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ORIGINAL ARTICLE

Hydroxychloroquine effects on miR-155-3p and miR-219 expression changes in animal model of multiple sclerosis

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Abstract

Multiple sclerosis (MS) is a chronic inflammatory disease of the central nervous system which causes chronic demyelination. Hydroxychloroquine (HCQ) possess immunosuppressive and anti-inflammatory properties. The aim of this study was to investigate the effect of HCQ on miR-219 and miR-155-3p expression changes in MS-induced model. The animal model was induced by the administration of cuprizone containing food pellets (0.2%). Briefly, C57BL/6 mice were randomly divided into five groups. Group 1 received normal food and water during the study. Group 2 received cuprizone pellets for 5 weeks (demyelination phase) following one-week normal feeding during the remyelination phase. The remaining three groups received HCQ (2.5, 10 and 100 mg/kg) via drinking water during the demyelination phase. At the end of each phase, mice were deeply anesthetized, perfused with PBS through the heart, and their brains were removed. Brain sections stained with luxol fast blue and the images were analyzed. Also, the expression levels of miR-219 and miR-155-3p were evaluated by quantitative Real-Time PCR in all samples. HCQ decreased the expression of miR-155-3p and increased miR-219 expression in animals treated with 100 mg/kg of HCQ compared to the control group ($p < 0.0001$) and the cuprizone group ($p < 0.0001$). LFB method revealed a gradual increment of myelination in animals treated with 10 and 100 mg/kg of HCQ compared to the cuprizone group. Based on the obtained results of this study, HCQ can decrease microglial activity and increase oligodendrocye production by altering the expression of disease-associated miRNAs.

Keywords miR-155-3p · miR-219 · Hydroxychloroquine · Multiple sclerosis · Myelination · Cuprizone

Introduction

Multiple sclerosis (MS) is an autoimmune chronic inflammatory disease of the central nervous system (CNS) which causes demyelination and axonal loss (Herz et al. 2010; Aliomrani et al. 2017). Oligodendrocyte cells are the main myelin-producing cells in the CNS and it is obvious that a loss of myelin occurs if the oligodendrocyte precursor cells (OPCs) do not differentiate into the mature oligodendrocytes (Dhib-Jalbut 2007). Recent immunology studies demonstrate that T cells, especially T helper type1 (Th1) and T helper type17 (Th17) cells, play an important role in the development of MS (Kebir et al. 2007; Sałkowska et al. 2020). In response to injury, infection, or inflammation, Th1 and Th17 cells can increase microglia activity (González et al. 2014; Holley and Kielian 2012). Activated microglia can produce various pro-inflammatory cytokines, including interleukin IL-1, IL-6, and tumor necrosis factor-α, as well as potentially neurotoxic compounds (Gottschall et al. 1995; Harry et al. 2002; Pinteaux et al. 2002; McPherson et al. 2011). Microglia also

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accelerate the process of disease by preventing the differentiation of OPCs into oligodendrocytes (Luo et al. 2017). Thus reducing microglial activation and increasing the differentiation of OPCs into oligodendrocytes are considered to be an effective therapeutic approach to inhibit the progression of MS (Domingues et al. 2016).

MicroRNAs (miRs) are a class of small single-stranded non-coding RNAs (~22 nucleotides) that regulate gene expression of mRNAs by including either translational repression or mRNA degradation. In recent years, many studies have shown the role of miRNAs in regulating numerous genes and pathways in the pathogenesis of inflammatory and autoimmune diseases such as MS (Fan et al. 2017).

In particular, miR-219 has been reported as a promoter of oligodendrocyte differentiation by targeting negative repressors of oligodendrocyte development such as PDGFRα, Sox6, Hes5, Foxj3, and Zfp238, all of which normally help promote OPC proliferation (Shin et al. 2009; Fan et al. 2017). These data indicate that the increment expression of miR-219 has a potential therapeutic role in demyelinating diseases such as MS.

Opposed to the miR-219, miR-155-3p increases the production of pro-inflammatory mediators, including NO, TNF-α, IL-1, and IL-6 by microglial activation, which targeted SOCS1 signaling pathways (Zheng et al. 2018). Also, miR-155-3p can activate Th17 cells by targeting the genes producing the proteins including Dnaja1 and Dnaja2 (Mycko et al. 2015). As mentioned, decreasing expression of miR-155-3p can prevent of microglial activation and the progression of MS. An antimalarial drug, Hydroxychloroquine (HCQ), used in autoimmune diseases such as systemic lupus erythematosus and rheumatoid arthritis because of its immunomodulatory effects (Ben-Zvi et al. 2012).

