Linagliptin attenuates thioacetamide-induced hepatic encephalopathy in rats: Modulation of C/EBP-β and CX3CL1/Fractalkine, neuro-inflammation, oxidative stress and behavioral defects

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\textbf{ABSTRACT}

The degree of neuroinflammation is correlated mainly with cognitive and motor dysfunctions associated with hepatic encephalopathy (HE). The current study was conducted to explore the possible protective potential of the antidiabetic drug; linagliptin (LNG; 10 or 20 mg/kg) against HE induced by thioacetamide (TAA) in rats. Animals received two consecutive intraperitoneal injections of TAA (200 mg/kg) on alternate days. Neurobehavioral tests were performed 24 h after the last injection, and rats were sacrificed 24 h later (48 h). The higher LNG dose more effectively protected against TAA-induced changes. Administration of LNG for 15 days before TAA notably mitigated TAA-induced acute liver injury and HE, as verified by the marked improvement in motor coordination, locomotor activity, and cognition function. LNG maintained both brain and liver weight indices and retracted the hyperammonemia with a prominent suppression in liver transaminases. This was accompanied by an evident modulation of hepatic and hippocampal oxidative stress markers; GSH and MDA. LNG attenuated both liver and hippocampal pro-inflammatory cytokine; IL-1β while augmented the anti-inflammatory one; IL-10. It noticeably reduced hepatic and hippocampal COX-2 and TNF-α and maintained hepatic and brain architectures. It also induced a marked decrease in the inflammation-regulated transcription factor, C/EBP-β, with a profound increase in hippocampus's anti-inflammatory chemokine, CX3CL1/Fractalkine. LNG modulated TAA-induced disturbances in hippocampal amino acids; glutamate, and GABA with a significant increase in hippocampal BDNF. In conclusion, the regulatory effect of LNG on neuroinflammatory signaling underlines its neuroprotective effect against progressive encephalopathy accompanying acute liver injury.

1. Introduction

Hepatic encephalopathy (HE) is a critical neuropsychiatric disorder usually affecting hepatic failure patients [1]. It is characterized by cognitive and motor deficits, personality changes, sleep disturbances, shortened attention, which may lead lastly to coma and death [2]. HE has a profound influence on patients' life quality and counts as a socio-economic burden [3].

While increased brain ammonia is identified as a chief etiological factor in this condition, various studies have documented the pivotal role of neuroinflammation in the pathogenesis of HE. [4]. Interestingly, the severity of HE is irrelevant to the degree of liver dysfunction and ammonia level while considerably related to inflammatory markers [5]. The degree of neuroinflammation was correlated mainly with motor and cognitive dysfunctions associated with HE following liver failure [6]. In both patients and several experimental models of liver failure, pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1β, and IL-6 are elevated. Regulation of pathogenic cytokines production in the brain is mainly a result of microglia stimulation through various signals such as that transduced by chemokines [7]. Fractalkine/CX3CL1 is the sole member of the CX3C chemokine class [8]. Fractalkine is highly expressed constitutively in normal neurons and

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activated astrocytes [9]. Upon cleavage of the native fractalkine [10], its soluble form binds to its receptor, CX3CR1, which is localized in the microglia [11]. Fractalkine/CX3CR1 signaling kept microglia in a quiescent state [12] and was revealed to decrease IL-1β production in microglia treated with lipopolysaccharide (LPS) to inhibit neuro-inflammation [13]. Fractalkine constitutive expression is significantly reduced due to neuronal damage, resulting in a lack of fractalkine/CX3CR1 signaling, which induces microglial activation and recruitment [14] and is implicated in cognitive impairment in a mouse model of obesity [15]. On the other hand, the inflammation-regulated transcription factor, CCAAT/enhancer-binding protein beta (c/EBPβ), was reported to drive a potent pro-inflammatory response under the circumstances involving microglial activation [16].

