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Pro-inflammatory influence of high sucrose consumption on experimental autoimmune encephalomyelitis mouse immunized with MOG_{35-55}

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Abstract

Experimental autoimmune encephalomyelitis (EAE) is the most often used animal model for the multiple sclerosis (MS). EAE is characterized by the complex symptoms of neuropathology and immunopathology leading to similar key symptoms of MS. In this study, we evaluated the influence of high sucrose (HS) consumption in the autoimmune disease pathogenesis. In brief, 7-8 weeks old C57BL/6J mice were randomly distributed into three following groups for 30 days: healthy control, EAE, and EAE+HS (20 % sucrose in a drink). To establish an EAE mouse model, all mice except those in healthy control were sensitized with MOG_{35-55}. The EAE in HS-induced mice was worsened as shown by higher clinical scores, infiltrated inflammatory cells, severe demyelination, making disease at the peak in brain and spinal cord compared to healthy control. Additionally, the chronic exposure of HS exhibited excessive lipid accumulation in the liver. Furthermore, mRNA expressions of TGF-β, IL-17A, and MCP-1 in central nervous system (CNS) were significantly higher in EAE+HS compared to EAE or healthy control. EAE+HS group also showed the exacerbation of inflammatory gene expressions, i.e., IFN-γ, TNF-α, IL-1β, IL-6, and IL-22 for the differentiation of Th1 and Th17 cells. Conclusively, the results indicate high sucrose consumption promotes axonal damage, inflammation, and demyelination in EAE mice.

Key words; Experimental autoimmune encephalomyelitis, Multiple sclerosis, High sucrose, Central nervous system

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List of abbreviations

ABC	Avidin Biotin Complex
APCs	Antigen Presenting Cells
ANOVA	One-way Analysis of Variance
BBB	Blood Brain Barrier
CFA	Complete Freund's Adjuvant
CNS	Central Nervous System
Ct	Cycle threshold
DAB	3, 3'- diaminobenzidine
DCs	Dendritic Cells
EAE	Experimental Autoimmune Encephalomyelitis
EDTA	Ethylene Diamine Tetra Acetic Acid
FAS	Fatty Acid Synthase
FITC	Fluorescein Isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GFAP	Glial Fibrillary Acidic Protein
H&E	Hematoxylin and Eosin
HC	Healthy Control
HFD	High Fat Diet
HS	High Sucrose
HSD	High Sucrose Diet
Iba-1	Ionized calcium binding adapter molecule 1

IHC	Immuno Histo Chemistry
IL	Interleukin
NF-H	Neurofilament-H
MBP	Myelin Basic Protein
MCP-1	Monocyte Chemoattractant Protein
MOG	Myelin Oligodendrocyte Glycoprotein
mRNA	Messenger Ribonucleic Acid
MS	Multiple Sclerosis
NAFLD	Non-alcoholic Fatty Liver Disease
PBS	Phosphate Buffer Solution
PLP	Proteolipid Protein
PT	Pertussis Toxin
ROR	Retinoid-related Orphan Receptor
ROS	Reactive oxygen species
RT-PCR	Real time PCR
SCD1	Stearoyl-CoA desaturase-1
T-bet	T-box Transcription Factor
TGF- β	Transforming Growth Factor Beta
Th1	T helper type 1
Th2	T helper type 2
Th17	T helper type 17
TNF	Tumor Necrosis Factor

1. Introduction

Multiple sclerosis (MS) is a chronic inflammatory demyelinating disease of central nervous system (CNS) that affects 2.5 millions of people worldwide and lacks of curative treatments (Milo and Kahana, 2010). Experimental autoimmune encephalomyelitis (EAE) is a mostly commonly used animal models for determining the immunopathogenic pathways that contribute to MS development (Sospedra and Martin, 2005). Ascending paralysis is a common EAE symptom, which begins with a weakening of tail tone and progresses to demyelination and inflammatory infiltration of the subpial and parenchymal nervous systems (Gold et al., 2000). Inflammation is a characteristic of both MS and EAE pathogenesis, which is marked by auto-reactive T cells and macrophage infiltration in the CNS. When antigen specific Th1, and Th17 cells penetrate the CNS, the disease will be initiated. These T cells are re-stimulated when they come into contact with antigens, and they produce inflammatory cytokines to recruit inflammatory macrophages and microglia resulting in an explosive inflammatory cascade and tissue damage, including demyelination and axonal injury (Sospedra and Martin, 2005, Bramow et al., 2010). EAE can be produced in mice by an assortment of immunization, but most often by the subcutaneous injection of CNS derived proteins, such as myelin oligodendrocyte glycoprotein (MOG), myelin basic protein (MBP) or myelin proteolipid protein (PLP, emulsified in complete freund's adjuvant (CFA) (Miller et al., 2010). The clinical course of EAE differs based on the antigen and mice strain. C57BL/6J mice sensitized with myelin oligodendrocyte glycoprotein peptide (MOG_{35-55}) develop chronic progressive paralysis, whereas SJL/J mice sensitized with proteolipid protein peptide 139-151 ($PLP_{139-151}$) exhibit relapsing-remitting paralysis (Croxford et al., 2011). It is extremely easy to generate gen targeted or immune-deficient animals so, several studies have

employed the MOG_{35-55} induced chronic EAE model in C57BL/6 mice widely. The SJL/J mouse strain, on the other hand, may be utilized to examine the relapsing-remitting illness phase after being immunized with the PLP peptide (Skundric, 2005).

Diet has long been a possible environmental risk factors for the rise in autoimmune disorders, the underlying processes are still unknown (Lerner and Matthias, 2015, Manzel et al., 2014, Zhang et al., 2019). Significant changes in dietary patterns in western countries have influenced the population to consume high-sugar, high-salt, and high-fat foods, resulting in an increase in the prevalence of obesity, metabolic syndrome, and cardiovascular diseases (Manzel et al., 2013). The western diet including a lot of fat and cholesterol, a lot of protein, a lot of sugar and a lot of salts, as well as a lot of processed and “fast meals” with industrial food additives, has been proven to raise the risk of many of these autoimmune disorders (Thorburn et al., 2014, Tilg and Moschen, 2015, Conlon and Bird, 2015, Chassaing et al., 2015). The prevalence of autoimmune diseases, such as Crohn's disease and multiple sclerosis (MS), has risen in recent decades, particularly in western countries (Bach, 2002, Selmi, 2010, Molodecky et al., 2012, Rees et al., 2016). Several recent research have uncovered additional undiscovered harmful consequences of a high-sugar diet. For example, according to one study, sugar-sweetened beverage consumption is connected to overall mortality and has a dose-dependent connection (Malik et al., 2019). In another research, researchers revealed that glucose fructose syrup can cause intestinal cancers in mice (Goncalves et al., 2019). Several recent studies have found that consumption of a high-fat, high-sugar diet is a major contributor to the rise in metabolic diseases, particularly non-alcoholic fatty liver disease (NAFLD) and insulin resistance (IR) (Ragab.et.al.,2015). The expression of the lipogenic genes fatty acid synthase (FAS), and stearoyl-coA desaturase-1 (SCD1), has been found to be influenced by a high simple carbohydrate diet (Dentin et al., 2005). Studies have revealed that when a high

simple carbohydrate meal is consumed, the levels of FAS, and SCD1 rise compared to a fasting control (Dentin et al., 2004). However, it is yet unknown whether high sucrose intake influences the development of EAE through T cell differentiation and effects on hepatic tissues.

Therefore, here, we adopted a mouse model of EAE to investigate the impacts of high sucrose on autoimmune disease development. Our findings revealed that excessive sucrose consumption accelerated disease progression, as evidenced by higher clinical scores, worsened demyelination, severe hepatic steatosis, and increased pro-inflammatory mediators in the central nervous system and liver. We demonstrated that high sucrose intake promotes the autoimmunity in experimental models of EAE enhancing the CNS inflammation featured by pathogenic T cells inflammatory mediators such as IL-1β, IL-6, IL-17A, IL-22, IFN-γ, TNF- α, and chemokine MCP-1 showing the involvement of Th1 and Th17 mediated immune response in CNS. We further elucidate that high sucrose develops the severe steatosis in hepatic tissues with the upregulated lipogenic genes like FAS, and SCD1 and inflammatory mediators such as IL-6, TNF-α, IFN-γ, and chemokine MCP-1.

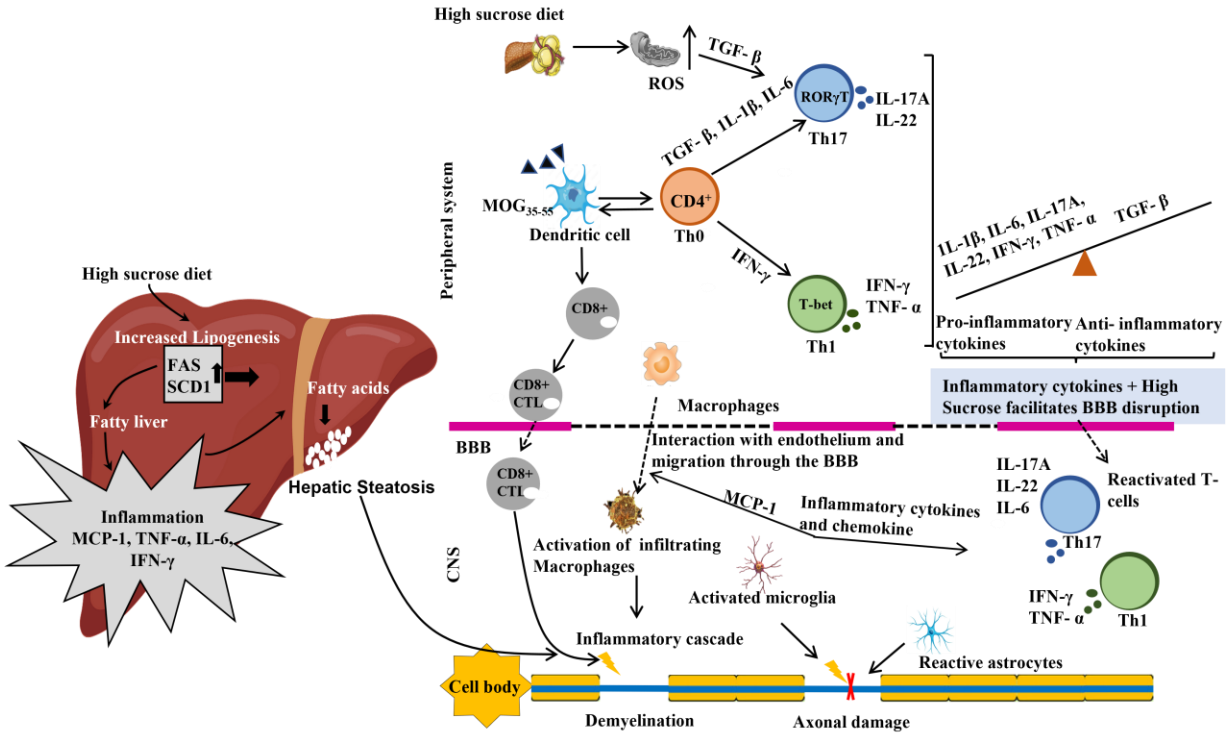
2. Research hypothesis

The animal is immunized with a subcutaneous injection of an emulsion containing the selected antigen and complete Freund's adjuvant (CFA) on the day of vaccination and an intraperitoneal dose of pertussis toxin two days later for a EAE induction (Bittner et al., 2014). Pathophysiology of EAE is occurred through the immunization with myelin antigen protein like myelin oligodendrocyte glycoprotein (MOG) (Baxter, 2007). Dendritic cells present myelin antigen in the EAE model, activating and proliferating $CD4^+$ T cells. Dendritic cells are activated by primed

