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Original Research

A potential therapeutic effect of miR-155 downregulation in an experimental model of demyelination

Anti microRNA-155 in demyelination model

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Abstract
Aim: The aim of this study was to investigate the possible effects of microRNA-155 inhibition in restoring remyelination after the establishment of lysolecithin-mediated demyelination in rats.
Material and Methods: Adult male albino rats were subjected to intrahippocampal injection of lysolecithin (LPC). LPC-treated rats received either antimicroRNA-155 NPs, blank NPs or phosphate buffer saline (PBS). One additional group was injected with PBS, acting as a negative control. After scarification of rats, histopathological examination of hippocampi, and quantitative PCR to detect mir-155 and myelin basic protein (MBP) were conducted.
Results: Our results showed that delayed treatment with antimicroRNA-155 nanoparticles was associated with histopathological improvement of demyelination score with no significant effect on biochemical markers.
Discussion: This study supports the promising therapeutic role of antimicroRNA-155-loaded nanoparticles in demyelinating disorders.

Keywords
Demyelination, miRNA 155, Multiple Sclerosis

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Introduction

Many demyelinating diseases affect the central nervous system (CNS) with heterogeneous etiologies, varying from metabolic, infectious, or autoimmune processes to genetic disorders. With an estimated 3 million patients globally, multiple sclerosis (MS) is the most common of these illnesses. Focal regions or demyelination plaques in the CNS, which are surrounded by areas of inflammation and neurodegeneration, are the pathological hallmarks of all types of MS [1]. More than 50% of MS patients, chiefly those with progressive disease, will experience depression, decline in cognitive functions, and gait disability [2]. MicroRNAs (miRNAs) are small ~22 nucleotide RNA sequences that control genetic expression and can significantly impact the important cellular processes related to CNS repair after neuropathological conditions such as ischemic stroke and neurodegenerative diseases [3]. Numerous miRNAs have been strongly associated with different pathological conditions of CNS. Among them is microRNA-155, a multifunctional microRNA associated with the regulation of various physiological and pathological processes. miRNA-155 plays a remarkable role in the immune system and its augmented expression is consistent with bad prognosis in patients with epilepsy and amyotrophic lateral sclerosis [4,5]. In several animal models, miR-155 down regulation was accompanied by declined inflammation and improved regeneration processes. At the same time, miRNA-155 upregulation was noticed in experimental models of stroke, while downregulation of miRNA-155 reinforced post-stroke recovery [6,7].

One of the main difficulties of microRNA-based therapeutic strategies is to achieve accurate, healthy and fruitful regulation of miRNAs. Although oral administration of microRNA inhibitors (or mimics) is ineffective, intravenous and subcutaneous delivery of oligonucleotides is also difficult due to their toxicity and limited bioavailability. Nanoparticles, viral vectors, and biodegradable polymers are used for effective administration of miRNA [8]. At the present time, Poly lactic-co-glycolic acid (PLGA) is considered one of the most employed synthetic polymers. Thanks to its biocompatible, biodegradable and simple functionality, PLGA NPs were chosen as a carrier device in this study [9]. In the current work, the efficacy of delayed treatment with antimir-155 loaded nanoparticles in enhancing remyelination process in lysolecithin-mediated demyelination model was studied.

Material and Methods

1. Material

Poly (D, L-lactide-co-glycolide) lactide: glycolide (50:50), mol wt 30,000-60,000 and L-α-Lysophosphatidylcholine from egg yolk (LPC) ≥99%, Type I, powder, protease inhibitor cocktail and β-actin antibody were purchased from Sigma Aldrich (St. Louis, MO, USA). Anti-rno-miR-155-5p miScript miRNA Inhibitor and negative control and miRNeasy Mini Kit were purchased from Qiagen (Hilden, Germany). TaqMan miRNA Reverse Transcription Kit, TaqMan microRNA assay system, TaqMan® Universal PCR Master Mix II, SuperScript II Reverse Transcriptase, 1XSYBR® Green PCR Master Mix.

2. Preparation of antimir-155 loaded nanoparticles

PLGA nanoparticles were formed using emulsion technique followed by incubation of 100 µL of the nanoparticles solution with 700 µL of polyethylenimine aqueous solution for 15 minutes [10]. Then the nanoparticles suspension was mixed with the antimiRNA solution and incubated for 30 minutes at room temperature to form nanoparticle/antimiRNA complexes. The loading efficiency of the miR-155 inhibitor on the nanoparticles was analyzed using UV-Vis Spectrophotometer NanoDrop (Thermo Scientific, USA) [11].