It has also been proposed that reducing inflammation has a therapeutic value in treating cognitive dysfunction in mild or severe HE [17]. Glucagon-like peptide-1 (GLP-1) regulates glucose homeostasis and insulin signaling. GLP-1 receptors are extensively expressed in the brain [18] and the neuroprotective and neurotrophic potentials of their activation have been tackled in several preclinical studies. GLP-1 analogs were reported to enhance brain-derived neurotrophic factor (BDNF) [19–22], a neurotrophic factor that promotes central and peripheral neurons survival, differentiation, and outgrowth [23] and its deficiency is mainly implicated in behavioral dysfunction in various neuropsychiatric disorders including HE [24,25]. Dipeptidyl peptidase-4 (DPP-4) inhibitors have established potent neuroprotective properties where saxagliptin, alogliptin, and linagliptin (LNG) were formerly stated to regulate neurons survival, differentiation, and outgrowth [23] and its deficiency was revealed to decrease IL-1β production [24]. Fractalkine/CX3CR1 signaling kept microglia in a quiescent state [12] and was revealed to decrease IL-1β production under the circumstances involving microglial activation [16].

LNG, a dipeptidyl peptidase-4 (DPP-4) inhibitor, is approved and is primarily regarded as the first-line treatment of patients with type 2 diabetes [30]. LNG markedly countered cognitive deficits in diabetic mice subjected to transient cerebral ischemia. It reduced reactive microglia count and preserved surviving neurons in hippocampi and cortices of diabetic mice [31]. Treatment of Human U937 monocytes with LNG repressed LPS-induced TNF-α and IL-6 production [32]. Moreover, LNG showed extended anti-inflammatory action in endothelial cells of human umbilical vein [33]. Therefore, the current work sought to evaluate the protective potential of LNG against thioacetamide (TAA)-induced acute liver failure (ALF) and HE in rats. It also extended to examine its modulatory effect on C/EBP-β and CX3CL1/Fractalkine as crucial regulators of inflammatory signaling and neurological complications in an attempt to underline the mechanisms implicated in its coveted neuroprotective effect.

2. Materials and methods

2.1. Animals

Adult male Wistar rats (200–250 g) were procured from the National Research Centre’s animal facility in Dokki, Giza. Rats were housed under ideal environmental conditions, including a temperature of (22 ± 2 °C) and a 12/12-h light/dark cycle. They were kept at a typical laboratory diet and allowed water ad libitum. The experiment was approved by the Medical Research Ethics Committee (MREC) of the National Research Centre (462/16) and the Research Ethics Committee of the Faculty of Pharmacy, Cairo University (REC-FOPCU) PT (2112).

2.2. Drugs and chemicals

TAA (Cat. #172502) and LNG (Cat. # ATE428009629) were obtained from Sigma Chemicals Co. (St. Louis, MO, USA).

2.3. Induction of acute hepatic encephalopathy

Hepatic encephalopathy was induced in rats as described earlier [34] by two successive intraperitoneal injections of TAA (200 mg/kg) in 48 h intervals on the 13th and 15th days of the experiment. TAA was dissolved in saline immediately before use for intraperitoneal (i.p.) injection. Along with TAA, all of the animals were given ringer lactate solutions (10 mg/kg/day, i.p.) and dextrose (5%) to protect them from hypoglycemia, renal failure, and electrolyte imbalance until the experiment was finished [35].

2.4. Experimental design

Animals (12 rats per group) were randomly allocated into four groups. Group I served as a normal control and received intraperitoneal saline injections for two days at 48 h-interval. Groups II: rats received two successive injections of TAA (200 mg/kg; i.p.) in 48 h intervals to induce acute liver injury and HE. Groups III and IV: rats treated orally with LNG (10 or 20 mg/kg/day) for 15 consecutive days [36–38] and received two successive injections of TAA (200 mg/kg; i.p.) in 48 h intervals on the 13th and 15th days of the experiment.

2.5. Behavioral assessment

2.5.1. Assessment of locomotor activity using activity cage

A grid floor activity cage (Model No. 7430, Ugo Basile, Comerio, Italy) was utilized to track the rats’ movements and measure their spontaneous locomotor activity. Movements that disrupted infrared beams were detected automatically, and the activity cage software processed the beam interruption information to count the horizontal movements. Before starting treatment, rats were given an hour to get used to the test room. The basal activity counts were obtained after each rat was introduced individually in the activity cage for a 5-min session. At the end of the session, each rat was gently removed from the test cage and returned to its home cage. Between sessions, the arena was cleaned with a 70% (v/v) alcohol solution in distilled water to avoid scent cues [39]. Each rat was reintroduced to the activity cage for a 5-min session twenty-four hours following the last dose of treatment, and the final activity counts were recorded to determine the percentage relative of locomotor activity for each rat as follows: Activity counts (%) = (final activity counts / basal activity counts) × 100 [40,41].