$CD4^+$ T cells, leading in clonal growth of cytotoxic T lymphocytes ($CD8^+$ CTL), which can cause direct damage to myelin sheaths and axons (Khan and Smith, 2014). High sucrose intake increases reactive oxygen species (ROS) in T-cells, which further leads to the induction of Th17 cells via TGF- β activation (Zhang et al., 2019). Activated T cells also produce pro- and anti- inflammatory mediators. Activated Th1 cells and Th17 cells are considered to be the major effector cells in EAE/MS that migrate towards the blood-brain-barrier (BBB), making BBB impaired and these T cells enters into CNS (McGinley et al., 2018). Autoreactive T cells are reactivated once within the CNS by antigen-presenting cells (APCs) unique to the CNS, such as microglia/brain resident dendritic cells (DCs). These events in turn cause the creation of inflammatory products with the product like high sucrose and cytokines, making BBB disrupted, which can cause myelin and axon damage. Activated microglia in the CNS generate factors that attracts more inflammatory cells to the area, perpetuating the inflammatory cascade (Denic et al., 2016). Microglial expression of cytokines and the chemokine monocyte chemoattractant protein (MCP1) enhances infiltration of T cells and macrophages, respectively. (Berman et al., 1996). These cascades might promote demyelination and axonal loss in CNS.

Moreover, our hypothesis shows that high sucrose diet increases the levels of lipogenic genes FAS, and SCD1, which rose compared to a fasting control resulting the hepatic steatosis with inflammatory mediators like IL-6, TNF-α, IFN-γ and MCP1.

We hypothesize that high amount of sucrose may increase the expression of inflammatory mediators in CNS through the Th1 and Th17 mediated immune response resulting severe demyelination and axonal damages. We also suppose that high sucrose intake may exacerbate the development of EAE by promoting hepatic steatosis and inflammation in liver.



(Modified Khan and Smith, 2014)

Figure 1. Hypothesis showing the effect of high sucrose intake in EAE pathogenesis

3. Materials and methods

3.1. Animal and diet

4 weeks old male and female C57BL/6 mice were bought from the Orientbio, Inc. (Gwanju, Korea) and were housed in individual ventilated cages under specific pathogen-free conditions. Mice were kept in cages with a 12 h dark/light cycle with ad libitum access to food and drinking water at constant temperature (23 ± 1.5ºC) and 55 ± 15 % humidity.

Mice were assigned at random to one of three experimental groups fed the control diet and drink either tap water or 20 % sucrose drink after one week of acclimation: healthy control (HC), EAE-

induced group (EAE), or EAE with 20 % sucrose drink (high sucrose, EAE+HS). Diet preparation was adapted from the AIN-93G diets (Reeves et al., 1993), and the nutritional content of the experimental diets is listed in Table 1. Mice were provided with the autoclaved water containing 20 % sucrose (weight/volume) or autoclaved tap-water in water to simulate the sweetened beverage consumption to the mice assigned to EAE+HS group (Kim et al., 2018). The chemical structure of sucrose is shown in Figure 2.

Table 1. Dietary composition of control diet

Ingredients	g/kg
Casein	200
L-Cysteine	3
Sucrose	0
Corn starch	600
Maltodextrin 10	50
Lard	10
Cholesterol	0
Soybean oil	39
Cellulose	50
Mineral mix	35
Calcium phosphate	4
Vitamin mix	10
Choline bitartrate	2
Carbohydrate	67.8
Protein	20.9
Fat	11.3
Total energy	1003 kcal

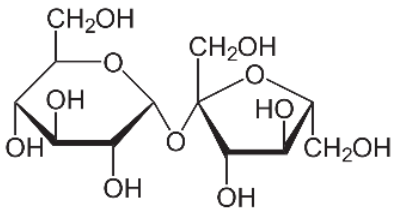


Figure 2. Chemical structure of sucrose

Diet components were described in Table 1 and were based on the AIN-93G diet (Reeves et al., 1993).

3.2. Induction and assessment of EAE

GL Biochem Ltd., (Shanghai, China) supplied the synthetic proteolipid protein MOG_{35-55} peptide (M-E-V-G-W-Y-R-S-P-F-S-R-V-V-H-L-YR-N-G-K). On day 1, mice were injected in upper and lower back subcutaneously (s.c.) with 200 μg of MOG_{35-55} peptide in complete Freund's adjuvant (CFA) (Difco, Detroit, MI) containing 500 μg of heat-inactivated mycobacterium tuberculosis, followed by intravenous (i.v.) injections of 200 ng of pertussis toxin (PT) on day 1 and 2 (List Biologic, Campbell, CA, USA). Clinical symptoms were noticed on a daily basis, and the following clinical grade evaluation of EAE was made 0 = no clinical symptom; 1 = tail limpness; 2 = limp tail and feeble hind legs; 3 = limp tail and complete paralysis hind legs; 4 = limp tail, partial front limp paralysis and full rear leg paralysis; 5 = moribund or reduced (Murugesan et al., 2012).

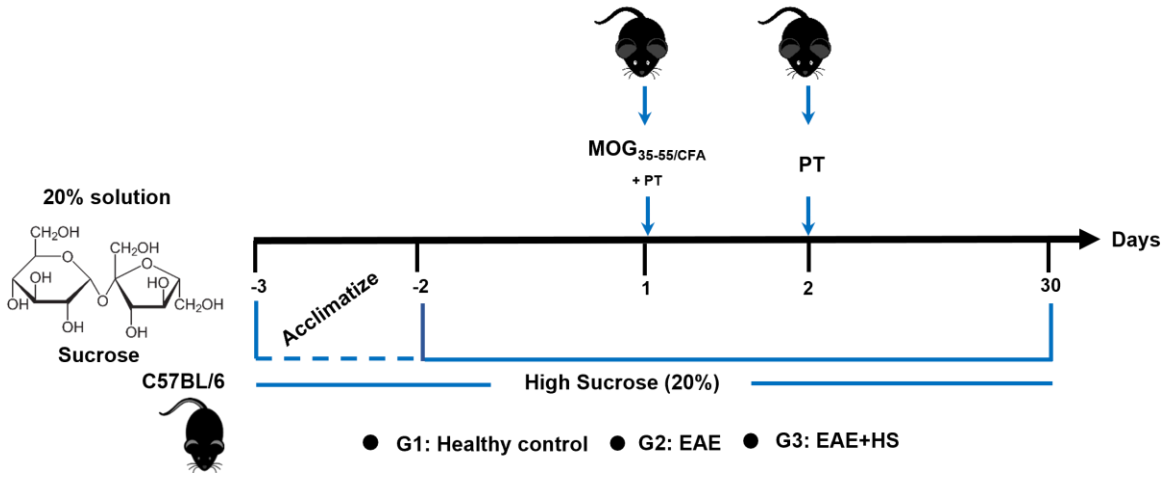


Figure 3. Mouse model of EAE

3.3. Preparation of tissue and histological analysis

During EAE's peak and recovery periods, spinal cords and brain were collected. Each group's experimental mice were sacrificed using diethyl ether anesthesia, and their brain and spinal cords were preserved in 10 % formalin and embedded in paraffin to create a 3 μm paraffin section. Three-micrometer paraffin sections were stained with hematoxylin and eosin (H&E) for visualization of inflammatory infiltrates. Luxol fast blue staining was performed to assess demyelination in spinal cord. The degree of inflammation and demyelination were determined by measuring 2-3 regions per tissue section of each mouse (n=3) using Olympus DP-72 microscope and expressing the results as mean ± SE. Histological results were classified into four groups for H&E staining 1= Infiltration of leptomeninges; 2 = Perivascular cuffing (mild); 3 = Significant perivascular cuffing; 4 = Significant parenchymal cell infiltration and widespread perivascular cuffing (Sakuma et al., 2004). Similarly, the demyelination was scored it as 1 = subpial demyelination traces; 2 = subpial and perivascular demyelination; 3 = perivascular or subpial demyelination confluent; 4 = perivascular and subpial demyelination affecting one half of the spinal cord, with cellular infiltrates in the CNS parenchyma; and 5 = The whole cord slice had significant perivascular and subpial demyelination, with cellular infiltrates throughout the CNS parenchyma (Zappia et al., 2005).

3.4. Immunohistochemistry

The brain and spinal cord were collected, fixed in 10 % formalin, and embedded in paraffin to acquire 3 μm thick sections. To inhibit endogenous peroxidase activity, deparaffinized sections were treated with 0.3 % H_2O_2 for 30 minutes. For demasking of antigens, the slides were then

treated in a microwave oven (1000 W), 20 min in sodium citrate buffer. After cooling to room temperature, the sections were incubated for 30 minutes with respective blocking serum (Vector Laboratories, Burlingame, USA). After blocking non-specific bindings, the sections were incubated overnight at 4 °C with primary glial fibrillary acidic protein (GFAP) antibody (1:500, Novus Biological LLC, Littleton, CO, USA), Phosphorylated neurofilament-H (NF-H) antibody (1:1000, Convance Inc., Carmegie Center Princeton, USA), and ionized calcium -binding adaptor molecule-1 (Iba-1) antibody (1:500, Santa cruz chemical USA Inc; Richmon, VA, USA). After washing, tissues were treated with biotinylated secondary antibody for 45 minutes, followed by avidin-biotin-peroxidase complexes (Vector Laboratories, Burlingame, USA). Diaminobenzidine (DAB) was used to stain the slides and are counterstained with hematoxylin (Dako) after the staining had established. Under an Olympus DP-72 (Olympus, Tokyo, Japan) microscope. Using Image J software (v1.46), positive zones were counted and expressed in graphs.

