

In vitro and *In vivo* neuroprotective study of solid lipid nanoparticles loaded with dimethyl fumarate

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Abstract

Introduction: Dimethyl fumarate (DMF) is a FDA approved oral medication used to treat patients with multiple sclerosis (MS). Biological half-life of DMF is about 1 h, and it is rapidly hydrolyzed to its active metabolite monomethyl fumarate. The objective of present study is to formulate and characterize DMF loaded solid lipid nanoparticles (SLNs) to overcome these limitations. **Material and Methods:** DMF loaded SLNs were prepared by hot microemulsion and sonication method. Stearic acid was used as lipid and soy lecithin was colipid. **Results and Discussion:** The synthesized SLNs offered an average particle size of 300 nm, polydispersity index <0.3, and zeta potential value of -34.89 mv. *In vitro* evaluation on human neuroblastoma SH-SY5Y cells confirmed to have a neuroprotective effect of these colloidal systems under oxidative stress. DMF loaded SLNs showed to increase cell viability under H₂O₂ induced cell apoptosis. Cuprizone mouse model for MS was used to evaluate the effect of DMF loaded SLNs on locomotion score. **Conclusion:** *In vitro* study also confirmed a better locomotion and motor coordination scores in cuprizone mouse model for MS.

Key words: Cuprizone model, dimethyl fumarate, multiple sclerosis, SH-SY5Y neuroblast cells

INTRODUCTION

The neurodegenerative process with damage to axons and oligodendrocytes is thought to be the cause of permanent neurological impairment and disability; this is a key feature of the disease multiple sclerosis (MS).^[1,2] At present, most available MS therapies are thought to exert their effects through immunomodulatory or immunosuppressive functions.^[3-5] Although these treatments are effective at inhibiting immune cell-driven inflammation and reducing the relapse rate, they are ineffective at controlling the predominantly neurodegenerative processes that occur later in the disease course.^[6] Fumaric acid esters have been used since 1959 as a treatment for psoriasis.^[7] Dimethyl fumarate (DMF) is currently approved by FDA as a first-line treatment for lowering relapse rates in MS.^[8-11] DMF and monomethyl fumarate were able to activate the transcription factor nuclear factor-erythroid 2-related factor 2 pathways and subsequently induce the expression of antioxidant proteins.^[12,13] Oxidative stress is one of the major factors in the pathogenesis of MS and is readily apparent within experimental autoimmune

encephalomyelitis, a mouse model of MS, and also in MS lesions.^[14] In recent years, many research has proved that new nanotechnology can be applied for the treatment and diagnosis of variety of immune-mediated diseases like MS. Solid lipid nanoparticles (SLNs) are a novel, nanocolloidal, biocompatible drug delivery systems with improved bioavailability and drug payload. These nanocolloidal systems have been recently explored for targeting central nervous system (CNS) and various neurological disorders. Taking cognizance to the challenges for neurological disorders, patient compliance, and enhanced efficacy, it was envisioned to formulate DMF loaded SLNs in an attempt to overcome these concerns. Further, the developed colloidal system was evaluated *in vitro* in human neuroblastoma cells and an established cuprizone animal model.

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MATERIALS AND METHODS

Materials

Stearic acid (m.p. 69.9 C), soy lecithin, tween 80, 1-butanol all are obtained from Chemsworth chemicals, Surat and DMF purchased from Alfa Aesar, a Johnson Matthey company. Dulbecco's Modified Eagle Medium, Ham's F12 (DMEM/HF12), fetal bovine serum (FBS), DMSO, 3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide (MTT), and Cuprizone were obtained from Aakaar biotechnology Pvt., Ltd., Lucknow. Other chemicals were used only in analytical grade.