2.5.2. Assessment of motor coordination and balance using rotarod

Rat motor coordination was tested using an accelerating rotarod (Model No. 7750; Ugo Basile, Italy). All rats received three training sessions at a set speed for three days prior to the start of the experiment to ensure consistent performance. Rats were located on the accelerating rod, and the falling latency time was recorded for each rat prior (basal) to the experiment as well as at its end (final) [42]. Finally, each rat’s results were reported as a percentage of relative falling latency time.

2.5.3. Assessment of cognitive function using T-maze task

Spatial memory is tested by the T maze using the rewarded alternation. Each trial in this test comprised two runs. The first one is the sample run: during which one of the goal arms was closed to force the rat to enter a particular arm, where it was rewarded with food. Before the consequent choice run, the second arm was opened, and the rat chose to enter either arm. If it entered the alternate arm, it received a food reward. Thus, correct performance depends mainly on memorizing which arm was entered in the sample run to guide proper response in the choice one. Each rat was subjected to five trials, with a 10-minute interval between them. The percentage of rewarded alternations was finally calculated [43,44].

2.6. Blood sampling and tissue preparation

Twenty-four hours after performing all behavioral tests, blood samples were withdrawn under light anesthesia. Serum was separated and stored at –80 °C to be used for biochemical analysis. The animals were decapitated after the blood samples were taken, and the brains and livers were immediately extracted and immersed in 10% formalin for histological studies.
were collected and weighed immediately to calculate brain and liver weight indices. Four brains and livers from each group were kept in 10% formalin for histological and immunohistochemical examinations. Brains of the remaining rats were sectioned into 2 hemispheres, then the hippocampi, as well as liver tissues, were kept at −80 to be used later in biochemical measurements

2.7. Biochemical measurements

2.7.1. Assessment of blood glucose, serum alanine aminotransferase, aspartate aminotransferase, and serum ammonia

Before the experiment, blood glucose was measured with a glucometer (Accu Chek, Roche Diabetes Care, Inc., USA) at a fixed time of day and after the last treatment. Blood samples were obtained via the orbital plexus to determine blood glucose levels, and a drop of blood was put on the glucometer strip installed in the equipment.

Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured in the sera [45] using Biodiagnostic kits (Egypt) (Cat #: AL 10 31 (45) and AS 10 61 (45), respectively). To estimate serum ammonia concentration, as directed by the manufacturer, BioVision test kits (USA) Cat #: K470 was used according to modified Berthelot assay procedures [46].

2.7.2. Determination of hippocampal malondialdehyde and reduced glutathione using HPLC

To make a 1 mM stock solution of malondialdehyde (MDA), 25 µl of 1,1,3,3-tetraethoxy propane (TEP) were dissolved in 100 ml water. 1 ml TEP stock solution was hydrolyzed in 1% sulfuric acid (50 ml) and kept for 2 h at room temperature to make the working standard. The resulting 20 nmol/ml MDA standard was then diluted with 1% sulfuric acid to obtain a final concentration of 1.25 nmol/ml, which was utilized to estimate total MDA [47].

The thiol components of reduced glutathione (GSH) were detected using HPLC according to the method of [48]. Sigma Chemical Company provided a reduced glutathione reference standard. 1 mg/ml stock dissolved in methanol (75%) and diluted prior to use.

2.7.3. Determination of hepatic malondialdehyde and reduced glutathione concentration

According to the manufacturer’s directions, the liver homogenates were tested for MDA and GSH levels using Biodiagnostic assay kits (Egypt) (Cat #: MD 25 29 and GR 25 11, respectively).

2.7.4. Assessment of liver and hippocampal inflammatory cytokines

Liver and hippocampal contents of IL-1β and IL-10 were measured using ELISA kits (R & D systems, MN, USA; Cat #: RLB00 and R1000, respectively).

2.7.5. Determination of hippocampal glutamate and GABA

Hippocampal tissue was homogenized in aqueous HPLC grade methanol (10% w/v) at a concentration of 75% [49]. The homogenate was swirled, and the supernatant was dried at room temperature using vacuum (70 Millipore) and used for GABA (gamma-aminobutyric acid) and glutamate estimation. HPLC was used to identify hippocampal glutamate and GABA utilizing the precolumn PITC derivatization procedure, as described by [50].