3.5. Flow cytometric analysis

Single cell suspensions of the spleen (contained 1×10^6 cells) in 24-well plates were stained with anti-mouse IgG (Caltag Laboratories, Burlingame, CA, USA) and then conjugated with fluorescein isothiocyanate (FITC) labeled anti-mouse CD3e (145-2C11), CD4 (H129.19) and CD8a (53-6.7) (BD Biosciences, San Jose, CA). Following staining, the cells (20,000 per sample) were examined with a CytoFLEX flow cytometer (Bio-Health Materials Core-Facility, Jeju National University) using CytExpert 1.2 software (Beckman Coulter, Inc., Kraemer Blvd, Brea, CA, USA).

3.6. Quantitative real-time PCR (RT-PCR)

Total RNA from central nervous system (CNS) were isolated using Trizol regent (Life technologies). Trizol reagent (Life technologies) was used to homogenize tissues from the central nervous system. The suspension was centrifuged at 15,000 × g for 10 minutes after adding 500 μL chloroform and thoroughly mixing it. To precipitate RNA, an equivalent amount of isopropanol was added to the supernatant and centrifuged at 12,000 × g for 10 minutes. The RNA pellets were rinsed in 70 % ethanol and allowed to dry at room temperature. The NanoVue spectrophotometer (GE Healthcare Life Sciences, UK). Single strand c-DNA was synthesized using promega A3500 DNA synthesis kit (St Louis, Cam, USA) according to manufacturer's guideline. Real-time PCR was performed with StepOnePlus realtime PCR system (Applied Biosysytems, Foster City, CA) using the Power SYBER Green PCR Master Mix (Applied Biosystems, USA). The fold changes in expression were calculated using 2-ΔΔCT method and endogenous control GAPDH was used for normalization. The primers that were utilized were indicated in Table 2.

Table 2. Quantitative PCR primer sequence

Gene	Sequence	
	Forward (5'-3')	Reverse (5'-3')
TGF-β	5'-GCCATCTATGAGAAAACCAAAG-3'	5'-TTAGTTCACACCTCGTTGTAC-3'
IL-1β	5'-GCTACCTGTGTCTTTCCCGTCG-3'	5'-TTGTCGTTGCTTGGTTCTCCTTG-3'
IL-6	5'-TGTGCAATGGCAATTCTGATTGTA-3'	5'-ATGGTCTTGGTCCTTAGCCACTCC-3'
IL-17A	5'-TCAACCGTTCCACGTCACCCTGGAC-3'	5'-TCAGCATTCAACTTGAGCTCTCATGC-3'
IL-22	5'-ACCTTTCCTGACCAAACTCA-3'	5'-AGCTTCTTCTCGCTCAGACG-3'
IFN- γ	5'-CTTCTTGGATATCTGGAGCAACTG-3'	5'-GGTGTGATTCAATGACGCTTATG-3'
TNF-α	5'-GGCAGCTTCTGTCCCTTTCACTC-3'	5'-CACTTGGTGGTTTGCTACGACG-3'
MCP1	5'-AACTGAAGCTCGCACTCTCG-3'	5'-TCAGCACAGATCTCCTTGGC-3'
T-bet	5'-ATGTTTGTGGATGTGGTCTTGGT-3'	5'-CGGTTCCCTGGCATGCT-3'
RORγt	5'-CACGGCCCTGGTTCTCAT-3'	5'-GCAGATGTTCCACTCTCCTCTTCT-3'
GAPDH	5'-AACGACCCCTTCATTGACC-3'	5'-TCAGATGCCTGCTTCACCC-3'

TGF- β: Transforming growth factor beta, IL: Interleukin, IFN- γ: Interferon gamma, TNF- α: Tumor necrosis factor- α, MCP1: Monocyte chemoattractant protein 1, T-bet: T-box transcriptional factor, RORγt: Retinoid-related orphan nuclear receptor gamma, GAPDH: Glyceraldehyde 3-phosphate dehydrogenase.

3.7. Statistical analysis

For each group, the numerical data were reported as means ± SEM. One-way analysis of variance (ANOVA) was used to determine statistical significance between groups. The non-parametric Mann-Whitney U-test was used in GraphPad prism software to compare clinical and histological scores. A p value < 0.05 was considered statistically significant.

4. Results

4.1. High sucrose intake upregulates the severity score of disease in EAE mice

To evaluate the pathological role of high sucrose in creating a EAE model, we sensitized C57/BL6 mice with MOG_{35-55} in CFA to induce EAE model. The progression and severity of clinical symptoms in two groups of mice (EAE, EAE+HS) were followed up on till day 60 after immunization (Figure 4). The EAE grouped mice have mild clinical symptoms throughout the course of disease and the symptoms appeared on day 13 after immunization (maximum clinical score, 1.6 ± 0.31). The mean severity score of the disease was higher in EAE+HS induction group that began from 11 days of immunization and score rose quickly and reached at maximal level on day 43, (maximum clinical score, 3.25 ± 1.01) characterized by progressive paralysis of the tail and hind limbs as well as severe neurologic impairment than EAE and healthy control mice. During 60 days observation period, these effects resulted in significant clinical symptom and disease exacerbation, indicating that EAE+HS aggravates EAE progression.

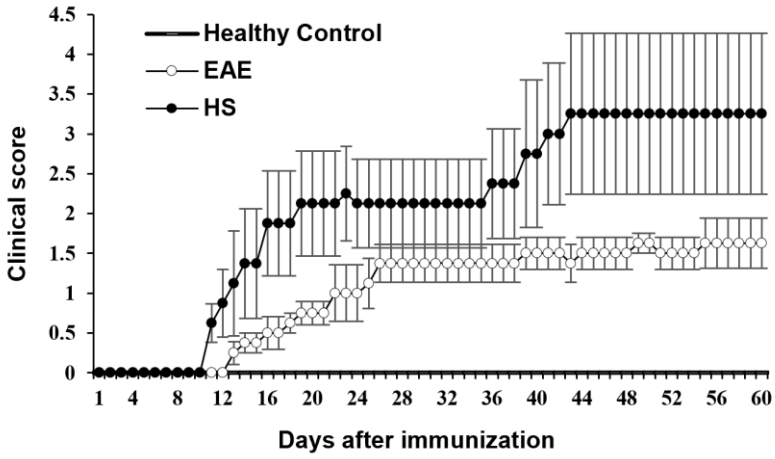


Figure 4. High sucrose intake increases severity in EAE mice

C57BL/6 mice were inoculated with MOG35-55 in CFA to induce EAE and fed with high sucrose diet (20 % sucrose in drink) for 30 days. Clinical disease score in group of mice (healthy control, EAE and EAE+HS) were monitored longitudinally till 60 days. Data were represented as mean ± SEM.

Table 3. Active clinical parameters of EAE

	Clinical status		
Group	Incidence (%)	Days of onset	Mean of maximal score
HC	0 (0/4)	0	0
EAE	75 (3/4)	18.0 ± 0.62	1.62 ± 0.12
EAE+HS	100 (4/4)	11.0 ± 0.62	3.25 ± 1.01*

EAE and EAE+HS mice were inoculated with MOG35-55 to induce EAE. Results are the mean values ± SEM of 4 animals per group from two independent experiments. Days of onset is denoted by onset of paralysis. *($p < 0.05$), represent as determined by Mann- whitney Utest.

4.2. High sucrose intake increases the area of lipid droplets in liver in EAE mice

Steatosis is defined as the liver inflammation due to the phenomenon of lipid accumulation over time, also known as non-alcoholic fatty liver disease (NAFLD). Lipid retention in hepatocytes leads to formation of droplets eventually displacing nuclei of the cells. Therefore, we evaluated the accumulation of lipid droplets in hepatic tissues of all groups with H&E staining. The accumulation of lipid droplets was found to be significantly increased in the liver tissue of EAE model and EAE+HS model (Figure 5). Liver sections from EAE disease model exhibited a slightly significant change in comparison with healthy control (by 4.99-fold) (Figure 5B and C). Conversely, the model with EAE+HS group exhibited highly significant dramatically larger area of lipid droplets accumulation in hepatocytes (increased by 33.33-fold as compared to healthy

control) (Figure 5C and D). The EAE+HS model led to a severe hepatic steatosis, with increased infiltration of immune cells in portal area and hepatic lobule. The above-mentioned results indicate the adverse influence of high sucrose diet for the development of steatosis in hepatic tissues.

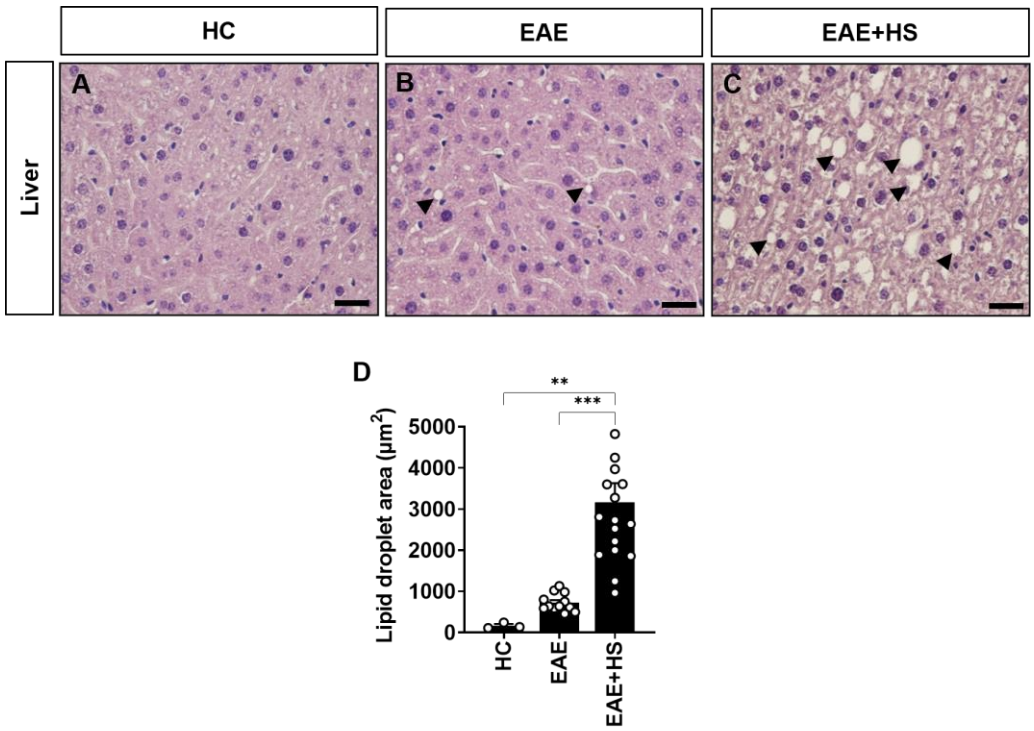


Figure 5. High sucrose intake promotes steatosis in hepatic tissues

Tissue sections of liver were stained with H&E staining. Black arrowhead indicates lipid droplets. The liver sections are indicated as; (A) healthy control, (B) EAE and (C) EAE+HS mice. The lipid droplets area in the liver was calculated from lipid droplet size, presented as a graph (D). Data were represented as the mean ± SD. **($p < 0.01$), ***($p < 0.001$) represents a significant increase compared to untreated control. Scale bars in (A, B and C) are 100 μm.