3. Experimental protocol

3.1 Experimental animals

This study was carried out on adult male Wistar rats (12 weeks old) weighing 200–250 g in the animal house of the Medical Physiology department Faculty of Medicine, Alexandria University. The animals were kept under standard laboratory conditions, maintained under a 12-h light–dark cycle with free access to food and water. All animal experiments complied with the Guide for the Care and Use of Laboratory Animals, Faculty of Medicine, Alexandria University (IRB NO: 00007555-FWA NO: 00018699). Further, the Ethics Committee of Alexandria Faculty of Medicine approved this study.

3.2 Stereotaxic surgery

For induction of demyelination, fifteen rats were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg) mixture via intraperitoneal injection. The cranium was exposed and a dental lab drill was used to drill a hole to the dorsal hippocampus (AP = −3.8 mm; ML = −2 mm; DV = −3 mm) according to the stereotactic coordinates of Paxinos and Watson's rat brain atlas [12]. Then, 1 µl of freshly prepared lysolecithin (LPC) in phosphate-buffered saline (PBS; ph 7.4) (1%) was injected bilaterally into the hippocampus of all rats [13]. One additional group was injected with an equivalent volume of PBS only into the same site as described above as a negative control. LPC injected groups were subdivided into 3 subgroups (five rats per group): the LPC group (untreated group), in which rats received bilateral intrahippocampal injection of 2 µl of phosphate buffer solution (PBS), blank NPs group, in which rats received 2 µl of blank nanoparticles and antimir-155 NPs group, in which rats received 2 µl of antimir-155 nanoparticles on day 7 after LPC injection. The syringe was left in situ for an additional 2 min before withdrawal to prevent solution leakage from backflow.

3.3 Tissue sampling and processing

Three days after the administration of the nanoparticles, the animals were sacrificed using ether anesthesia. After decapitation and craniotomy, the whole brain was removed and washed with ice-cold saline and the hippocampi were dissected. One hippocampus was fixed in formalin for histopathological examination, and the other one was stored at -80°C for molecular analysis.

3.4 Histopathological examination using hematoxylin & eosin (H&E)

Hippocampal samples were fixed overnight at 4 °C with 4% paraformaldehyde, and specimens were placed in a 10% neutral formalin solution, processed and embedded in paraffin for histological examination. Coronal sections (5 µm thick) were cut with microtome, and stained with hematoxylins & eosin (H&E) [14].

3.5 Assessment of demyelination score using Luxol fast blue (LFB) stain

Demyelination was assessed using LFB staining and scored as described: 0 = Normal, 1 = One small focal area of demyelination, 2 = 2-3 areas, 3 = 1-2 large areas of demyelination and 4 = Extensive demyelination involving >=20% of the white matter [13]. The extent of demyelination, as the ratio of lesion size per total area, was determined using and analyzed using a Leica Application Suite, Version 4.12.0 (Leica Microsystems CMS GmbH) image analysis system [14].

3.6 Quantitative reverse transcription PCR (qRT PCR) for expression of miR-155

TaqMan microRNA assay system was used for quantification of miRNA -155 using TaqMan® Universal PCR Master Mix II (Applied Biosystems, USA). Amplification was performed in a real-time PCR system StepOne (Applied Biosystems, USA). StepOne™ Software v2.3 was used for data analysis [15].

3.7 Quantitative reverse transcription PCR (qRT PCR) for expression of Myelin Basic Protein (MBP)

Reverse transcription was done using 100 ng of total RNA. The amplification of MBP cDNA was done in duplicate using a real-time PCR system StepOne using 1XSYBR® Green PCR Master Mix. Data analysis was performed using StepOne™ Software v2.3 using the comparative CT method for gene expression relative to the housekeeping gene GAPDH [15].

Statistical analysis

IBM SPSS software package version 20.0. (Armonk, NY: IBM Corp) was used for data analysis. Quantitative data were described using the mean and Standard Error of Mean (SEM), and the significance of the obtained results was judged at the 5% level. F-test (ANOVA) was used for normally distributed quantitative variables, to compare between more than two groups, and Post-Hoc test (Tukey) for pairwise comparisons.

Results

Anti-microRNA-155/ nanoparticles loading efficiency

To ensure the incorporation of anti-miRNA into the nanoparticles, the loading efficiency of anti-miRNA was measured as the percentage of adsorbed anti-miRNA to the total amount of anti-miRNA added and it was found to be around 70 %.

Effect of antimiR-155-NPs on hippocampal tissue using H&E stain.

Histological examination of the brain exhibited the normal architecture of the hippocampus in the control group. In contrast, the hippocampal tissue in LPC group revealed the presence of hippocampal and subcortical inflammatory plaques and infiltration by mononuclear inflammatory cells. On the other hand, antimir-155 treated group showed hippocampal and subcortical healed remyelinated lesions (Figure 1).

Effect of antimiR-155- NPs on the demyelination score.