2.7.6. Assessment of hippocampal brain-derived neurotrophic factor

Hippocampal BDNF was assayed using the ChemiKine BDNF Sandwich ELISA (Millipore, Merck Germany; Cat #: CYT306).

2.7.7. Assessment of hippocampal CCAAT/enhancer-binding protein-beta and fractalkine

Hippocampal C/EBP-β was evaluated using rat ELISA Kit (Assay Genie, Inc.TM, Dublin, Ireland; Cat #: RTEB0421). Likewise, the hippocampal homogenate was used to estimate fractalkine content by a specific ELISA kit (MyBioSource, Inc., San Diego, CA, USA; Cat #: MBS177370) according to the manufacturers’ instructions.

2.8. Histopathological studies

Samples of hepatic and brain tissues from each group were cautiously isolated and fixed for 24 h in 10% buffered neutral formalin, followed by their routine processing to get 4-5um paraffin sections. The sections were stained with hematoxylin and eosin (H&E) [51] and blindly examined with a light microscope (Olympus, Germany).

2.9. Immunohistochemical studies of cyclooxygenase-2 and tumor necrosis factor-α

For detection of TNF-α and cyclooxygenase-2 (COX-2) expression in livers and hippocampi of all groups, the immunohistochemical technique was performed on paraffin slides using avidin-biotin-peroxidase as reported earlier [52]. In summary, the slides were deparaffinized, exposed to antigen retrieval, and then incubated with monoclonal antibodies of COX-2 (Abcam Co., Cambridge, UK; Cat#: ab179800) and TNF-α (Abcam Co., Cambridge, UK; Cat#: ab220210), followed by different reagents of avidin-biotin-peroxidase technique (Vactastain ABC peroxidase kit, Vector Laboratories, Burlingame, CA, USA; Cat#: PK-4000) to detect the antigen-antibody complex. Chromagen 3,3-diaminobenzidine tetrahydrochloride (DAB, Sigma Chemical Co.; Cat #: D7304) was utilized to visualize the expression of both markers. The optical density of the positive brown color of both markers expression in 5 randomly chosen microscopic fields in each organ was quantified and averaged using image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA). To eliminate bias, all histopathological examinations were done by a qualified investigator who was unaware of the samples’ identities.

2.10. Statistical analysis

The results are expressed as mean ± SD, then subjected to One-way analysis of variance (ANOVA), followed by the post hoc Tukey’s multiple comparison test. GraphPad Prism v. 8.0 was used to analyze the data (GraphPad Software, Inc., CA, USA). When the P-value is <0.05, the difference is considered to be significant.

3. Results

3.1. LNG protects against TAA-induced behavioral changes in rats

Intraperitoneal administration of TAA (200 mg/kg) on the 13th and 15th was associated with a decrease in falling latency time and locomotor activity by about 62% and 74% (P < 0.0001), respectively, as compared to the control group. Oral treatment with LNG (10 & 20 mg/kg) for 15 consecutive days showed an increment of falling latency time by 110% and 144% (P < 0.0001), respectively, reaching normal time with the large dose (P = 0.405), along with an increase in locomotor activity by 62% and 89% (P < 0.0001), as compared to TAA group (Fig. 1a and b).

Administration of LNG (200 mg/kg) twice was accompanied by a decrease in the percentage of rewarded alternations of rats in the T-maze apparatus by 69% (P < 0.0001), as compared to the control group. Treatment with LNG (10 & 20 mg/kg) showed a marked increment in rats’ rewarded alternations by about 136% and 163% (P < 0.0001), respectively as compared to TAA-injected rats, reaching normal value with the large dose (P = (0.198)) (Fig. 1c).

3.2. LNG protects against TAA-induced liver and brain weight indices changes in rats

Thioacetamide (200 mg/kg) injection on the 13th and 15th was
accompanied by an increase in liver and brain weight indices by 54% and 22% (\(P < 0.0001\) and 0.01), respectively, as compared to the control group. Oral treatment with \(\text{LNG (10\&20 mg/kg)}\) maintained normal liver and brain weight indices as compared to TAA-injected rats (\(P = 0.419\) and 0.159, respectively) (Table 1).