4.3. High sucrose intake increases the mRNA expression of lipogenic genes and inflammatory cytokines in liver of EAE mice

To investigate possible mechanism underlying the differences in hepatic lipid accumulation, we further measured the changes in the expression of genes involved in lipogenesis by quantitative PCR. Compared with healthy control and EAE mice, Relative mRNA expression of lipogenic genes in liver (FAS, SCD1) were up dramatically regulated in EAE+HS mice (Figure 6A and B). This shows that, high sucrose increases the mRNA expression of lipogenic genes in liver as compared to EAE and healthy control mice. Similarly, pro-inflammatory cytokines/chemokines have been implicated to play important role in the pathogenesis of liver. So, we further analyzed whether the high sucrose affects the expression of inflammatory genes in the liver during EAE by quantitative real-time PCR (Figure 6C, D, and E). We found the expression of IL6, TNF-α, INF-γ were elevated in EAE+HS as compared to EAE and healthy control. The expression of chemokine monocyte chemoattractant protein (MCP1) is also slightly increased in EAE+HS as compared to EAE (by 2.3-fold) and healthy control (by 2.6-fold) (Figure 6F). These increased lipogenic genes and inflammatory genes may be responsible for the severe hepatic steatosis in liver.

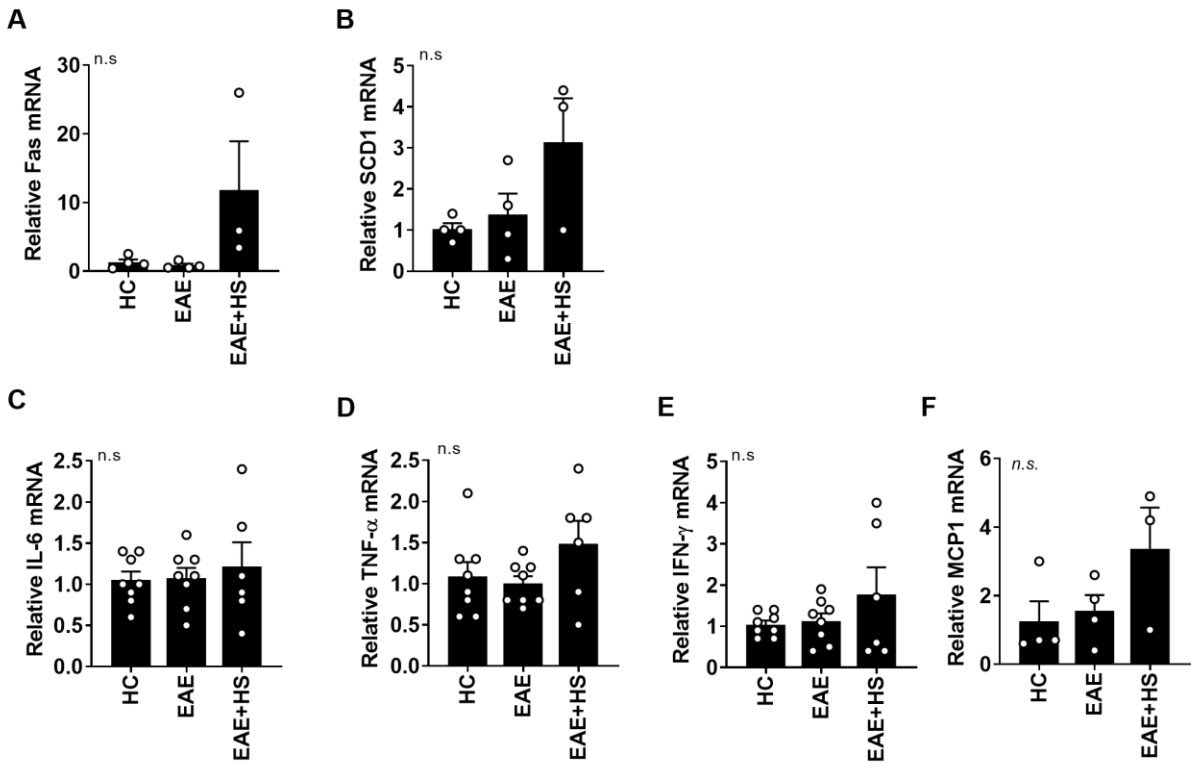


Figure 6. High sucrose intake increases the expression of lipogenic genes and pro-inflammatory mediators in liver

C57BL/6 mice were fed with high sucrose diet (20 % sucrose in drink) at week 7 - 8, gene expression was assessed by real-time PCR. Lipogenic genes (A) FAS, (B) SCD1, inflammatory genes (C) IL-6, (D) TNF-α, (E) IFN-γ and (F) chemokine, MCP1. Data are expressed as mean ± SEM and statistical significance was determined using one way analysis of variance (ANOVA).

4.4. High sucrose intake upregulates the activation and infiltration of inflammatory cells in brain of EAE mice

Increase in the infiltration of inflammatory cell in CNS seems to be a hallmark of pathogenesis of EAE. Therefore, we tested the regulating effect of high sucrose on immune cell infiltration into the brain of EAE mice by H & E staining (Figure 7). As shown in figure, cellular infiltration in the brain of mice in EAE+HS group was increased dramatically as compared to that of EAE (by 1.25-fold) and healthy control (by 4.37-fold) (Figure 7I and J), ($p < 0.001$), which showed apparent vascular cuff like changes and diffused inflammatory cell infiltration. This result suggest that high sucrose shows severity of inflammation in the brain promoting pathogenesis of EAE.

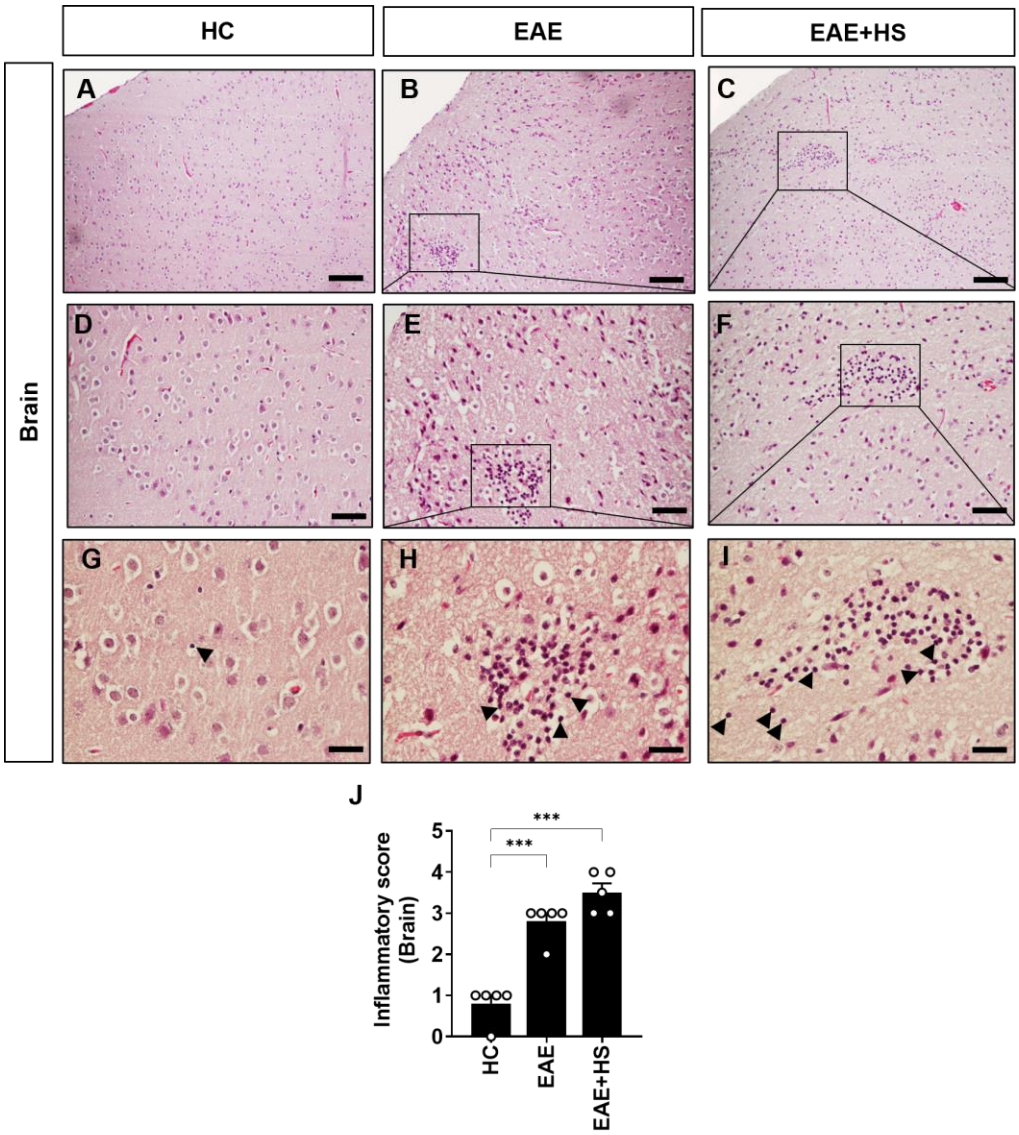


Figure 7. High sucrose upregulates severity of inflammation in brain

H&E staining was performed for the inflammatory cell infiltration in brain (A-I). (A, D, G) Healthy control, (B, E, H) EAE, (C, F, I) EAE+HS and inflammatory score in brain (J). All values were shown as mean ± SEM. ***($p < 0.001$) represents a significant up regulation as compared to untreated control. Black arrow heads indicate the inflammatory cells infiltration in brain. Scale bar of (A-I) are 25 μm.

4.5. High sucrose upregulates the activation and infiltration of inflammatory cells in spinal cord of EAE mice

We further tested the regulating effect of high sucrose on immune cell infiltration in the spinal cord. Like that in brain, as expected, we also found widespread cell infiltration and cuffed vessel in spinal cord in EAE+HS group as compared with EAE (by 1.53-fold) and healthy control (by 2.39-fold) (Figure 8I and J) ($p < 0.001$). This result indicates that, high sucrose shows severity of inflammation in spinal cord that promotes the development of pathogenesis of EAE.