Although the mode of this drug action is uncertain, it has been shown that HCQ treatment can reduce the production of pro-inflammatory cytokines including, TNF- α, IL-1, and IL-6 by downregulating the expression of miR-155-3p (Castro-Villegas et al. 2015). Also, it was suggested that HCQ treatment could affect microglia cells and/or oligodendrocytes activity (Koch et al. 2015).

In the present study, we investigated the possible involvement of miR-219 and miR-155-3p expression changes in MS and the effect of HCQ exposure via drinking water on myelination during cuprizone induced animal model.

Materials and methods

Animals

C57BL/6 mice were purchased from the Royan institute (Isfahan, I.R. Iran). All mice were used following the Guidelines for the Care and Use of Laboratory Animals and housed in individually ventilated cages under standard conditions of 12-h light/dark photoperiod and controlled humidity with *ad libitum* access to food and water. Changes in mice's weight, water, and food intake were measured weekly in each group. All procedures were approved by the Iran National Committee for Ethics in Biomedical Research (IR.MUI.RESEARCH.REC.1397.388) which was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals.

Experimental design

Forty-six C57BL/6 mice aged 6–8 weeks and weighing 18–22 g were randomly divided into five groups. Mice were fed with cuprizone (Sigma-Aldrich, USA) containing pellets 0.2% (w/w) for 5 weeks to induce demyelination phase of MS model in the corpus callosum followed by feeding with normal pellets for one additional week (remyelination phase). Briefly, Group 1 (negative control, n = 05) received normal food pellets during the study and served as a healthy group. Group 2 composed was performed to evaluate the effect of cuprizone administration on demyelination after 5 weeks (the first group sacrificed at this point) and remyelination (another group) after one week without any particular treatment. Group 3–5 received 2.5, 10, and 100 mg/kg/BW HCQ respectively via drinking water at the first 5 weeks of cuprizone exposure (Gómez-Guzmán et al. 2014; Capel et al. 2015; Virdis et al. 2015). In each week, the drug concentration in the water was adjusted to maintain the dose. All groups composed of two subgroups of 5 animals each (n = 02 × 05). The first subgroup sacrificed at the end of the demyelination phase and the second subgroup was fed with normal food pellets and tap water for one additional week.

Cardiac perfusion

At the end of each phase, mice were deeply anesthetized with ketamine/xylazine (80/10 mg/kg) and were transcardially perfused with 5 mL phosphate-buffered saline (PBS) followed by 4% neutral formalin (5 ml). At the end of perfusion, the brain tissues were immediately removed and were cut coronally. The anterior part of the midbrain fixed in cold formalin (overnight) and the posterior part of the samples were stored in -70˚C for checking the miRNAs expression (Tao-Cheng et al. 2007).

Tissue processing

Samples were placed in a tissue processor (Sakura Tissue Tek VIP E150, Sakura, Japan) and dehydrated in an ethanol series followed by a xylene clearing step to replace trapped alcohols inside the tissue. After that, the samples embedded in hot

paraffin and sections were cut serially on a RM 2255 microtome (Reichert S, Leica, USA) with a thickness of 8 μm from the mid part of the brain (de Santana Nunes et al. 2012).

Luxol fast blue staining

Myelin was detected using luxol fast blue (LFB, Sigma, USA). Samples were deparaffined and then transferred through 95% ethanol to a 0.1% solution of LFB in 95% ethanol and 0.05% acetic acid and incubated for 24 h at 60 °C. The next day, stained sections were rinsed in 95% ethanol followed by distilled water to remove the excess blue stain. The blue color was trapped in myelinated white matter after placed slides in 0.05% aqueous lithium carbonate for 20 s. The slides were washed with 70% ethanol for 5 min. The sections were observed under an inverted microscope (Zeiss MicroImaging GmbH, Germany). Myelinated fibers were visualized with blue in this technique (Mohammadi-Rad et al. 2019).

Imaging and myelin quantification

Images were taken using Nikon Eclipse 55i light microscope (Nikon, Japan) with a 4 × objective magnification and were processed with Fiji ImageJ software (National Institute of Health, Bethesda, Maryland, USA). To evaluate myelin changes at first background was subtracted from images and then colors were separated through a defined region of interest using the color deconvolution plugin. The density of all samples corpus callosum was quantified three times with the equation below:

$$Myelin\ \ percent = 1 - \frac{(X1 - Xs)}{X1} \times 100$$

where X1 is the mean of measured integrity density of normal control and Xs is the same region of interest integrated density of each sample (Ruifrok and Johnston 2001).