### 3.3. LNG protects against TAA-induced blood glucose, and serum biochemical parameters changes in rats

Injection of TAA twice at the end of the experiment showed a hypoglycemic effect expressed by a 32% decrease in the blood glucose level (\(P < 0.0001\)). Moreover, TAA exhibited a dramatic elevation in the serum level of ammonia by 65%, indicating a state of hyperammonemia (\(P < 0.0001\)) compared to control rats. TAA-induced HE in rats was associated with a prominent elevation in the serum activities of ALT and AST by about 128% and 91% (\(P < 0.0001\)), respectively. However, LNG (10\&20 mg/kg; p.o) attenuated this hypoglycemic effect (\(P < 0.05\)) and succeeded to reduce serum ammonia level showing a decrease by 27.5% and 36% (\(P < 0.0001\)), respectively, as compared to TAA-treated rats, reaching the normal level with the large dose (\(P = 0.77\)).

Treatment with LNG (10\&20 mg/kg; p.o) counteracted the elevated liver function parameters i.e. ALT by 20% and 37% (\(P < 0.001\) and 0.0001), respectively as well as AST by 22% and 40% (\(P < 0.0001\)), respectively in comparison to TAA-injected rats (Table 2).

### 3.4. LNG protects against TAA-induced hepatic and hippocampal malondialdehyde and reduced glutathione changes in rats

Rats with HE displayed a dramatic oxidative stress status; TAA showed a marked decrease in the hepatic and hippocampal GSH by about 61% and 50% (\(P < 0.0001\)), respectively, and an elevation in the hepatic and hippocampal MDA by 143% and 50% (\(P < 0.0001\)), respectively, as compared to control group.

On the other hand, LNG (10\&20 mg/kg; p.o) diminished the oxidative status accompanied with HE evidenced by the prominent elevation in the liver GSH by 57% and 120% (\(P < 0.05\) and 0.0001), respectively, and hippocampal GSH by 70% (\(P < 0.0001\)) for LNG 20-
3.5. **LNG protects against TAA-induced hepatic and hippocampal inflammatory markers changes in rats**

HE displayed an elevation in the hepatic and hippocampal pro-inflammatory mediator; IL-1β by 165% and 136% (P < 0.0001), respectively and a reduction in the anti-inflammatory mediator, IL-10 in the liver and hippocampus by 70% and 58% (P < 0.0001), respectively, in comparison to control group. LNG (10 and 20 mg/kg; p.o) reduced hepatic IL-1β by 15% and 31% (P < 0.001 and 0.0001), respectively, along with a decrement in hippocampal IL-1β by 46.5% and 50% (P < 0.0001), respectively. Contrariwise, LNG (10 & 20 mg/kg; p.o) elevated hepatic IL-10 by 108% and 137% (P < 0.0001), receptively, along with hippocampal IL-10 by 34% and 101%, (P < 0.001 and 0.0001) respectively, as compared to TAA-treated rats (Fig. 2a, b, c & d).

3.6. **LNG protects against TAA-induced hippocampal amino acids (GABA and Glutamate) changes in rats**

The results are presented as mean ± SD (n = 8). One-way ANOVA was performed for the statistical analysis, followed by Tukey’s Multiple Comparison test, where, *p < 0.05 vs. control, **p < 0.001 vs. control, ***p < 0.0001 vs. control, ****p < 0.0001, respectively, as compared to the control group. LNG (10&20 mg/kg) reduced the hippocampal GABA by about 10.5% and 17.5% (P < 0.0001), respectively, as compared to the TAA group, reaching the normal level with the large dose (P = 0.0314). Hippocampal glutamate was reduced by about 19% and 22% (P < 0.0001) after LNG treatment at 10 or 20 mg/kg, as compared to TAA-injected rats, respectively reaching normal level (P = 0.926 and 0.851) (Fig. 3a & b).

3.7. **LNG protects against TAA-induced hippocampal BDNF changes in rats**

TAA-induced HE exhibited a prominent reduction in the hippocampal BDNF by 63.5% (P < 0.0001) compared to the control group. Treatment with LNG (10 & 20 mg/kg) elevated the hippocampal BDNF by 70.5% and 156% (P < 0.0001), respectively, as compared to TAA-injected rats (Fig. 3c).