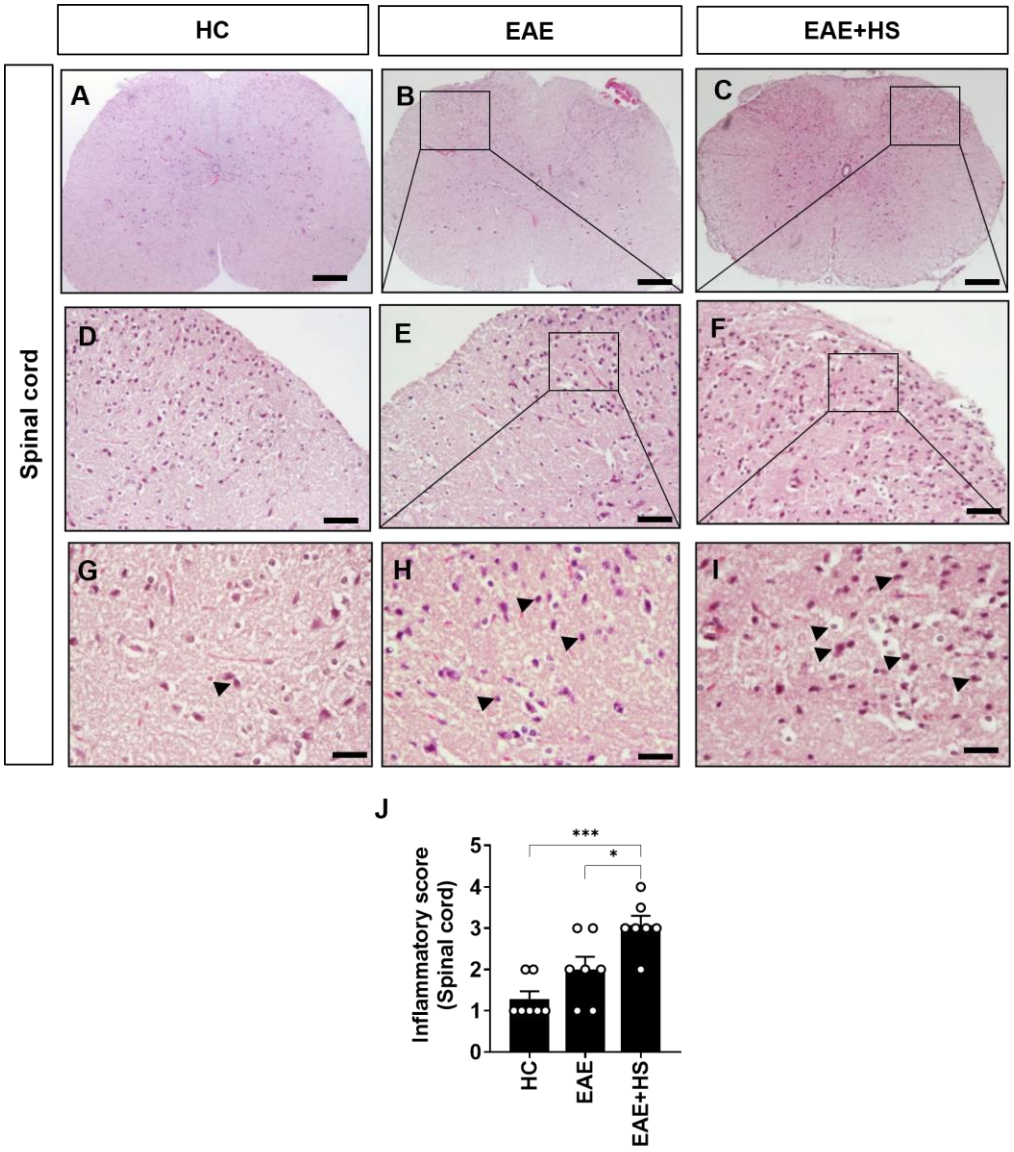


Figure 8. High sucrose upregulates severity of inflammation in spinal cord

H&E staining was performed for the inflammatory cell infiltration in spinal cord (A-I). (A, D, G) Healthy control, (B, E, H) EAE, (C, F, I) EAE+HS and inflammatory score in spinal cord (J). All values were shown as mean ± SEM. *($p < 0.05$), ***($p < 0.001$) represents a significant up regulation as compared to untreated control). Black arrow heads indicate the inflammatory cells infiltration. Scale bar of (A-I) are 25 μm.

4.6. High sucrose intake upregulates helper T cells and cytotoxic T cells infiltration in EAE mice

The molecular mechanism of pathophysiology of high sucrose exacerbated EAE was evaluated to identify the involvement of $CD3e^+$ $CD4^+$ helper T cells and $CD3e^+$CD8a cytotoxic T cells in splenocytes. The flow cytometric results exhibited a significant up-regulation of $CD3e^+$ $CD4^+$ helper T cell in EAE and EAE+HS (26.67 % and 31.09 % in EAE and EAE+HS respectively) as compared to healthy control (18.23 %) (Figure 9A and B). In addition, $CD3e^+CD8a^+$ cytotoxic T cells population was also increased in EAE model and EAE+HS (21.26 % and 25.87 % in EAE and EAE+HS respectively) as compared to the healthy control (14.10 %) (Figure 9A and C). Taken together, high sucrose diet exacerbates the activation of the severe inflammatory cell infiltration by activation of $CD3e^+$ $CD4^+$ helper T cells and $CD3e^+CD8a^+$ cytotoxic T cells population in EAE induced mice.

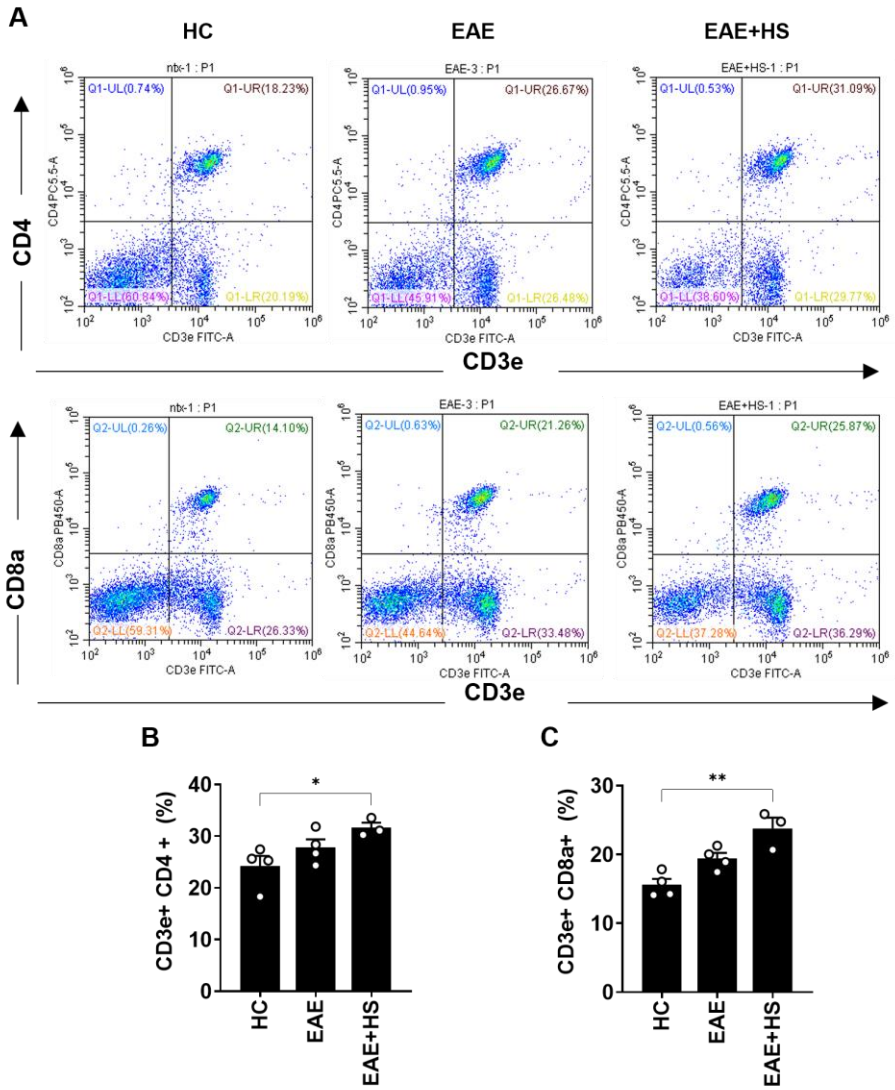


Figure 9. High sucrose on T-helper and cytotoxic T cells population in EAE mice

The effects of high sucrose exacerbated EAE on the population of $CD3e^+$ $CD4^+$ T-helper cells and $CD3e^+CD8^+$ T-cytotoxic cells was performed using FACS analysis (A). The splenic cell population's average value of $CD3e^+$ $CD4^+$ T-helper cells and $CD3e^+CD8^+$ T-cytotoxic cells were presented in (B) and (C) respectively. Data were expressed as the mean ± SEM (n = 4). *($p < 0.05$) and ** ($p < 0.01$) represent significant increase compared to untreated control.

4.7. High sucrose intake increases the expression of GFAP positive cells in brain and spinal cord in EAE mice

To determine whether the high sucrose regulate the inflammatory responses, we performed immunohistochemistry analysis of glial fibrillary acidic protein (GFAP) positive cells in brain and spinal cord for the localization of astrocytes. We found increased number of GFAP positive cells in EAE+HS as compared to EAE (by 1.33-fold) and healthy control (by 2.43-fold) (Figure 10C and G) in brain. Similarly, likewise in brain, as expected we found the increased number of GFAP positive cells in EAE+HS as compared to EAE (by 1.66-fold) and healthy control (by 3.04-fold) (Figure 10F and H). This result suggests that high sucrose intake upregulates the GFAP positive cell in brain and spinal cord of EAE induced mice.