Methods

Preparation of SLNs

Hot microemulsion method was used to develop SLNs with slight modifications.^[15] Accurately measured quantity of Stearic acid was heated until the entire lipid melts completely on water bath at about 75°C. To this molten lipid, an another colipid soy lecithin was added. Tween 80 (as per formula) was dissolved in distilled water separately. Aqueous phase was also maintained at 75°C. The lipid phase (1 ml) was added to the aqueous phase, and after each addition, the hot emulsion was vortexed at 1200 rpm for about 5–10 min or till clarity of solution. To remove turbidity sample was sonicated for sufficient time at 75°C. The above clear dispersion was poured into distilled water kept at 2–5°C. The ratio of lipid microemulsion to cold water was kept as 1:20.^[16]

Characterization of optimized batch of DMF loaded SLN

Average particle size and zeta potential

The optimized batch of DMF loaded SLNs was characterized for its average particle size, polydispersity index (PDI), zeta potential, % entrapment efficiency (EE), and % cumulative drug release. Particle size, PDI and zeta potential were measured with a Photon correlation spectroscopy DelsaNano C (Beckman Coulter Counter, USA) particle size analyzer. The samples of formulated SLNs were placed in a disposable cuvettes for average size and zeta potential measurement. The SLNs were dispersed in an appropriate volume of HPLC grade water at 25°C, at a detection angle of 90° for measuring the average size and PDI and an angle of 120° for its zeta potential measurement.

Percent EE

The % EE was determined as the per previously reported procedures. Optimized batch of DMF loaded SLNs was separated from its colloidal dispersion by ultracentrifugation at 12000 rpm for 1 h. DMF content in the supernatant was

analyzed with ultraviolet (UV)-visible spectrophotometer at 208 nm. % EE was calculated using the formula given below.^[17,18]

(% EE = Total amount of DMF added-Free DMF in supernatant/Total amount of DMF added * 100)

In vitro drug release

Profile

In vitro drug release study of the DMF loaded SLNs was carried out with the help of an equilibrium dialysis process at 37 ± 1°C.^[16,19,20] Accurate amount of SLNs (equivalent to 1 mg DMF) was suspended in 5 mL phosphate buffer solution (PBS) having pH 7.4, and this suspension was placed in a dialysis membrane bag. The dialysis membrane bag with DMF loaded SLNs suspension was then placed in 500 mL PBS and agitated at a speed of 50 rpm with an attached stirrer. Throughout dissolution experiment, sink condition was maintained, and at regular time intervals, 5 mL of the aliquots were withdrawn. The volume of dissolving media was made constant by replacing an equal volume of fresh PBS after each withdrawal. To separate colloidal nanoparticles, the collected aliquots were then centrifuged at 12000 rpm. The supernatant was analyzed with the help of UV-visible spectrophotometer at 208 nm to calculate the % cumulative DMF release.

Cell culture and MTT assay

In vitro neuroprotective effect was determined by MTT assay.^[21] SH-SY5Y human neuroblast cells were obtained from CDRI, Lucknow, and seeded in 96 well plate at a density of 5000 cells/well. The cells were allowed to grow for 24 h in DMEM/HF12 medium containing 10% FBS and antibiotic/actinomycin solution (1%).

Determination of optimum concentration of H₂O₂

To study the effect of DMF loaded SLNs on SH-SY5Y cells H₂O₂ was used to induce cell apoptosis.^[22,23] Optimum concentration of H₂O₂ which was able to produce 60% cell death was selected. Cytotoxicity of H₂O₂ toward SH-SY5Y was tested with freshly prepared H₂O₂ at concentrations 0, 50, 100, 200, 250, and 300 μM.

Determination of neuroprotective activity

To observe and evaluate neuroprotective effect of DMF loaded SLNs, cells were treated with different concentrations of DMF loaded SLNs for 24 h. Thereafter, cells were treated with H₂O₂ (250 μM) and incubated for another 24 h followed by addition of MTT solution (5 mg/ml in PBS) in each well. The plate was allowed to stand for 4 h, and the formazan crystals formed inside the viable cells were solubilized in DMSO, and the absorbance was measured at 570 nm using a microplate reader.^[24]

In vivo studies

The optimized batch of DMF loaded SLNs was selected for *in vivo* studies. *In vivo* experiments were carried out as per the protocol of Institutional Animal Ethical Committee. Wistar rats, which were present in the institutional animal house, were randomly selected and placed under set environmental conditions, i.e., $22 \pm 2^\circ\text{C}$ temperature and 12 h light/dark cycles. The rats weighing 150–200 g were then grouped into three groups ($n = 18$) as shown in Table 1. In various literature, the range of cuprizone dose was between 0.4 and 2% in powdered rat chow.^[25-27] In the present study, 1% cuprizone dose was selected and was given after mixing with powdered rat chow for 4 weeks to develop the disease. The treatment group received DMF loaded SLNs from very 1st day at a dose equivalent to 50 mg/kg of DMF along with the same amount of cuprizone toxin. All the experiments were carried out in daylight.