RNA extraction

Total RNA was extracted from the brain samples to evaluate the expression of miR- 219 and miR-155-3p. About 60 mg of frozen tissue was homogenized by RNX-plus solution (Sinaclone, I.R. Iran) according to the manufacturer's instruction and incubated at room temperature for 5 min, then 200 ml chloroform added to each tube. After Centrifugation at 12,000 × g for 15 min at 4 °C, the supernatant was transferred into a new tube with the addition of 3 ml isopropanol. After centrifugation at 12,000 × g for 15 min at 4 °C, the RNA pellet washed with70% ethanol and suspended with DEPC water. The concentration of RNA was quantified by UV spectrum measurement of $OD_{260/280}$ nm (Sun et al. 2009).

cDNA synthesis and Quantitive Real-Time PCR

Reverse transcription of RNA was performed according to the manufacturer's instruction (Biomir, I.R. Iran) by adding 1 μg of total RNA, 1.5 ml RT stem-loop, 1.5 ml stem-loop (control group), and DEPC water incubated at 70 °C for 5 min and immediately cooled on ice. Then, each component was added in the indicated order 4 μl of (5×) first strand RT buffer, 1 μl dNTP, and 0.5 μl RT enzymes and total samples were amplified in the thermocycler (BioRad, USA) at 37 °C for 60 min and 70 °C for 5 min (Rachagani et al. 2010).

The expression levels of miRNA-219 and miRNA-155-3p were measured for treated and control groups according to the Biomir recommended protocol. The qPCR reactions were set up by the denaturation step of 10 min at 95 °C, followed by 40 cycles of amplification, which were performed according to the following thermocycling profiles: denaturation for 10 s at 95 °C and annealing and development for 40 s at 60 °C. Fluorescence data were acquired during the last step. Dissociation protocol with a gradient (0.5 °C every 30 s) from 65 to 95 °C was used to investigate the specificity of the qPCR reaction and the presence of primer dimers. Finally, the $2^{-\Delta\Delta Ct}$ method was applied for the relative gene expression of selected microRNAs (Matoušková et al. 2014).

Statistical analysis

LFB data are expressed as a Means±standard deviation (SD). Differences between the groups were analyzed by one-way analysis of variance (ANOVA) test, followed by Bonferroni's Multiple Comparison post-test using GraphPad Prism software ver 8 (Graphpad Software, Inc., San Diego, CA). Spearman correlation test was used to determine the relationship between measured variables. Expression data were analyzed with two-way ANOVA using Tukey's multiple comparisons post-hoc-test. P values ≤ 0.05 were considered statistically significant.

Results

The clinical analysis showed that the cuprizone group had the signs of disease induction including reduced physical activity, and abnormal walking and posture. The group treated with 2.5 mg/kg of HCQ exhibited no clinical improvement, but the animals treated with 10 and 100 mg/kg of HCQ exhibited normal walking and posture.

The body weights of the mice were recorded weekly to evaluate the effect of HCQ on mice with cuprizone-induced demyelination. During the first two weeks after the beginning of the study, the cuprizone group and the group treated with 2.5 mg/kg HCQ lost their body weight as compared with the control group and the animals treated with 100 mg/kg of HCQ

($p < 0.05$). However, all groups gained a gradual weight during the 6 weeks of the study and a positive correlation between time and weight gain was observed (r, 0.75–0.93) as shown in Fig. 1a. The violin plots as shown in Fig. 1b and c demonstrate the differences in the probability distribution of the food and water intake in cuprizone-treated animals and the control group. The results of food and water intake indicated no significant difference among all of the groups of animals.

To confirm the difference in the extent of demyelination within the corpus callosum, luxol fast blue (LFB) stained images of brain sections was performed at the end of 5 weeks cuprizone feeding following one-week normal feeding as shown in Fig. 2a and i. The animals treated with cuprizone exhibited a significant decrease in myelin density of corpus callosum in comparison with the control group after 5 weeks of exposure to cuprizone, however, lower demyelination was observed after one-week normal feeding (Fig. 2a and c). Treatment with 2.5 mg/kg of HCQ did not alter the density of white matter fibers of the corpus callosum in relation to the cuprizone group (Fig. 2d and e). However, in the animals treated with 10 mg/kg of HCQ, remyelination was observed (Fig. 2f,g) and in the animals treated with 100 mg/kg of HCQ, LFB staining was similar to that found in the control group (Fig. 2h and i).