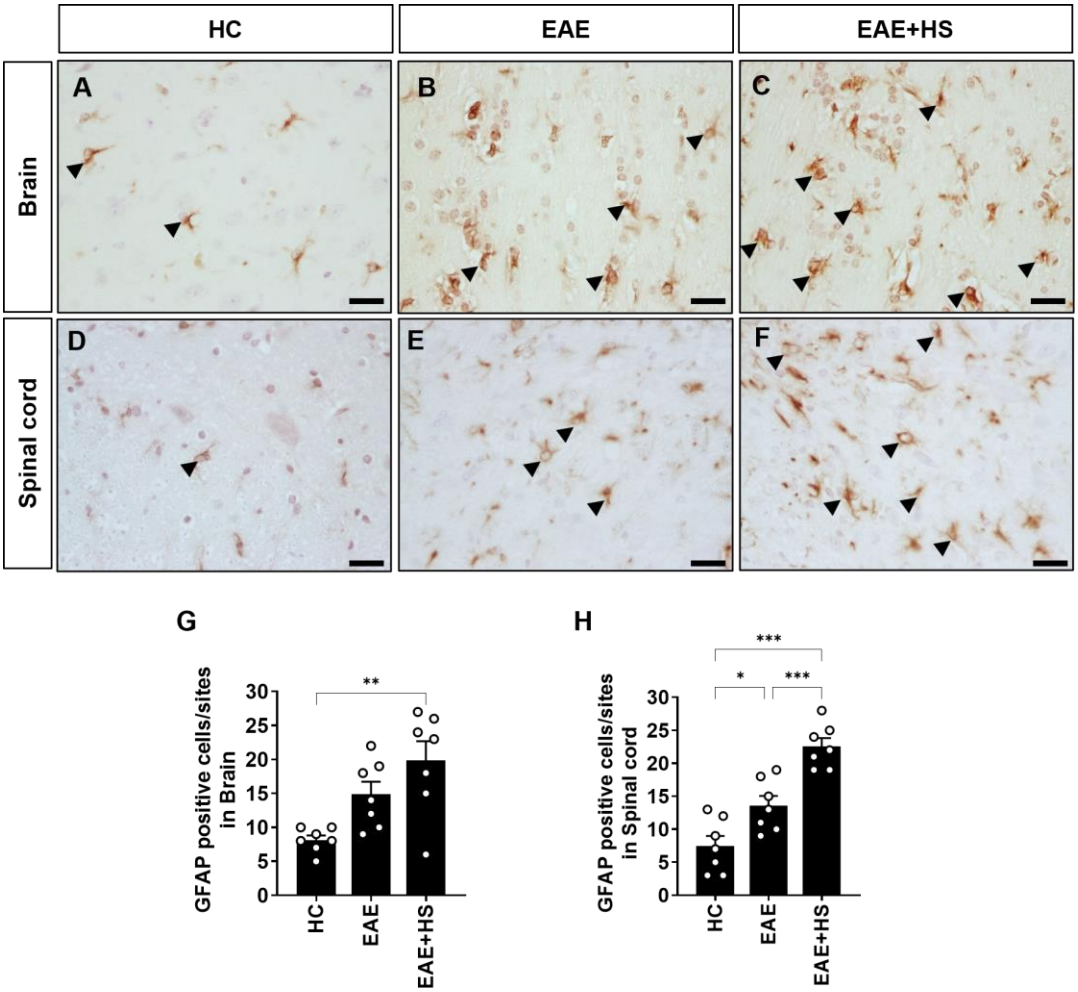


Figure 10. High sucrose increases the expression of GFAP positive cells in brain and spinal cord in EAE mice

Representative images of IHC represents the GFAP- positive cells in (A-C) brain and (D-F) spinal cord sections. (G) GFAP positive cells in brain and (H) GFAP positive cells in spinal cord. Arrow indicates positive cells in brain and spinal cord. Data are represented as the mean ± SEM of changes in values. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$). Scale bars of (A-F) are 25 μm.

4.8. High sucrose intake upregulates the mRNA expression of inflammatory mediators in CNS in EAE mice

The release of cytokines and chemokines that consolidate the disease is linked to the pathogenesis of EAE. Therefore, we use quantitative real-time PCR to see whether high sucrose changes the expression of inflammatory genes in the CNS during EAE. As depicted in (Figure 11), the mRNA expression of growth factor TGF-β for differentiation and induction of Th17 cells increased dramatically in EAE+HS as compared to EAE (by 15.01-fold) and healthy control (by 48.47-fold) (Figure 11A). Similarly, proinflammatory cytokines (IL-1β, IL-6, IFN-γ, and TNF-α) for the differentiation of Th1 and Th17 cells were upregulated in the CNS of EAE+HS group mice as compared to EAE and healthy control. In addition, the expression of IL-17A secreted by Th17 was increased significantly in EAE+HS group as compared to EAE (by 10.25-fold) and healthy control (by 11.94-fold) (Figure 11D). Furthermore, the expression of IL-22 secreted by Th17 cells, was also increased in EAE+HS as compared to EAE (by 1.52-fold) and healthy control (by 3.02-fold) (Figure 11E). These results suggest that high sucrose exacerbates the severity of disease through the secretion of Th1 and Th17 in EAE mice.

We further evaluated the relative mRNA expression of MCP 1 in high sucrose induced EAE mice. We found that the expression of chemokine MCP1 was surprisingly increased in EAE+HS as compared to EAE (by 2.86-fold) and healthy control (by 10.36-fold) (Figure 11H).

Moreover, we confirmed the effects of high sucrose induced EAE expression of T-bet, and ROR-γt master transcription factor for the differentiation of Th1, and Th17 cells respectively. The expression of T-bet mRNA was increased in EAE+HS as compared to EAE and healthy control. Similarly, the relative mRNA expression of ROR-γt was also increased in EAE group as compared

to healthy control. But interestingly, in addition of high sucrose in EAE further increases the mRNA expression (by 2.73-fold) (Figure 11J).

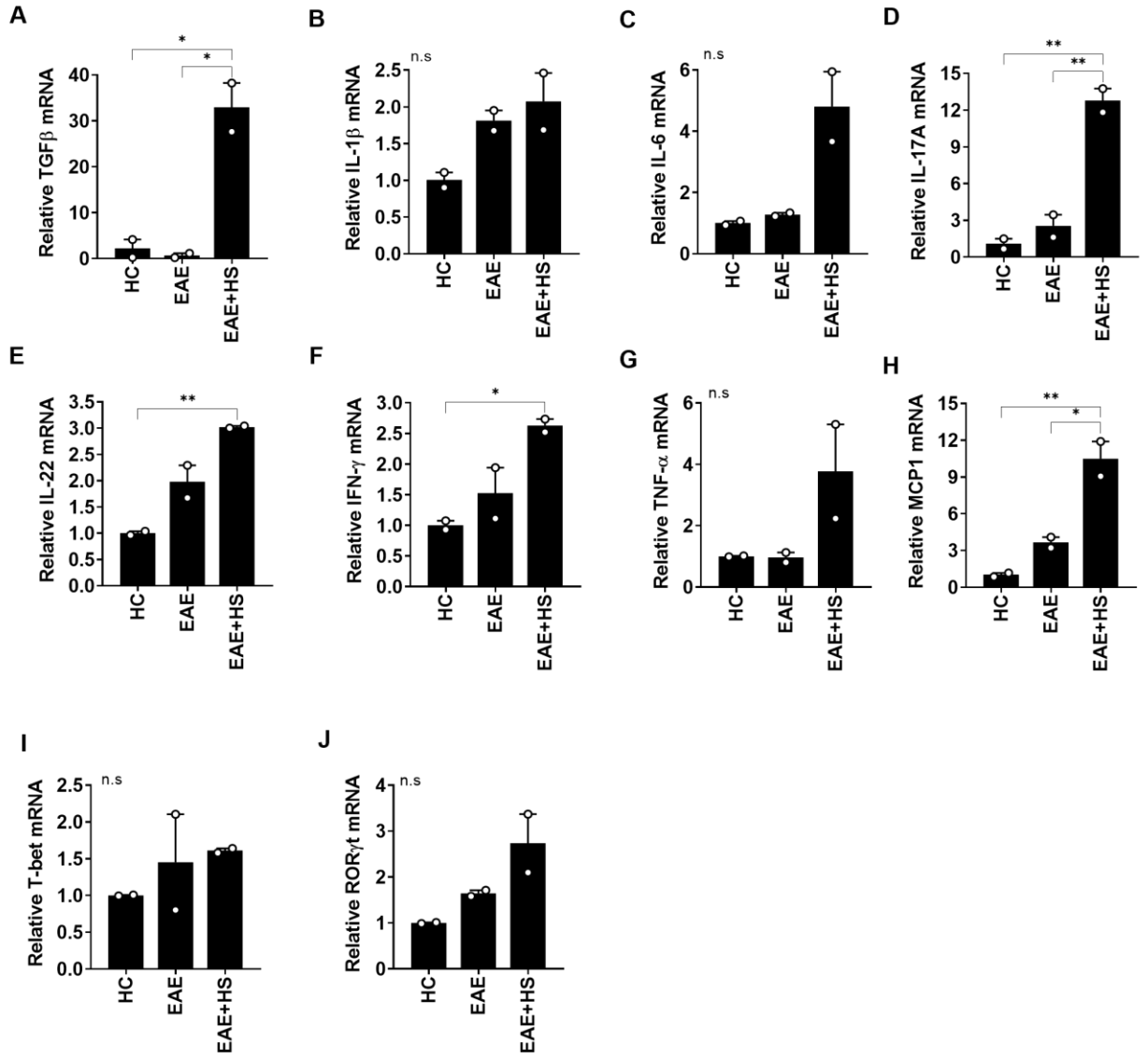


Figure 11. High sucrose upregulates the inflammatory cytokines in CNS in EAE mice

Quantitative PCR analysis to determine the relative mRNA expression levels of genes, growth factor (A) TGF- β, inflammatory cytokines (B) IL-1β, (C) IL-6, T cells regulatory cytokines (D) IL-17A, (E) IL-22, (F) IFN-γ, (G) TNF- α, (H) MCP1, (I) T-bet, and (J) RORγt. *(p<0.05), **(p<0.01) represents significant increase compared to healthy control.

4.9. High sucrose increases Iba-1 positive cells in brain and spinal cord of EAE mice

The activation of microglia may in turn causes neuronal damage through the release of potentially cytotoxic molecules such as proinflammatory cytokines. Therefore, we performed immunohistochemistry analysis of Iba-1 positive cells in brain and spinal cord to detect the activation of microglia in neuron. In brain we found, significantly increased number of Iba-1 positive cells in EAE+HS as compared to EAE (by 1.16-fold) and healthy control (by 2.45-fold) (Figure 12C and G). Similarly, likewise in brain, as expected we found the increased number of Iba-1 positive cells in EAE+HS as compared to EAE (by 1.7-fold) and healthy control (by 4.0419-fold) in spinal cord (Figure 12F and H). This result suggests that high sucrose intake promotes the activation of microglia in brain and spinal cord in EAE induced mice.