Locomotor activity

The locomotor activity can be referred as a movement from one location to other and is involved in many behavioral and physiological functions. This activity can be studied using actophotometer and open field apparatus. Animals from each group ($n = 6$) were placed in actophotometer individually, and the basal locomotor activity score was recorded and compared.

Motor coordination (Rotarod test)

The rotarod test equipment had a rod associated with drum of 7.0 cm diameter. The speed of rod was set at 20 revolutions/ min during the experiment. 6 rats from each group were selected and placed on the rod the number of falls from the rod was counted for 5 min.^[28]

Open field test

Open field apparatus made up of plywood was used with a dimension 72 cm \times 72 cm \times 42 cm. The apparatus was open from top and bottom, and the floor was divided into 25 similar squares. Animals from each group were kept at one end of the apparatus and number of squares crossed by them in 3 min was noted.

RESULTS AND DISCUSSION

Characterization of DMF loaded SLNs

Average particle size, polydispersity index and zeta potential of optimized batch of DMF loaded SLN were found to be 300 nm, <0.3 and -34.89 mv, respectively. The % EE was found to be 59%, and drug loading was 15%. Better EE and

drug loading assure a good drug carrying capacity which is one of most desirable characteristics of SLN. Transmission electron microscopy (TEM) microphotograph [Figure 1] also confirmed smooth and spherical particles with non-aggregation. Zeta potential value is related with the kinetic stability of SLNs formulations and indicates a balance between attractive and repulsive forces of suspended particles. Zeta potential values lower than -30 mv and higher than $+30$ mv indicates a stable colloidal formulation. Thus, the present formulation with a zeta potential value of -34.89 mv is a stable colloidal dispersion [Figure 2]. *In vitro* drug release profile confirms a prolonged therapy with a % cumulative drug release $70.46 \pm 1.17\%$ and 71.79 ± 0.79 in 0.1 N HCl and 7.4 pH phosphate buffer respectively over a period of 24 h. The *in vitro* release profile of DMF loaded SLNs is depicted in Figure 3. The initial drug release of more than 25% within 2 h is also a prime requisition for an early onset of action.

MTT assay

The result demonstrated an increase in intracellular reactive oxygen species with an increase in H_2O_2 concentration [Figure 4]. As the concentration of H_2O_2 was increased absorbance decreased. H_2O_2 concentration of 250 μM produced 60% cell death and was selected as an optimum concentration for further neuroprotective study. Treatment with DMF loaded SLNs affects the viability of SH-SY5Y cells significantly. The percentage of cell viability increased as compared to the cells treated with H_2O_2 alone [Figure 5]. Percent cell viability was shown to be increased at different concentrations of DMF and SLNs at a dose equivalent to 12.5 μM showed max cell viability of 85.21 %. The results of this study supported the previous data of neuroprotective effects of DMF and showed the same results with formulated DMF loaded SLNs. Present formulation showed the neuroprotective effect in H_2O_2 induced SH-SY5Y cell apoptosis and the concentration of 12.5 μM DMF showed the best neuroprotective effect.

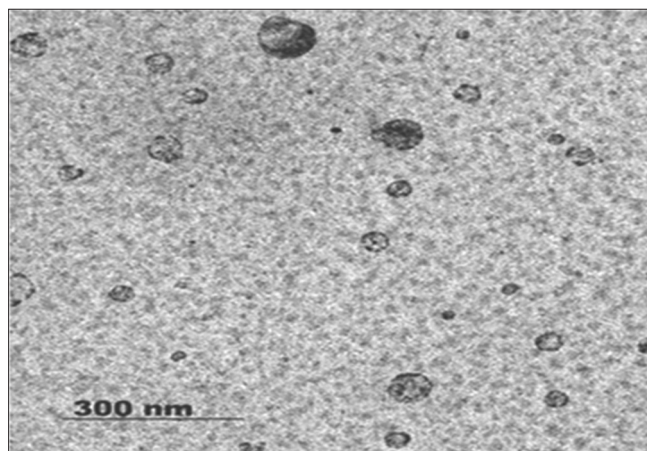


Figure 1: Transmission electron microscopy microphotograph of dimethyl fumarate loaded solid lipid nanoparticles