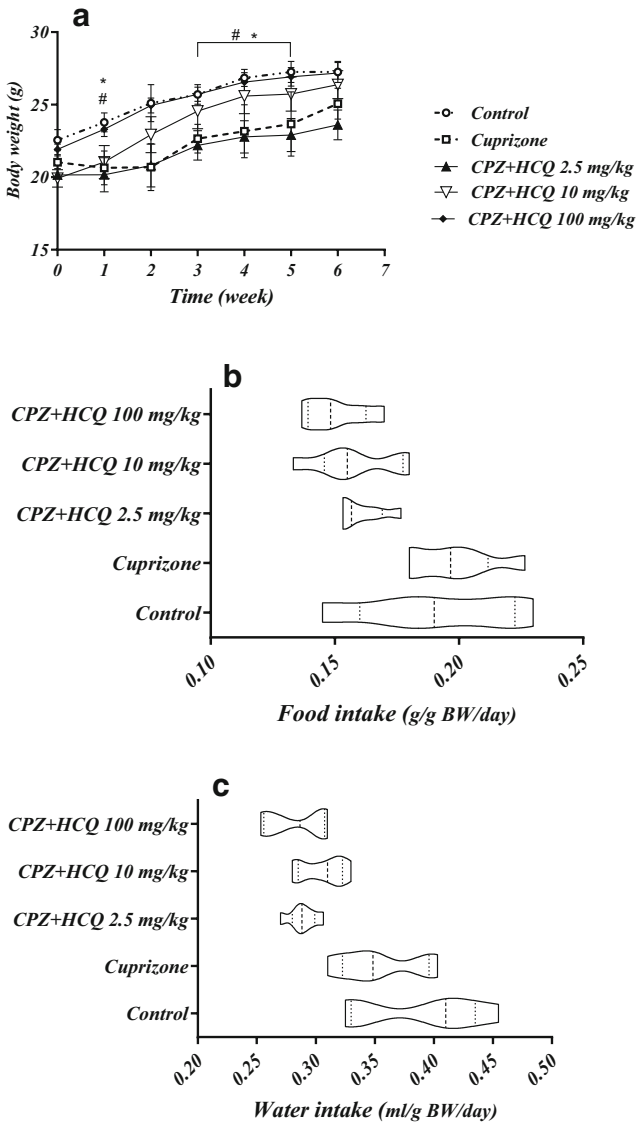

Fig. 1 Effect of HCQ (2.5, 10, and 100 mg/kg in drinking water) administration during and after cuprizone exposure on (**a**) body weight, (**b**) food, and (**c**) water intake of C57BL/6 mice multiple sclerosis model. Control: animals that received normal food and water, cuprizone (CPZ): animals that received normal water and cuprizone food. * shows the significant difference ($p < 0.05$) between the group treated with 2.5 mg/kg of HCQ and the control group. # indicates the significant difference ($p < 0.05$) between the animals treated with 2.5 and 100 mg/kg of HCQ

The min to max plot as shown in Fig. 3a revealed the differences in the distribution of the myelin percent in cuprizone-treated animals and the control group. Statistical comparison of all groups of animals showed a significant decrease in the myelination intensity of median corpus callosum compared with the control group ($P < 0.0001$). However, treatment with 100 mg/kg of HCQ indicated no significant difference as compared to the control group. After 5 weeks of cuprizone feeding (demyelination phase), a significant increment of myelination intensity was observed in the animals treated with 10 and 100 mg/kg of HCQ as compared to the cuprizone group and the group treated with 2.5 mg/kg of HCQ ($p < 0.0001$) as shown in Fig. 3b. Treatment with 100 mg/kg of HCQ improved the remyelination and decreased myelinated axons in comparison with the cuprizone group in the remyelination phase ($p < 0.05$).

The expression level of miR-219 in demyelination and remyelination phase for each group is shown in Fig. 4. The reduction of miR-219 expression was observed in the cuprizone group in de- and remyelination phase as compared to the control group. A significant increment of miR-219 expression was observed in the animals treated with 100 mg/kg of HCQ when compared with the control group in the demyelination phase ($p < 0.0001$) and remyelination phase ($p < 0.001$). There is no significant difference between the cuprizone group and the group treated with 2.5 mg/kg of HCQ.