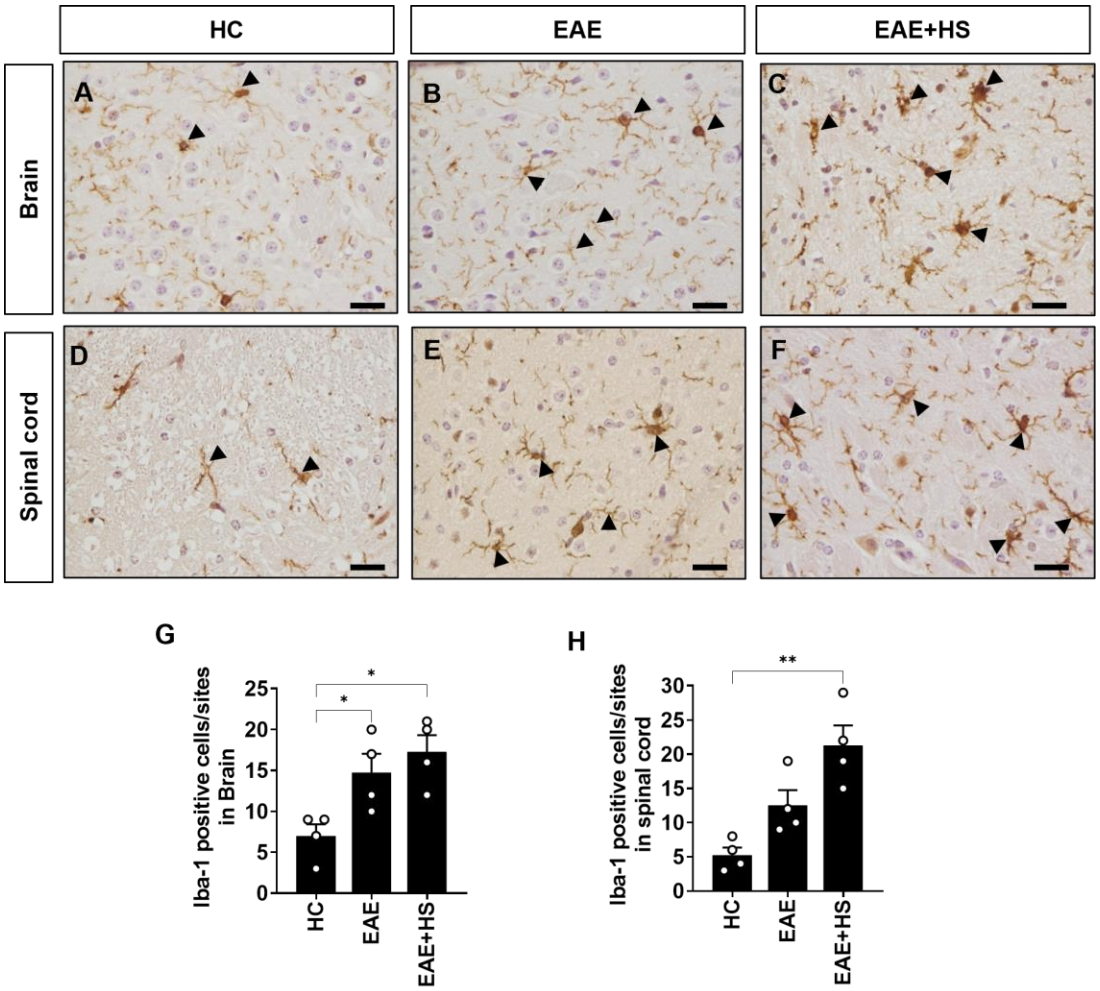


Figure 12. High sucrose increases the expression of Iba-1 positive cells in brain and spinal cord in EAE mice

Representative images of IHC positive cells in (A-C) brain and (D-F) spinal cord sections. (G) Iba-1 positive cells in brain and (H) Iba-1 positive cells in spinal cord. Arrow indicates positive cells in brain and spinal cord. Data are represented as the mean ± SEM of changes in values. * ($p < 0.05$), ** ($p < 0.01$) represents significant increase compared to healthy control. Scale bars of (A-F) are 25 μm.

4.10. High sucrose intake upregulates the demyelination in spinal cord in EAE mice

Demyelination of the spinal cord is the hallmark of disease such as multiple sclerosis (MS), an immune-mediated inflammatory disease of the central nervous system that leads to the loss of axonal condition and clinical symptoms. Therefore, we evaluated whether the high sucrose affects the score of nucleated cells in the white matter and for demyelination. As shown in figure, demyelination score in the spinal cord of mice in EAE+HS group was increased surprisingly as compared to that of EAE (by 1.46-fold) and healthy control (by 5.49-fold) (Figure 13I and J), ($p < 0.001$), which showed the marked subpial and perivascular demyelination. This result suggest that high sucrose shows severity of demyelination in the spinal cord promoting pathogenesis of EAE.

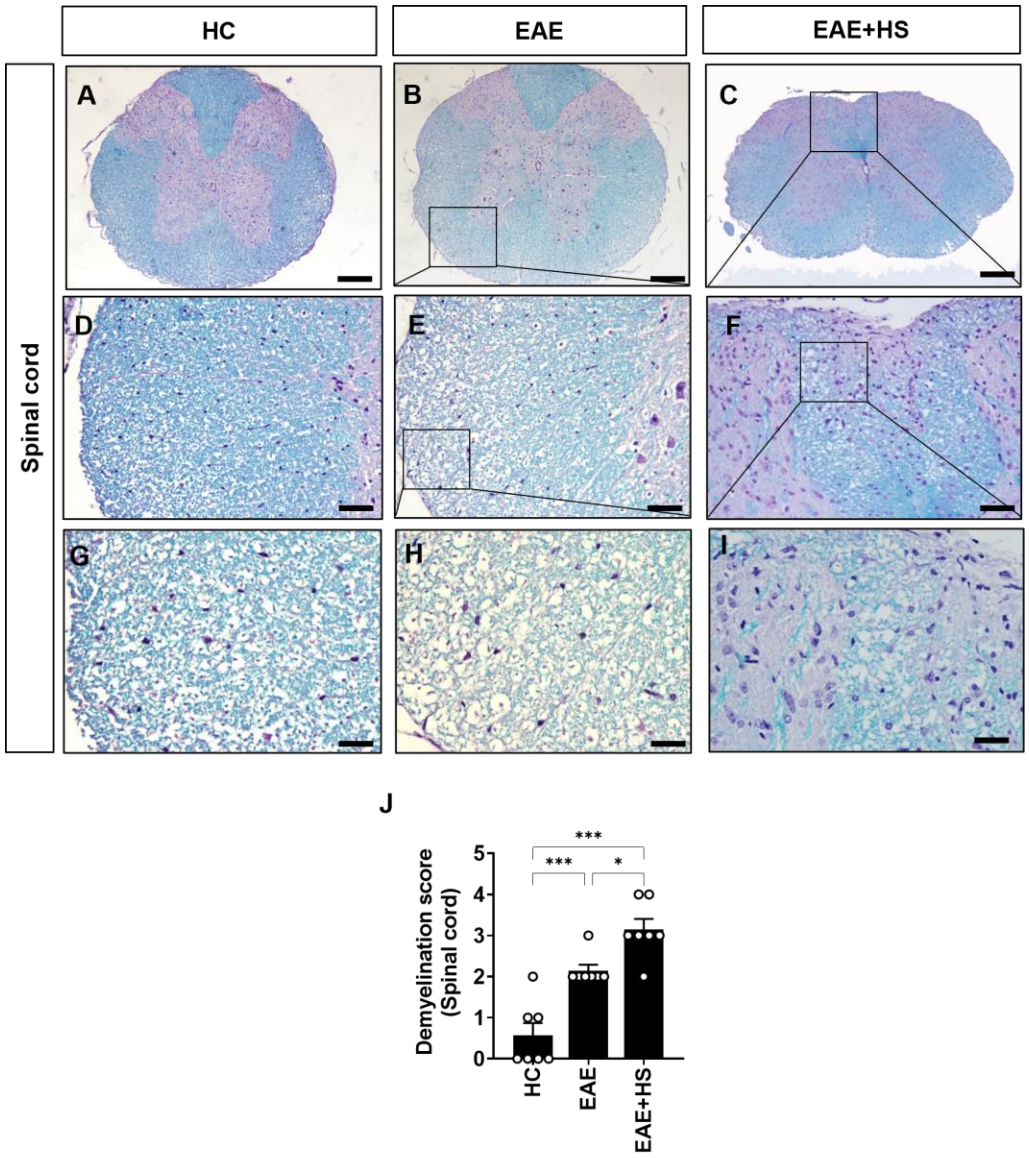


Figure 13. High sucrose upregulates demyelination of spinal cord in EAE mice

Luxol fast blue staining (LFB) was performed to access the demyelination in spinal cord (A-I). (A, D, G) Healthy control, (B, E, H) EAE, (C, F, I) EAE+HS, and demyelination score in spinal cord (J). All values were shown as mean ± SEM, *($p < 0.05$), ***($p < 0.001$) represents a significant up regulation as compared to untreated control. Scale bar of (A-I) are 25 μm.

4.11. High sucrose intake increases the expression of NF-H positive cells in brain and spinal cord in EAE mice

Inflammatory axonal damage is a fundamental pathogenic process in multiple sclerosis that leads to progressive neurological impairment. Therefore, we performed immunohistochemistry analysis of NF-H positive cells in brain and spinal cord to detect axonal damage in neuron. In brain we found, significantly increased number of NF-H positive cells in EAE+HS as compared to EAE (by 1.35-fold) and healthy control (by 2.87-fold) (Figure 14C and G). Similarly, likewise in brain, as expected we found the increased number of NF-H positive cells in EAE+HS as compared to EAE (by 1.5-fold) and healthy control (by 4.0-fold) in spinal cord (Figure 14F and H). This result suggests that high sucrose intake promotes the severe axonal damage in brain and spinal cord of EAE induced mice.