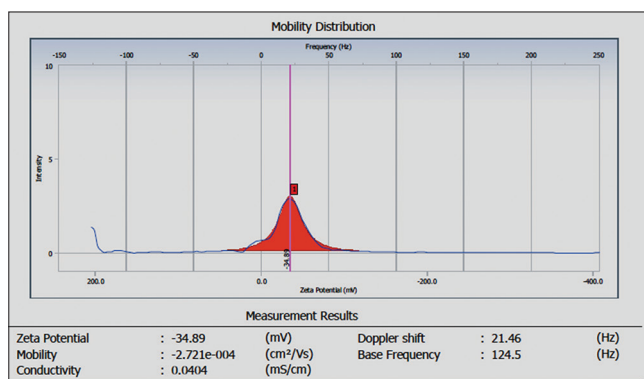


Figure 2: Zeta potential of dimethyl fumarate loaded solid lipid nanoparticles

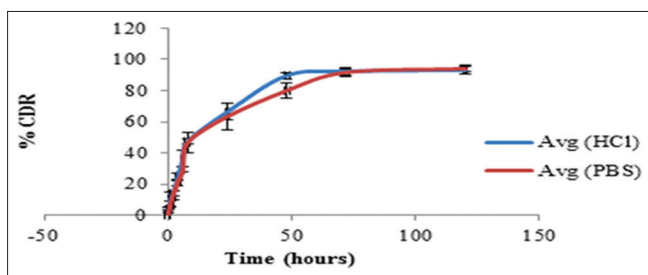


Figure 3: *In vitro* % cumulative drug release profile

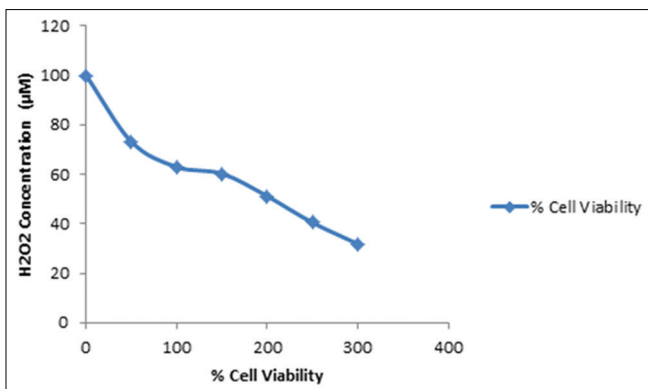


Figure 4: Effect of H₂O₂ concentration on % cell viability

In vivo activity

Cuprizone is a toxin, and the model was used to induce demyelination by killing oligodendrocytes in white and gray matter of CNS. The neurobehavioral scores (such as neurological deficit score, grasping ability, forelimb strength, and motor function) were significantly improved with treatment of DMF loaded SLNs in cuprizone model of rodents as compared to the diseased group. The results of locomotor scores and number of falls from rotarod are depicted in Table 2. Improvement in locomotor function and neurobehavioral scores is an indication of successful neuroprotection treatment. Decrease in number falls in treated groups from rod also states that DMF treated groups show better neurological behavior than the diseased groups. The same results were observed in open field test, and the number of squares crossed by the treated groups was significantly high as compared to diseased groups [Figure 6]. The current *in vivo* study suggests that the present formulation

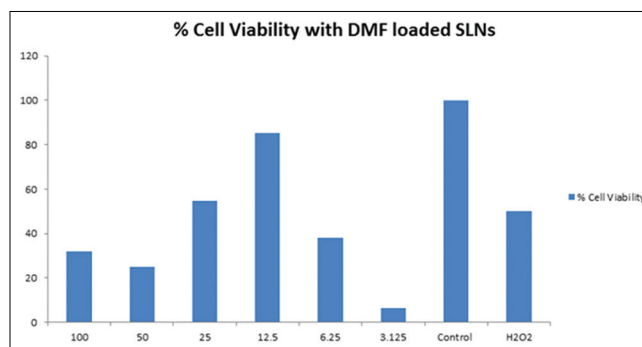


Figure 5: Effect of dimethyl fumarate loaded solid lipid nanoparticles on H₂O₂ induced cell apoptosis