The significant increment of miR-155-3p expression was observed in the cuprizone group in the demyelination phase as compared to the control group ($p < 0.001$) as shown in Fig. 5. Moreover, the animals treated with 10 and 100 mg/kg of HCQ showed a significant reduction of miR-155-3p expression as compared with the control. A significant difference between all treated groups and the cuprizone group was observed ($p < 0.0001$).

Discussion

The present study evaluated the administration of HCQ on corpus callosum neuroinflammation and demyelination in

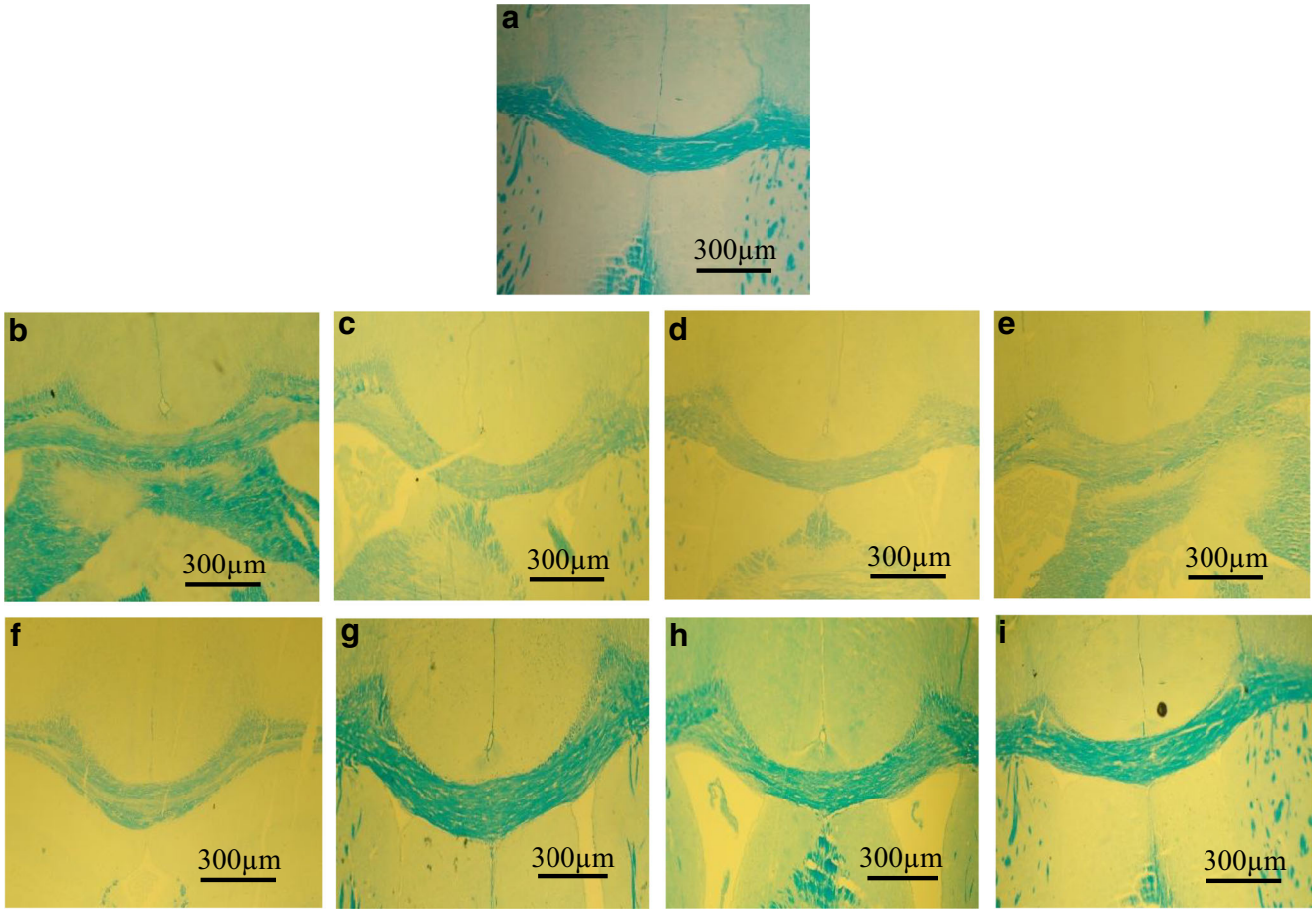

Fig. 2 Effect of HCQ (2.5, 10, and 100 mg/kg, in drinking water) treatment on cuprizone-induced demyelination after 5 weeks (demyelination phase) or one week of remyelination (remyelination phase). In comparison with the (**a**) control group, significant myelin loss shows in the cuprizone group at the end of the demyelination phase (**b**) and remyelination phase (**c**). (**d**) indicates the animals treated with 2.5 mg/kg of HCQ at the end of the demyelination phase. (**e**) indicates the animals treated with 2.5 mg/kg of HCQ at the end of the remyelination phase. Significant restoration of myelin was observed in animals treated with 10 mg/kg of HCQ at the end of the demyelination phase (**f**) and remyelination phase (**g**). The animals treated with 100 mg/kg of HCQ in the demyelination phase (**h**) and remyelination phase (**i**) exhibited uniform myelin organization and LFB staining intensity similar to the control group

MS mice model induced by cuprizone. We have demonstrated that HCQ altered the level of microglia and oligodendrocytes by modifying the expression of miR-219 and miR-155-3p.