3.8. **LNG protects against TAA-induced hippocampal C/EBP-β and fractalkine changes in rats**

The liver of control animals exhibited normal histological architecture (Fig. 5ia). On the other hand, rats that received TAA showed severe hepatocellular degeneration and necrosis with marked vacuolar and ballooning degeneration (Fig. 5ib). The portal triads revealed severe inflammatory reactions with the proliferation of biliary duct epithelial cells and fibrous proliferation with its peripheral extension. The latter resulted in bridging fibrosis and parenchymal pseudolobulation (Fig. 5ic). Dense lymphocytic infiltration was observed along with apoptosis (Fig. 5id). Livers of LNG (10 and 20 mg/kg; p.o.) treated rats showed a dose-dependent decreased fibrosis and decreased intensity of hepatocellular necrobiotic changes with limitation of the fibrous proliferation to the portal areas and an apparent absence of parenchymal pseudolobulation (Fig. 5ie and f).

Hippocampi of control rats showed normal histological structure (Fig. 5ia and b), while those of TAA-injected rats revealed marked neuronal cell damage. The dentate gyrus (DG) of TAA rats showed a marked vacuolar degeneration and pyknosis of the small pyramidal cells with degeneration of scattered granular cells (Fig. 5ic). The CA demonstrated neuronal degeneration, with some necrotic cells and marked vacuolar degeneration and pyknosis of the small pyramidal cells (Fig. 5id). However, treatment with LNG showed a decrease in neuronal cells necrobiotic changes either with the use of low dose (Fig. 5ie and f) or the high dose (Fig. 5if and g) as presented by a mild degree of neuronal degenerative and scattered necrosis of the DG and CA areas of the hippocampi of those rats.
3.10. LNG protects against TAA-induced hepatic and hippocampal COX-2 and TNF-α changes in rats

Immunohistochemical examination revealed hepatic and hippocampal areas of normal rats exhibiting negative expression of COX-2 (Fig. 6 Ia and IIa) and TNF-α (Fig. 7 Ia and IIa). Liver tissues and hippocampi of TAA rats showed a marked expression of COX-2 ($P < 0.0001$) (Fig. 6 Ib and IIb) and TNF-α ($P < 0.0001$) (Fig. 7 Ib and IIb).

On the other hand, rats treated with LNG (10 and 20 mg/kg) showed a dose-related decreased expression of both COX-2 (Fig. 6 Ic, d, and IIc, d) and TNF-α (Fig. 7 Ic, d, and IIc, d), respectively in livers and hippocampi as presented in Figs. 6 Ie, Ile, and 7Ie, Ile ($P < 0.0001$), as compared to TAA group.

4. Discussion

This investigation reveals the efficiency of LNG in guarding against behavioral and neurological exacerbations associated with TAA-induced HE. This effect was manifested by the largely preserved integrity of hippocampal neurons detected in LNG-treated rats with enhanced locomotor activity, motor coordination, and retrieval of spatial memories.

In parallel, liver and brain indices were kept at the normal range following treatment with LNG. Aminotransferases (ALT and AST) were significantly reduced in LNG groups compared to TAA-treated rats. LNG also attenuated the hypoglycemic effect of TAA and decreased blood level of ammonia, verifying its hepatoprotective activity and improvement of the hepatic detoxification process. Based on the current results, both doses (10 and 20 mg/kg) of LNG have verified hepatoprotective as well as neuroprotective potentials. However, the highest dose (20 mg/kg) was more effective in preserving the endpoints in almost normal levels. Coherent with these results, the hepatoprotective potential of LNG in various liver diseases was previously reported and was attributed to its DDP-4 inhibition activity [53,54].

Several lines of evidence strongly suggested a synergistic interplay between inflammatory cytokines, hyperammonemia, reactive oxygen species (ROS) in astrocyte activation, brain edema, and behavioral deficits observed in HE [55]. Ample experimental models of acute liver injury have reported increased circulating inflammatory mediators and pro-inflammatory cytokines [56,57]. These cytokines stimulate the BBB’s vascular endothelium, releasing a variety of inflammatory mediators into the brain [58]. Furthermore, circulating pro-inflammatory cytokines can pass through defective BBB areas or cross the BBB directly to activate astrocytes and microglia, resulting in the generation of a full repertoire of cytokines such as IL-1, IL-6, and TNF-α, resulting in cerebral inflammation [59]. In parallel, elevated ammonia level during HE induces neuroinflammation via direct activation of astrocytes and microglia [17,60] contributing to cognitive and motor impairment [60]. Further, blocking peripheral inflammation with an anti-TNFα prevented neuroinflammation in rats with HE, advocating that peripheral inflammation triggers neuroinflammation in HE [61].