Microscopic examination showed normal myelination of the hippocampi in control group. Hippocampal tissue in LPC group revealed the presence of hippocampal and subcortical demyelinated plaques (mean demyelination score 1.33, mean extent of demyelination area 9%) with a significantly higher demyelination score compared to the negative control group. However, antimiR-155 NPs treated group showed tiny hippocampal and subcortical remyelinated patches (mean demyelination score 0, mean extent of demyelination area 2%). The demyelination score was significantly lower compared to LPC groups and with no significant difference compared to the control groups (Figure 2).

Effect of antimiR-155-NPs on tissue expression of miRNA-155. Although the expression of miRNA-155 in hippocampal tissue was 1.95 ± 0.18 fold change in LPC untreated group and decreased to 1.59 ± 0.24 fold change in antimir-155 NP group, which received the treatment with antimir-155 nanoparticles after the establishment of demyelination, there was no significant difference in the levels of miRNA-155 expression in the hippocampal tissue in the treated group when compared to LPC group (Figure 3A).

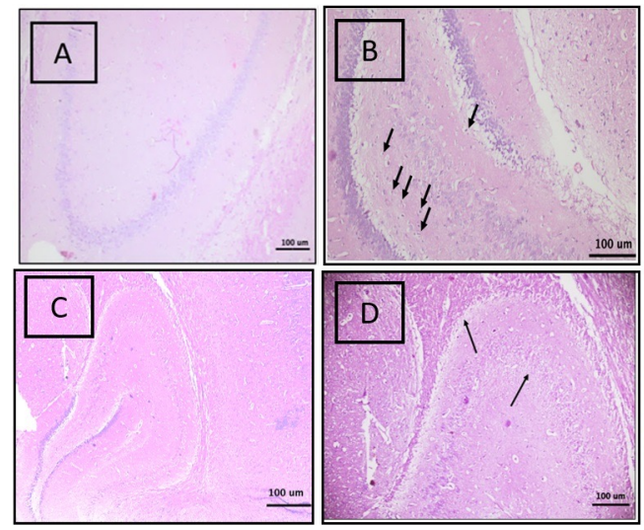

Figure 1. Light micrograph of hippocampal tissue sections stained with H&E in (A) control group showing a normal histological appearance with absence of demyelination. (B) A high power view of LPC rat hippocampus showing mild rarefaction in the white matter (arrows) with inflammatory infiltration, (C,D) A high power view of a antimir-155 NP rat hippocampus showing healed patches (arrows) with absence of white matter rarefaction.

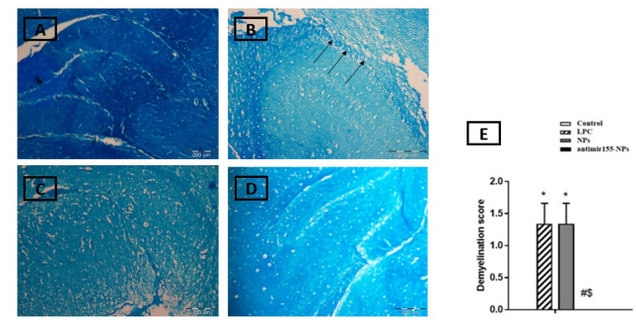

Figure 2. Light micrograph of hippocampal tissue sections stained with luxol fast blue (LFB) in (A) control group showing normal hippocampal myelination, (B) LPC group showing area of demyelination (arrows), (C) NPs group, (D) antimir-155 NP group showing completely remyelinated white matter with absence of pale blue areas. (E) Quantitative analysis and statistics of demyelination scores. Values are mean ± SEM from n = 3/group. Significant post hoc test is indicated by asterisks * p ≤0.05 versus control group, # p ≤0.05 versus LPC group, $ p ≤0.05 versus NPs group.

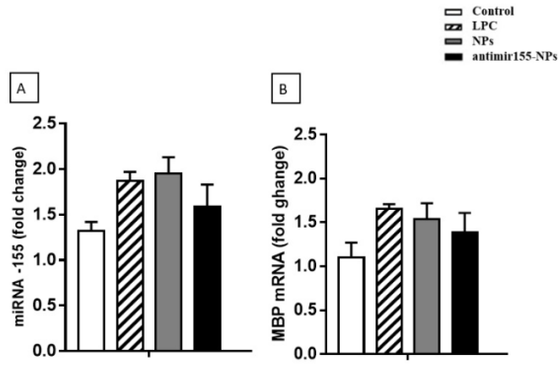

Figure 3. Effect of anti-miR-155 loaded nanoparticles treatment on microRNA-155 expression (fold change) (A) and MBP mRNA expression (fold change) (B) in the hippocampal tissue homogenate in different studied groups using RT- qPCR. Values are expressed as mean ± SEM. Significant post hoc test is indicated by asterisks * p ≤0.05 versus control group, # p ≤0.05 versus LPC group, $ p ≤0.05 versus NPs group.

