6 vs. 9 ± 4), CD 45 (64 ± 5.4 vs. 53.3 ± 5.2) and CD34 (75 ± 6.2 vs. 60 ± 4.8) were significantly increased among DMD patients compared to controls. In conclusion cognitive function decline in DMD patients is associated with increased levels of $A\beta_{42}$, which is suggested to be the cause of brain damage in such patients. The significant increase plasma NFG and in the number of mononuclear cells bearing CD_{34, CD45} and nestin indicates that regeneration is an ongoing process in these patients. However, this regeneration cannot counterbalance the damage induced by dystrophine mutation and increased $A\beta_{42}$.

Key words: Duchenne muscular dystrophy, neural damage, cognitive function

Introduction

Duchenne muscular dystrophy (DMD) represents an X-linked recessive disorder related to mutations in the dystrophin gene which is located on chromosome Xp21.1 (1). It is one of the most common and severe form of dystrophinopathies, characterized by progressive and disabling muscle weakness affecting approximately 1 in 3000 to 4000 male births (2). The disease is character-

ized by ongoing degeneration and regeneration of skeletal muscle that leads to replacement of muscle by connective tissue and fat (3).

In addition to the profound skeletal muscle lesions, DMD is associated with mild to severe cognitive deficits and poor academic achievement, which are independent from the muscular handicap or clinical environment (4). Full-scale intelligence quotient (IQ) scores of DMD patients are distributed in accordance with the assumption that the cognitive defect results from the same mutations that cause myopathy (5). In fact, about one third of DMD boys have IQ scores below 70 and display mental retardation. Deficits affect both receptive and expressive language skills, with alterations in auditory comprehension, phonological knowledge and language, and delayed acquisition of reading, which has been partly attributed to a form of developmental dyslexia, that is, dysphonetic dyslexia (5). Impaired short- and long-term memory performances are consistently reported and include defective recall, working memory, memory span, and visuo-spatial skills (5, 6-8).

Amyloid beta peptide ($A\beta$) is a proteolytically processed fragment of the amyloid precursor protein (APP) (9). It occurs in different length variants with peptides of 40 amino acid residues ($A\beta_{40}$) and 42 amino acid residues ($A\beta_{42}$), the latter is the most prevalent. The accumulation $A\beta$ plaques is a key feature in the brains of Alzheimer Disease (AD) patients and is implicated in the disruption of normal cellular processes leading to neurodegeneration (10). $A\beta$ is secreted into the extracellular space allowing its detection in the CSF and plasma (11). Functional studies have demonstrated that oligomeric $A\beta$ species can impair long-term potentiation (LTP) and synaptic function in mature neurons (12). The magnitude of amyloid plaque deposition in the brain correlates poorly

with cognitive decline, and emerging evidence suggests that A β oligomers may be the major culprits in this regard (13).

NGF is a neurotrophin, shown to support the survival and differentiation of neurons during brain development (14), and reduces neural degeneration (15) and promotes peripheral nerve regeneration in rats (16). Lately, it has been shown that different subtypes of neural stem/progenitor cells respond differently to traumatic brain injury, which induces their activation reflecting the induction of innate repair and plasticity mechanisms by the injured brain (17, 18), where during such process nestin and CD34 expression increases and is serum level dependent (19, 20). It was reported that CD34 cells are present in DMD patients for tissue regeneration (21). It has been demonstrated that CD45 subset comprise juvenile protective factors for the maintenance of brain microvascular health (22).

During the last two decades, the role of dystrophin in the CNS has been investigated in DMD boys and the dystrophin deficient mdx mouse (model of DMD), and have demonstrated a range of abnormalities in CNS function, from behavioral and cognitive dysfunction to alterations in the clustering of ion channels in single identified neurons (23). Accordingly, this study was conducted in order to investigate markers related to neural damage and repair in DMD patients. The study investigated levels of CD 34, CD 45 and nestin in an attempt to investigate markers of regeneration in blood of DMD patients and degeneration in terms of A β ₄₂ in relation to IQ.

Subjects and methods

Subjects were 60 boys diagnosed clinically and at the molecular level as having DMD (mean of age (8.1 ± 1.9)), versus 20 age and socioeconomic matching healthy boys (mean of age (8.2 ± 2.2)). Patients and controls were chosen to be free from any infection and receiving no thera-

peutic treatment known to increase the oxidative stress. Blood samples were drawn after their parents' consent.

Biochemical Investigations

A β ₄₂

This was carried out using Amyloid Beta (A β) ELISA Kit (Millipore catalog number EZHS42 (24).

CD45, CD34 and Nestin Quantification

To quantify EPCs in circulation, peripheral mononuclear cells were first isolated from the blood samples (0.5 mM EDTA). The isolated cells were labeled with the phycoerythrin (PE)-conjugated monoclonal nestin antibody and Fluorescein isothiocyanate (FITC) conjugated CD34 (Macs). The stained cells were washed with phosphate buffered saline and /BSA and then analyzed by flow cytometry at the Faculty of Medicine, Cairo University (25).