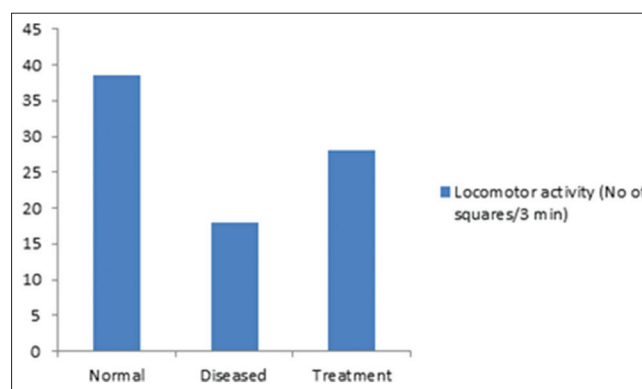


Figure 6: Mean score in open field method

is effective in cuprizone model of Wistar rat in MS. From the results of *in vivo* and *in vitro* studies, we can conclude that DMF loaded SLNs possess neuroprotective activity.

CONCLUSION

In the present study, colloidal SLNs containing DMF was fabricated by hot microemulsion method with the use of probe sonicator. The produced nanoparticles were spherical in shape with non-aggregation as evidenced by TEM image. Further, the *in vitro* drug release profile also confirmed a sustain release profile of the drug. DMF loaded SLNs also proved its neuroprotective activity on SH-SY5Y neuroblast

Table 1: Groups of Wistar rats for *in vivo* studies

Group name	Diet specification
Group 1 (control)	Under normal diet
Group 2 (diseased)	Cuprizone (0.4%)
Group 3 (treatment)	Cuprizone (0.4%)+DMF loaded SLN at a dose equivalent to 50 mg/ kg DMF

DMF: Dimethyl fumarate, SLN: Solid lipid nanoparticle

Table 2: Activity score in actophotometer and rotarod

Group	Score locomotor activity	Time in seconds (to fall from rotating rod)
Control	195.12±11.24	112.34±8.12
Diseased	107±12.36	56±10.45
Treatment	178±12.45	84±12.87

Results are expressed as mean±standard deviation (n=6)

cell under oxidative stress. In cuprizone model of Wistar rat for MS, the present formulation showed better locomotion scores. Taken together, our findings suggest that the present formulation have a significant potential in management and treatment of the disease MS.

CONFLICTS OF INTEREST

The authors declare that there is no conflict of interest.