Cuprizone, a copper-chelating agent, is widely used to study demyelination and remyelination by damaging mitochondrial function and decreasing oligodendrocyte production in the corpus callosum (Skripuletz et al. 2008). In the prior studies, demyelination in mice fed with 0.2% cuprizone was observed from the second week of treatment and after 5 weeks (phase I), when cuprizone treatment was stopped, the process of demyelination ceased and allowing myelination to return (Phase II) (Lindner et al. 2008; Gudi et al. 2014). Hiremath et al.. confirmed several crucial points for this model, such as the age of the animal (8–10 weeks), the dosage of cuprizone in pellets, and the duration of treatment (5–6 weeks) (Hiremath et al. 1998). In this study, behavioral deficiencies and weight loss were observed in the cuprizone-treated animals compared with the control animals after 2 weeks of cuprizone treatment, a finding later confirmed by Morell et al. (1998).

In this study, we used three doses of HCQ (2.5, 10, and 100 mg/kg) in drinking water to evaluate the effect of HCQ in myelination and expression of miR-219 and miR-155-3p. The recent studies on the experimental autoimmune encephalomyelitis (EAE) animal model show that HCQ treatment can reduce the production of pro-inflammatory cytokines such as TNF-α and IL-6 by inactivating macrophages and lymphocytes (Jang et al. 2006). The same protecting effects against TNF-α secretion was observed in LPS stimulated RAW 267 macrophage cells treated with chloroquine (Jeong and Jue 1997). Also, HCQ has an anti-proliferative effect and prevents protein synthesis by incorporating DNA and RNA polymerase (Chafin et al. 2013). Importantly, HCQ can accumulate in tissues including the brain and it might be effective in the