Effect of antimiR-155-NPs on tissue expression of myelin basic protein (MBP)

There was no statistically significant difference between MBP mRNA expression in all groups when compared to control group. Delayed treatment with antimir-155 NPs after establishment of demyelination did not lead to any significant change in MBP mRNA expression in the treated group when compared to the LPC group (Figure 3B).

Discussion

Despite major progress in different treatment modalities of MS, to date, immunotherapy cannot sufficiently prevent progression of clinical disability. In addition, currently available treatments for MS do not facilitate remyelination nor improve axonal repair in the central nervous system. There is strong evidence from disease models that upregulation of miRNA-155 leads to neuroinflammation and eventual neurodegeneration, while silencing of miRNA-155 has neuroprotective impact making the modulation of its expression an important target for the treatment of demyelinating disorders [4]. In this work, lysophosphatidylcholine (LPC) was chosen to induce toxic hippocampal demyelination due to its accurate temporal regulation, the definite anatomical location, and the consistency of LPC-induced demyelination [16]. In the current research, histopathological examination of the hippocampi revealed the presence of demyelinating lesions in the rats following stereotaxic injection of LPC. These histological findings are similar to the pathological features of LPC-induced demyelinated lesions, previously described [17].

Currently, one of the methods used to attenuate miRNA activity is administration of anti-miR antisense oligonucleotides into cells. These anti-miR molecules can be delivered to the cells using either viral or non-viral vectors. While viral vectors previously used to deliver genes in injured animal models of diseases showed evidence of toxicity and immunogenicity, non-viral vectors, mainly nanoparticles delivery systems, showed lower toxicity, lower immunoresponsiveness and easy handling properties [18]. In the present study, PLGA nanoparticles were chosen since they offer several advantages such as biodegradability, biocompatibility, low cytotoxicity, long-standing biomedical applications and targeted delivery [19]. A loading efficiency of about 70 % was achieved in this study, which is consistent with previous studies that support PLGA nanoparticles as encouraging non-viral vectors in miRNA-related diseases such as tumors and neurodegenerative diseases [20,21].

Previous studies on experimental demyelination models such as experimental encephalomyelitis revealed that microRNA-155 knockout mice had a delayed onset, reduced disease severity and less CNS inflammation [22]. In addition, other researchers found that downregulation of microRNA 155 resulted in prolongation of survival in ALS-model mice [4]. In the present study, although delayed injection of anti-mir-155 nanoparticles after establishment of demyelination decreased the level of expression of microRNA-155, it did not significantly affect the level of microRNA-155 expression when compared to the control or untreated groups. These results can be due to the spontaneous remyelination process, which occurs in LPC demyelination model, in which extensive demyelination is followed by robust remyelination [16]. The results of this study support previous researche, which proved that normal levels of microRNA-155 expression are essential for efficient remyelination process. Also, this finding may highlight the importance of the time factor in the protocol of intervention during treatment of demyelination via inhibition of miRNA-155. Interestingly, the administration of antimir-155 nanoparticles was associated with significant histopathological improvement, which was evident by more compact hippocampal myelin structure and significantly decreased demyelination score when compared to the untreated group.

Myelin basic protein (MBP) is one of the most abundant myelin proteins in CNS myelin sheaths and is expressed in both immature and mature oligodendrocytes [23]. Oligodendrocytes are the myelinating cells of the CNS corresponding to the Schwann cells in the peripheral nervous system. Oligodendrocyte progenitor cells (OPCs) originally come during development from neural stem cells inside the ventricles in the brain and spinal cord. The results of this study showed increased levels of MBP up to normal levels in all groups compared to the control group. Also, delayed treatment with anitmir-155 nanoparticles did not significantly affect the level of myelin basic protein expression compared to the LPC group. Similar findings were observed in previous studies, which can be explained by the migration of oligodendrocyte precursor cells to the lesion site during remyelination phase and then their differentiation to mature oligodendrocytes. Also, the MBP expression is affected by the age of myelinating cells; hence, the presence of younger cells in the area of remyelination is associated with a higher level of MBP expression [24, 25].

In conclusion, although delayed treatment with antimir155 nanoparticles after induction of demyelination was not associated with a significant improvement in biochemical markers, there was evident histopathological improvement that deserves further studies to investigate the different treatment strategies via modulation of microRNA-155 expression.

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Scientific Responsibility Statement
The authors declare that they are responsible for the article's scientific content including study design, data collection, analysis and interpretation, writing, some of the main line, or all of the preparation and scientific review of the contents and approval of the final version of the article.

Animal and human rights statement
All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. No animal or human studies were carried out by the authors for this article.

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Conflict of interest
None of the authors received any type of financial support that could be considered potential conflict of interest regarding the manuscript or its submission.

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