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REFERENCES

- Stangel M. Neuroprotection and neuroregeneration in multiple sclerosis. *J Neurol* 2008;255:77-81.
- Kamm CP, Uitdehaag BM, Polman CH. Multiple sclerosis: Current knowledge and future outlook. *Eur Neurol* 2014;72:132-41.
- Lucchinetti C, Nruock W, Parisi J, Scheithaue BR, Rodriguez M, Lassmann H. Heterogeneity of multiple sclerosis lesions: Implications for the pathogenesis of demyelination. *Ann Neurol* 2000;47:707-17.
- Hemmer B, Archelos JJ, Hartung HP. New concepts in the immunopathogenesis of multiple sclerosis. *Nat Rev Neurosci* 2002;3:291-301.
- Pluchino S, Martino G. The therapeutic plasticity of neural stem/precursor cells in multiple sclerosis. *J Neurol Sci* 2008;265:105-10.
- Ben-Hur T. Immunomodulation by neural stem cells. *J Neurol Sci* 2008;265:102-4.
- Schweckendiek W. Treatment of psoriasis vulgaris. *Med Monatsschr* 1959;13:103-4.
- Moharreggh-Khiabani D, Linker RA, Gold R, Stangel M. Fumaric acid and its esters: An emerging treatment for multiple sclerosis. *Curr Neuroparmacol* 2009;7:60-4.
- Fox RJ, Miller DH, Phillips JT, Hutchinson M, Havrdova E, Kita M, *et al.* Confirm study investigators. Placebo-controlled phase 3 study of oral BG-12 or glatiramer in multiple sclerosis. *N Engl J Med* 2012;367:1087-97.
- Gold R, Giovannoni G, Phillips JT, Fox RJ, Zhang A, Meltzer L, *et al.* Efficacy and safety of delayed-release dimethyl fumarate in patients newly diagnosed with relapsing-remitting multiple sclerosis (RRMS). *Mult Scler* 2015;21:57-66.
- Phillips JT, Fox RJ. BG-12 in multiple sclerosis. *Semin Neurol* 2013;33:56-65.
- Albrecht P, Bouchachia I, Goebels N, Henke N, Hofstetter HH, Issberner A, *et al.* Effects of dimethyl fumarate on neuroprotection and immunomodulation. *J Neuroinflamm* 2012;9:2-10.
- Li J, Calkins MJ, Johnson DA, Johnson JA. Role of nrf2-dependent ARE-driven antioxidant pathway in neuroprotection. *Methods Mol Biol* 2007;399:67-78.
- Van HJ, Drexhage JA, Flor T, Gerritsen W, Van der Valk P, De Vries HE. Nrf2 and DJ1 are consistently upregulated in inflammatory multiple sclerosis lesions. *Free Radic Biol Med* 2010;49:1283-9.
- Kossena GA, Boyd BJ, Porter CJ, Charman WN. Separation and characterization of the colloidal phases produced on digestion of common formulation lipids and assessment of their impact on the apparent solubility of selected poorly water-soluble drugs. *J Pharm Sci* 2003;92:634-48.
- Muthu MS, Rawat MK, Mishra A, Singh S. PLGA nanoparticle formulations of risperidone: Preparation and neuropharmacological evaluation. *Nanomedicine* 2009;5:323-33.
- Ojha S, Kumar B. Formulation and optimization of chitosan nanoparticles of dimethyl fumarate using box-behnken design. *Int J Appl Pharm* 2016;8:10-7.
- Li S, Yunna C, Yali Z, Dongdong G, Yufan F, Fangyan G, *et al.* Preparation of 5-fluorouracil-loaded chitosan nanoparticles and study of the sustained release *in vitro* and *in vivo*. *Asian J Pharm Sci* 2017;12:418-23.
- Seju U, Kumar A, Sawant KK. Development and evaluation of olanzapine-loaded PLGA nanoparticles for nose-to-brain delivery: *In vitro* and *in vivo* studies. *Acta Biomater* 2011;7:4169-76.
- Chang KS, Ji-Eun C. Potential of nano carriers as cancer cell-specific drug delivery systems in photodynamic therapy. *Asian J Pharm Sci* 2016;11:8-9.
- Ting Y, Lingzhen Q, Zhouhua W, Jinyuan N, Zhefei G, Ge L, *et al.* Solid lipid dispersion of calcitriol with enhanced dissolution and stability. *Asian J Pharm Sci*

- 2013;8:39-47.
22. Han SM, Kim JM, Park KK, Chang YC, Pak SC. Neuroprotective effects of melittin on hydrogen peroxide-induced apoptotic cell death in neuroblastoma SH-SY5Y cells. *BMC Complement Altern Med* 2014;14:1-8.
 23. Benjamin NT, Ling AP, Koh RY, Chye SM, Wong YP. Neuroprotective effects of orientin on hydrogen peroxide-induced apoptosis in SH-SY5Y cells. *Mol Med Reports* 2014;9:947-54.
 24. Mosmann T. Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *J Immunol Methods* 1983;65:55-63.
 25. Love S. Cuprizone neurotoxicity in the rat: Morphologic observations. *J Neurol Sci* 1988;84:223-37.
 26. Silvestroff L, Bartucci S, Pasquini J, Franco P. Cuprizone-induced demyelination in the rat cerebral cortex and thyroid hormone effects on cortical remyelination. *Exp Neurol* 2012;235:357-67.
 27. Song SY, Kato C, Adachi E, Moriya-sato A, Inagawaogashiwa M, Umeda R, *et al.* Expression of an Acyl-CoA Synthetase, lipidosin, in astrocytes of the murine brain and its up-regulation during remyelination following cuprizone-induced demyelination. *J Neurosci Res* 2007;85:3586-97.
 28. Franco-Pons N, Torrente M, Colomina MT, Vilella E. Behavioral deficits in the cuprizone-induced murine model of demyelination/remyelination. *Toxicol Lett* 2007;169:205-13.

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