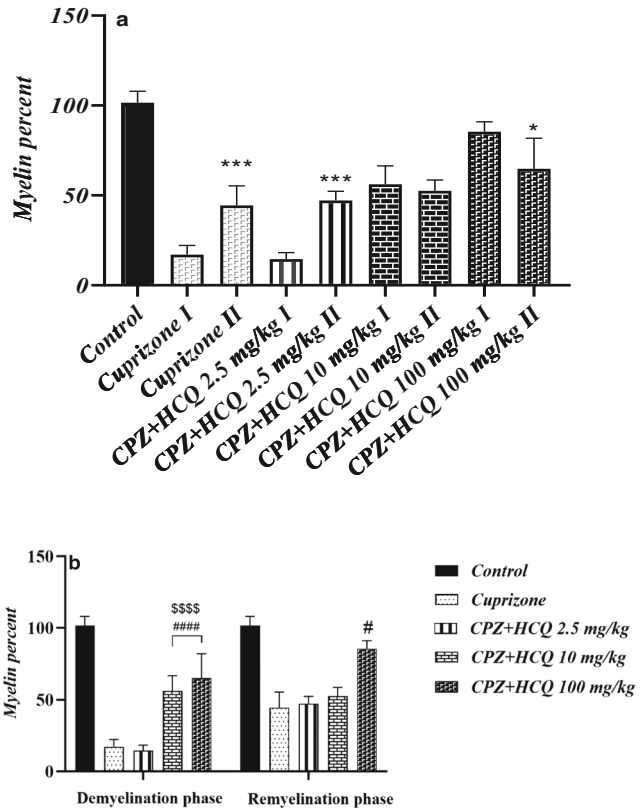

Fig. 3 Effect of HCQ (2.5, 10, and 100 mg/kg, in drinking water) treatment on myelin percent after 5 weeks (demyelination phase) or one week of remyelination (remyelination phase). Control: animals that received normal food and water, cuprizone (CPZ): animals that received normal water and cuprizone food. Data expressed as mean ± SD. * shows a significant difference between phase I and II (*$p < 0.05$, and *** $p < 0.001$). # indicate significant differences compared with the group treated with 2.5 mg/kg of HCQ ($^{\#}p < 0.05$, $^{\#\#\#\#}p < 0.0001$). $shows a significant difference in comparison with the cuprizone group ($^{\$\$}P < 0.0001$)

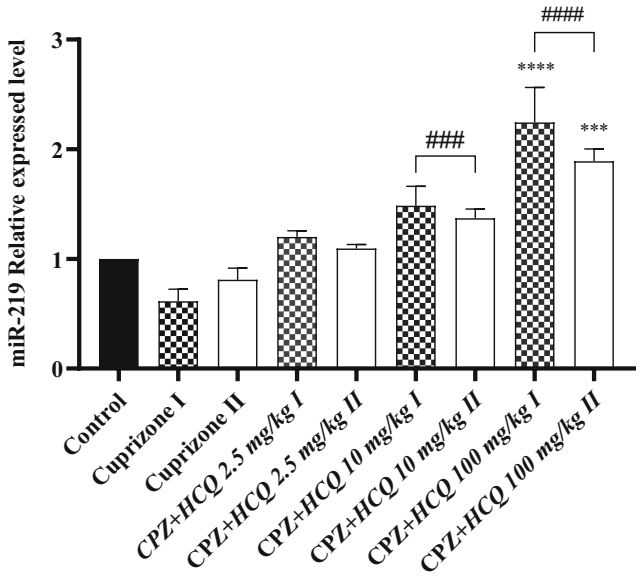

Fig. 4 The expression level of miR-219. Control: animals that received normal food and water, cuprizone (CPZ): animals that received normal water and cuprizone food. Data represented as mean ± SD. *** $p < 0.001$, **** $p < 0.0001$ indicate significant differences in comparison with the control group. $^{\#\#\#}p < 0.001$, $^{\#\#\#\#}p < 0.0001$ show significant differences compared with the cuprizone control in demyelination phase

treatment of MS since the blood-brain barrier is always been a difficult barrier to achieve sufficient drugs level (Browning 2014).

We observed that the animals treated with 2.5 mg/kg of HCQ exhibited weight loss and abnormal walking and posture similar to the cuprizone group during the second week after the beginning of the study. We hypothesized that HCQ may affect the appetite of mice during cuprizone-feeding and reduce consumption in CPZ + HCQ groups and evaluated the animals' food intake. Our results demonstrated that the food consumption in the mice was not changed by HCQ during 5 weeks of cuprizone feeding. In addition, the water intake was not significantly changed in all the groups treated with and without HCQ as a supportive study Shukla et al. (2015).

LFB staining was performed to obtain the myelination intensity in the median corpus callosum (Vincze et al. 2008). Our LFB results show that treatment with 100 mg/kg of HCQ did not reveal a significant decrease in the myelination intensity of median corpus callosum compared with the control group, thus, this dosage of HCQ can be a good candidate for the treatment of MS. In addition, there is no significant difference between the group treated with 2.5 mg/kg of HCQ and