Nerve Growth Factor

This is an enzyme-Linked immunosorbent assay, which employs an antibody specific for human for β -NGF coated on 96 well plate (26).

IQ

This was carried out using the Wechsler Intelligence Scale for Children third edition (WISC III): It provides scores for Verbal IQ, Performance IQ and Full Scale IQ (27).

Results

Results showed that A β ₄₂ (21.9 ± 6.7 vs. 12.13 ± 4.5) was significantly increased among DMD patients compared to controls (Table 1) and that it has a significant negative relation with IQ of the patients (Fig. 1). NGF

Table 1. Markers of neural damage among DMD compared to controls.

	DMD	Controls	t	P
Amyloid Beta Peptide 42	21.9 ± 6.7	12.13 ± 4.5	4.3	$P < 0.001$
Mean of IQ	74.8 ± 9.3	95.4 ± 10	10.9	$P < 0.00001$

Table 2. Markers of neural regeneration among DMD compared to controls.

	DMD	Controls	t	p
Nestin	18.9 ± 6	9 ± 4	12.3	$P < 0.0001$
CD34	75 ± 6.2	60 ± 4.8	9.1	$P < 0.0001$
CD45	64 ± 5.4	53.3 ± 5.2		$P < 0.001$
-NGF (pg/ml)	165.8 ± 72	89.8 ± 35.9	4.6	$P < 0.001$

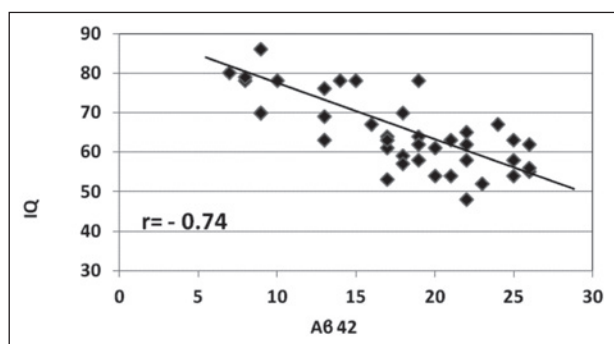


Figure 1. Correlation between $A\beta_{42}$ and IQ among DMD patients.

(165.8 ± 72 vs. 89.8 ± 35.9) and mononuclear cells expressing nestin (18.9 ± 6 vs. 9 ± 4), CD 45 (64 ± 5.4 vs. 53.3 ± 5.2) and CD34 (75 ± 6.2 vs. 60 ± 4.8) were significantly increased among DMD patients (Table 2).

Discussion

Results of the present study showed that $A\beta_{42}$ was significantly higher among DMD patients compared to controls and that a significant negative correlation exist between $A\beta_{42}$ and IQ of such patients. Data regarding levels of $A\beta_{42}$ in DMD are null. However, it has been shown that in patients carrying mutations predicted to affect dystrophin isoforms expressed in the brain, are associated with higher risk of cognitive impairment (28) and since $A\beta_{42}$ has been shown to be associated with cognitive function impairment, the present study assumed that $A\beta_{42}$ levels might be increased in DMD patients compared to controls. Supporting this assumption is that: a direct relation between the deposition of insoluble $A\beta_{42}$ after traumatic brain injury and the changes in brain interstitial fluid $A\beta$ levels has been reported, where the disruption of the blood brain barrier has been shown to play an important role in the pathogenesis of epilepsy (29). Partial or generalized epilepsy has been reported in DMD (30). Also the mdx mice were shown to be susceptible to seizure among administration of convulsing drugs (31) and brain edema and severe alterations of the glial and endothelial cells have recently been demonstrated in such mice (32).

Our recent finding that $A\beta_{42}$ was significantly higher among Down syndrome (DS) patients compared to controls (20 ± 5.1 vs. 11.9 ± 3.4) (33) provides further proof that mental retardation is associated with increased levels of $A\beta_{42}$ in blood and gives clue that DMD mental retardation is associated with increased levels of $A\beta_{42}$. Previous studies have shown that individuals with DS have increased levels of $A\beta_{40}$ and $A\beta_{42}$ peptides in plas-

ma together with increased risk for Alzheimer's disease (AD), neuropathology and clinical dementia (34-38).

In recent years there has been a substantial increase in the understanding of the role of dystrophin in the CNS. These studies have been largely carried out on DMD boys and the dystrophin deficient mdx mouse and have demonstrated a range of abnormalities in CNS function, from behavioral and cognitive dysfunction to alterations in the clustering of ion channels in single identified neurons (39). Dystrophin is considered the central component of a scaffold of proteins expressed in a variety of tissues including the brain, where it is involved in the clustering of several membrane receptors and ion channels and in the modulation of cellular signal integration and synaptic plasticity (30). Normally, in the cerebellum, dystrophin appears to play a role in normal neuronal function or development. Two carboxy-terminal dystrophin proteins (Dp), Dp71 and Dp140, are both expressed in the brain, in addition to full-length central nervous system dystrophins, and are initiated between exons 62 and 63, and upstream from exon 44, respectively (40-42). Rearrangements in the second part of the dystrophin gene tend to be more commonly associated with cognitive impairment, and several reports described mutations in the Dp71 coding region as a factor that contributes to the severity of mental retardation (42-44). It is suggested that a lack of the Dp140 isoform is thought to play a significant role in cognitive performances in Duchenne muscular dystrophy (45, 46) and mutations involving the Dp71 region are often associated with severe cognitive impairment (47, 48).