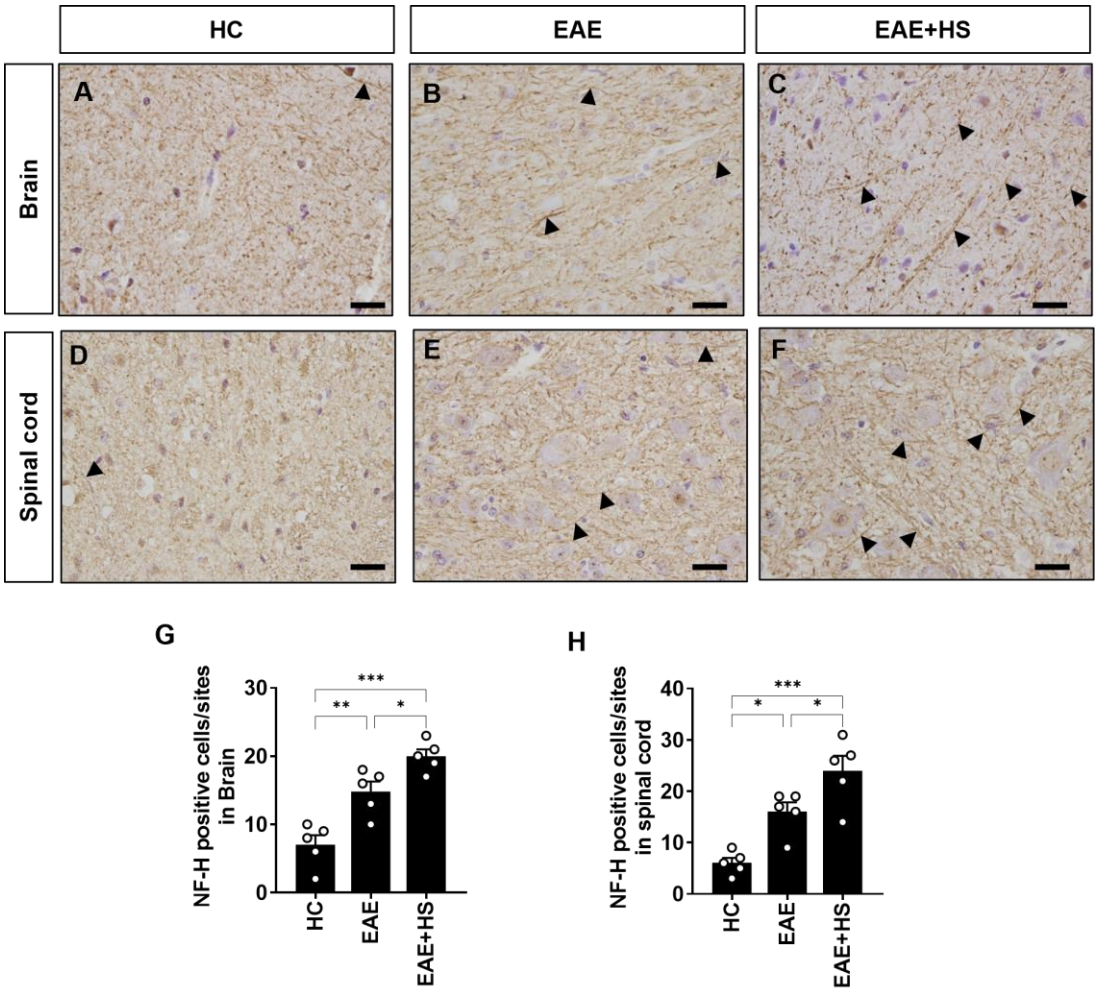


Figure 14. High sucrose increases the expression of NF-H positive cells in brain and spinal cord in EAE mice

Representative images of IHC positive cells in (A-C) brain and (D-F) spinal cord sections. (G) NF-H positive cells in brain and (H) NF-H positive cells in spinal cord. Arrow indicates positive cells in brain and spinal cord. Data are represented as the mean ± SEM of changes in values. * ($p < 0.05$), ** ($p < 0.01$), *** ($p < 0.001$) represent significant increase compared to healthy control group. Scale bars of (A-F) are 25 μm.

5. Discussion

Since long dietary components have been recognized to alter the course of illness in multiple sclerosis (MS) and its animal model EAE (Riccio and Rossano, 2015, Bagur et al., 2017). Majority of studies revealed about a sweetened diet, for instant a high glucose consumption, increases the risk of disease, such as obesity, cardiovascular disease, cancer, and type 2 diabetes (Goncalves et al., 2019, Hu et al., 2019). However, no evidence available to date clearly indicates the involvement of high sucrose drinking with EAE progression. In this research, we investigated the impact of high sucrose on the development of autoimmune illness MS using a mouse model of high sucrose diet (HSD). We have demonstrated that, the mice fed with HSD developed a worsened EAE with worse clinical score and pathological signs, than the control diet fed mice.

We produced high sucrose in EAE mice to establish a precise role of high sucrose in CNS autoimmune illness and discovered that the severity of EAE was surprisingly elevated. Greater inflammatory load and demyelination were linked to increased disease severity in EAE mice. T cells, particularly $CD4^+$ T cells, are the key mediators in EAE, the higher clinical symptom in high sucrose induced EAE animals might be ascribed to enhanced infiltration of T cells into the CNS lesion.(Goverman, 2009).

Previous studies support the notion that T helper type 1 (Th1) cells were widely known to be the predominant effector T cell fraction responsible for inflammation of immune system (Segal, 2003). Myelin-reactive T cells enter the CNS during MS/EAE demyelination by releasing proinflammatory cytokines and attracting and activating macrophages, as well as by releasing proinflammatory cytokines (Bauer et al., 1995). The pathogenic involvement of T helper type 17 (Th17) cells in the development and pathophysiology of EAE and MS has lately been emphasized

in various research (Rangachari and Kuchroo, 2013, Rostami and Ciric, 2013). In EAE and MS, $CD4^+$ T helper (Th) type, Th1 and Th17 cells are expected to initiate and regulates immune responses that results in tissue damage; on other hand, regulatory $CD4^+$ cells, and to some extent Th2 cells, are expected to be anti-inflammatory and protective. However, considering these and other emerging T-cell subsets, the immunological situation of immune-mediated CNS injury is more complicated (O'Brien et al., 2010). T-box transcription factor (T-bet) is a needed for controlling IFN- γ production in Th1 cells ROR-γt is the dominant transcriptional regulator of Th17 cells due to its capacity to induce the expression of IL-17A and IL-22 (Awasthi and Kuchroo, 2009). In the current work, we discovered that the CNS of high sucrose caused EAE mice had significantly greater levels of transcriptional factors for Th1 and Th17, as well as the production of Th1 and Th17 cytokines in $CD4^+$ T cells, suggesting that the presence of high sucrose in the CNS environment directly altered CNS-infiltrating effector T cells, resulting in an increase in EAE severity.

Our finding shows that in the CNS, IL-1β, IL-6, IFN- γ, and TNF α are upregulated in a disease manner during the start of the illness. These findings support the theory that Th1 and Th17 are responsible for EAE pathogenesis. Th1 cells release IFN- γ, which in turn activates macrophages to release TNF- α and IL-1β (t Hart et al., 2011). Furthermore, IL-6 is known to enhance the development of EAE by driving the differentiation of Th1 and Th17 cells.

Furthermore, this study showed that HSD-induced EAE is important for the activation of inflammatory cell. Their activation causes Th1 and Th17 cells to generate the effector cytokines IFN- γ, TNF-α, IL-17A, and IL-22, which can cause macrophage activation and neutrophil migration to the inflammatory areas, speeding up the lesion. Given the critical role of Th1 and Th17 $CD4^+$ cells in the course of EAE, an increase in both Th1 and Th17 is thought to aggravate

illness in our study's HSD-fed EAE mice. We discovered that in HSD-fed animals, both IFN- γ producing Th1 and IL-17A-producing Th17 cells were considerably increased, indicating that Th1 and TH17 cells are responsible for illness aggravation. The chemokine, MCP-1 facilitate recruitment of T-cells and macrophages into the CNS which increased surprising in our study. Hence therapeutic strategies aimed at inhibition of MCP-1 have potential as treatments for MS-associated neuropathic pain. The above data indicate that high sucrose has an effect of enhancing EAE. Astrocytes are the resident cells in the CNS. It has been reported that astrocyte participate in the inflammatory process, leading to the onset of EAE (Gao et al., 2017, Hofman et al., 1989). In previous studies it was shown that, astrocytic activation, also known as astrogliosis, is a critical component of the body's response to most or all neurological traumas, including infections, stroke, and neurodegeneration. The extent of astrogliosis can influence long-term recovery, and the response of astrocyte to different insults is likely to be graded and complex (Sofroniew and Vinters, 2010, Kang and Hébert, 2011). In our study, the number of increased astrocyte cell in high sucrose induced EAE mice reveals that, high sucrose may be responsible for a neurological disorder like neurodegeneration.

The high consumption of high-fat diet (HFD) and sugar-sweetened beverages are risk factors for developing obesity, insulin resistance, and fatty liver disease (Softic et al., 2017). Several recent studies have found that consumption of a high sugar diet is a major contributor to the rise of metabolic disease, particularly non- alcoholic fatty liver disease (NAFLD) and insulin resistance (IR) (Ragab et al., 2015). The expression of the lipogenic genes FAS, and SCD1, has been found influenced by a high simple carbohydrate diet (Dentin et al., 2005). However, it is yet unknown whether high sucrose intake influences the development of EAE through T cell differentiation and effects on hepatic tissue. In the current study, we have investigated the connection between the

dietary high sucrose on the pathogenesis of fatty liver disease as well as investigated the role inflammatory mediators in the process of pathogenesis. It has been found that the mice drinking with 20 % sucrose in water develops the severe liver steatosis as compared with regular water. It has been found that the expression of the lipogenic genes FAS and SCD1 rise as compared with the control. These results indicate that the sucrose component of dietary sugar is uniquely associated in accumulation of lipid droplets in liver with increased lipogenesis in hepatic tissues. To further confirm hepatic inflammation, we measured gene expression of inflammatory cytokines, IL-6, MCP-1 and TNF-α and we found higher expression in EAE+HS as compared to EAE and healthy control.

6. Conclusion

This study categorically shows the impacts of high sucrose level in EAE mice and observed various effects on brain and spinal cord. Induction of high sucrose in EAE mice showed higher clinical scores and infiltrated inflammatory cells, making disease at the peak in brain and spinal cord compared to healthy control mice. The high sucrose intake upregulates the demyelination activity, increasing the number of glial fibrillary acidic protein, and regulate the inflammatory response on brain and spinal cord, including inflammatory axonal damage lading to the progressive neurological impairment resulting to promote pathogenesis in EAE mice. Similarly, the chronic exposure of high sucrose exhibited excessive lipid accumulation in the liver and may cause hepatic steatosis in liver and activate severe inflammatory cells infiltration by activation of $CD3e^{+}$ $CD4^{+}$ helper T cells and $CD3e^{+}CD8a^{+}$ cytotoxic T cells population. Furthermore, mRNA expressions of IL-17A, TGF-β, and MCP-1 in CNS were significantly higher in EAE mice induced by high

sucrose. Also, such mice have the exacerbation of inflammatory gene expressions, i.e., IL-6, TNF-α, IFN-γ, IL-1β, and IL-22 resulting in revealing high consumption of sucrose with severe physiological impacts on EAE mice, which may be applicable on other mammalian species including human beings.

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