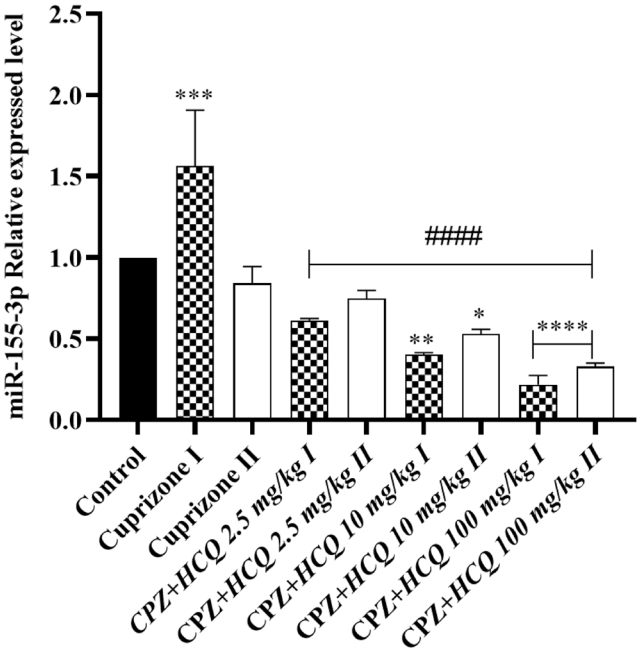

Fig. 5 The expression level of miR-155-3p. Control: animals that received normal food and water, cuprizone (CPZ): animals that received normal water and cuprizone food. Data represented as mean ± SD. * $p < 0.05$, ** $p < 0.005$, *** $p < 0.001$, **** $p < 0.0001$ indicate significant differences in comparison with the control group. $^{\#\#\#\#}P < 0.0001$ shows significant differences between all animal groups and the cuprizone group

cuprizone group. Our results is in accordance with the previous published study about suggesting the protecting effects of HCQ on progression phase in multiple sclerosis EAE model (Faissner et al. 2018).

Recent studies illustrated that several miRNAs have been able to differentiate oligodendrocytes such as miR-219, miR-29, miR138, miR-23, and miR-388 (Shin et al. 2009; Cui et al. 2012). In addition, some miRNAs help to activation of microglia including miR-155 and miR-221 (Lu et al. 2011). It was suggested that miR-219 expression related to the oligodendrocytes maturation and acts as a potential biomarker of multiple sclerosis progression (Bruinsma et al. 2017). *In vitro* and *in vivo* studies demonstrate the role of miR-219 in promoting oligodendrocyte differentiation and myelination by targeting the negative repressors of oligodendrocyte development such as PDGFRα, Sox6, Hes5, Foxj3, and Zfp238 (Dugas et al. 2010; Hudish et al. 2013). It was observed that the increment of miR-219 and miR-338 expression could increase oligodendrocytes production (Zhao et al. 2010; Wang et al. 2017). In another study, proved that miR-219 differentiates oligodendrocyte and decreases demyelination in the cuprizone model induced by regulating the expression of MCT1 (Liu et al. 2017). According to these studies, miR-219 up-regulation leads to differentiate OPCs to oligodendrocytes and help the myelination process. Our study shows that treatment with 100 mg/kg of HCQ significantly increases miR-219 expression in the corpus callosum.

Our results indicate that HCQ treatment decreases miR-155-3p expression in the white matter of corpus callosum. Overexpressed miR-155 has been reported in the white matter lesions of the brain tissue of MS patients (Noorbakhsh et al. 2011). Mir-155 has been shown to regulate the genes producing Dnaja1 and Dnaja2 to activate Th17 cells (Mycko et al. 2015). The previous study shows that Th17 cells increase microglial activation by releasing INF-γ (Jadidi-Niaragh and Mirshafiey 2011). Microglia reacts to injury to protect the neurons in the CNS and activated microglia cause neurogenesis (Nikolakopoulou et al. 2013). However, chronically activated microglia produce free radicals, cytokines, and mediators lead to myelin degeneration (Liu et al. 2013). Inhibition of miR-155 and 155-3p inhibited activation of microglia and decreased the production of pro-inflammatory cytokines and mediators such as NO, TNF-α, IL-1, and IL-6 from the active microglia as research by Zheng et al. (2018). According to these findings, miR-155-3p down-regulation supress microglia cells activation so promote the myelination process. Our study shows that treatment with 10 and 100 mg/kg of HCQ significantly decreases miR-155-3p expression in the corpus callosum.

Conclusion

In summary, HCQ treatment enhances myelination process by targeting miR-155-3p down-regulation more than miR-219 up-regulation following exposure to cuprizone in the corpus callosum of mice. This finding explains the critical role of HCQ in oligodendrocyte differentiation by regulating the expression of miR-155-3p and miR-219. These pharmacological properties of HCQ make it a suitable candidate to use in multiple sclerosis patients.

Author contribution FM and MAO conceived of the presented idea, FM and MM carried out the experiment, SME and MAO helped supervise the project, all authors provided critical feedback and helped shape the research, analysis and manuscript.

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Compliance with ethical standards

Conflicts of interest/Competing interests All authors declare no conflict of interest.

Ethics approval All procedures were approved by the Iran National Committee for Ethics in Biomedical Research (IR.MUI.RESEARCH.REC.1397.388) which was performed in accordance with the Guidelines for the Care and Use of Laboratory Animals.

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