Putative alterations of the brain vascular permeability have been suggested by some studies, which may also participate to behavioral deficits in mdx mice (31). Initial observations of mdx brains revealed severe alterations of endothelial cells with open tight junctions surrounded by swollen glial processes and enhanced vascular permeability suggesting brain blood barrier (BBB) breakdown (48). Follow-up studies suggested that this results partially from hypoxic condition leading to the activation of hypoxia inducible factor-1 α contributing to both BBB opening and compensatory angiogenesis, along with changes in expression of matrix metalloproteinases, nerve and vascular growth factors (32). Hence, the hypothesis that a progressive decline in respiratory function due to muscle degeneration, could worsen the brain and cognitive impairments in advanced DMD patients through a reduction in cerebral oxygenation and BBB disruption (49).

NFG was significantly higher in blood of DMD compared to controls in the present study. Although, NFG in blood of DMD studies are scarce, a previous study has shown by means of immunohistochemistry, that regenerating muscle fibers from DMD patients consistently

express NGF, as do myofibroblasts and mast cells (50). By contrast, rest fibers from dystrophic patients, as well as muscle fibers from healthy, control patients and even regenerative muscle fibers in polymyositis do not show NGF immunoreactivity (51, 52). Supporting this finding is a study carried out on mdx dystrophic mouse that demonstrated, by western blotting and real time polymerase chain reaction (RT-PCR), a higher expression of NGF and its receptor mRNA and protein in mdx brain as compared to controls (53). NFG was markedly elevated in the male mdx mouse at 8 and 11 weeks of age (54).

In the present study the numbers of mononuclear cells bearing CD 45, CD34 and nestin markers were significantly increased compared to controls, indicating that regeneration is an ongoing process in DMD patients. It can be expected that CD34 cells are present in DMD patients for tissue regeneration (21), but their capacity for muscle regeneration is hindered. CD34 is also important for vascular repair, and in rat model for traumatic brain injury (TBI). CD34 has been shown to be mobilized from the bone marrow to peripheral blood and brain tissue, a process critical for vascular repair (55). The recruitment of hematopoietic progenitor cells from the bone marrow into the peripheral blood after acute ischemic stroke when no thrombolytic treatment was given was identified in human studies, suggesting that increased progenitor cell recruitment might be caused by so far unknown signaling stimuli of the ischemic brain for stem cell mobilization (19).

Results of the present study showed that mean of mononuclear cell expressing nestin surface marker was significantly higher among DMD patients compared to control. An in vitro previous study, reported that nestin was found specifically in myopathic muscle fibers in Duchenne/Becker muscular dystrophy and myositis but was absent in controls (56). Nestin-Cre/DG null mice have been shown to exhibit earlier and more widespread disruptions of neuronal migration and developed hydrocephalus (57). Nestin has been also shown to play an important role in remodeling and repairing in the postnatal and adult central nervous system in rat models (58) and that a subset of neural progenitors bearing nestin becomes active after injury and can compensate for the injury-induced loss of granular neurons (59). The present study cannot confirm, whether the increased expressing nestin surface marker in circulating blood is due to muscle damage or brain damage in DMD.

Mononuclear cells expressing CD45 was significantly increased among DMD patients compared to controls. Cells expressing CD45 are regarded as muscle regenerating cells (60, 61). Their number increases in the presence of muscle damage (61, 62). It has been demonstrated that CD45 subset comprise juvenile protective factors

whose quantitative and qualitative normalization can attenuate the progression of ischemic-hemorrhagic stroke pathogenesis in rat model likely through the maintenance of brain microvascular health (22). It has been demonstrated that genetic loss of CD45 (1) accelerates cerebral amyloidosis (2), causes brain accumulation of soluble oligomeric A β species and reduction in plasma-soluble A β (3), promotes proinflammatory and anti-A β phagocytic microglial activation (4), and leads to mitochondrial dysfunction and neuronal loss in *mice model of Alzheimer Disease* (63).

In conclusion cognitive function decline in DMD patients is associated with increased levels in A β ₄₂, which is suggested to be the cause of brain damage in such patients. The significant increase plasma NFG and in the number of mononuclear cells bearing CD_{34, CD45} and nestin indicates that regeneration is an ongoing process in these patients. However, this regeneration cannot counterbalance the damage induced by dystrophine mutation

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