LNG exhibited a remarkable anti-inflammatory activity evidenced by a marked rise in the anti-inflammatory cytokine; IL-10, and a decline in the inflammatory mediators, IL-1β, TNF-α, and COX-2 of both liver and hippocampus of TAA-treated rats. Coherent with these results, several in
vitro and in vivo studies demonstrated the robust anti-inflammatory effect of LNG [32,33,62-64].

During HE, neuroinflammation could impair motor and cognitive functions by altering neurotransmission. A previous in vitro study correlated the ensued inflammation with increased GABAergic tone in hyperammonemia and HE. Neuroinflammation boosted GAT-3 transporters expression, which mediates GABA release from activated astrocytes under pathological conditions, leading to increased extracellular GABA. The rise in GABA promoted motor defects and diminished the glutamate-nitric oxide-cyclic guanosine monophosphate pathway.

Fig. 3. LNG protects against TAA-induced hippocampal GABA (a), glutamate (b) and BDNF (c) changes in rats. The results are presented as mean ± SD (n = 8). One-way ANOVA was performed for the statistical analysis, followed by Tukey’s Multiple Comparison test, where, ***p < 0.001 vs. control, ****p < 0.0001 vs. control, @@@@p < 0.0001 vs. TAA-treated group, ##p < 0.01 vs. LNG-10, ####p < 0.0001 vs. LNG-10.

Fig. 4. LNG protects against TAA-induced hippocampal C/EBPβ (a) and fractalkine (CX3CL1) (b) changes in rats. The results are presented as mean ± SD (n = 8). One-way ANOVA was performed for the statistical analysis, followed by Tukey’s Multiple Comparison test, where, ****p < 0.0001 vs. control, @@p < 0.01 vs. TAA-treated group, @@@@@p < 0.0001 vs. TAA-treated group, ##p < 0.01 vs. LNG-10, ####p < 0.0001 vs. LNG-10.
Similarly, neuroinflammation could also hinder astrocytic glutamate uptake induced by TAA injection in rats. Interestingly, LNG exerts a significant antioxidant activity evidenced by the increase in GSH and the decrease in MDA in the hippocampus in TAA-treated rats. The antioxidant and anti-inflammatory potentials exerted by LNG in the current investigation were highly reflected on its modulatory effect on ammonia levels along with hippocampal glutamate and GABA concentration to guard against motor incoordination as well as locomotor and cognitive dysfunctions in rats with HE. In agreement with our results, the current investigation was highly reflected on its modulatory effect on ammonium levels along with hippocampal glutamate and GABA concentrations in rats with HE. In agreement with our results, LNG mitigated cognitive impairment in diabetic mice [31,71] and in a rat model of AD [72] by curbing microglial activation and oxidative stress. Likewise, it guarded against demyelination and behavioral deficits induced by cuprizone in mice via virtue of its antioxidant and anti-inflammatory properties [73]. In addition, LNG effectively restored hippocampal BDNF in TAA-treated rats to ensure neuronal recovery.

Deprivation of the neurotrophic BDNF could exacerbate inflammatory response through upregulation of C/EBPβ [74], which regulates genes crucial to glial activation [75]. Furthermore, the expression of C/EBPβ has been induced in mouse cortical astrocytes by pro-inflammatory cytokines [76]. In parallel, interference with BDNF neurotrophic downstream signaling reduced the gene expression of fractalkine and its receptors in the hippocampi of normal mice, inducing severe cognitive deficits [15]. The current study revealed a noticeable increase in hippocampal C/EBP-β along with a decreased level of fractalkine in TAA-treated rats, confirming the role of microglia activation and astrocyte dysfunction in the development of HE-associated neuroinflammation and neurobehavioral disorders [77]. Consistent with these results, C/EBPβs have been involved in CNS inflammation [78]. Moreover, experimentally induced ALF was accompanied by neurobehavioral insult due to dysregulation between anti-inflammatory chemokines (CX3CL1) and inflammatory chemokines, causing microglial activation and HE progression [79].

Of note, the neuroprotective effect of LNG was further confirmed by the reduction of hippocampal C/EBP-β and the restoration of hippocampal Fractalkine/CX3CL1 to pin down its anti-inflammatory potential in the current study. Modulation of C/EBP-β was reported to alleviate brain injury and inflammation [80] with memory and learning function improvement [81]. In addition, restoration of hippocampal Fractalkine/CX3CL1 implies neuroprotective effect via preventing microglial activation and subsequent inflammatory cytokine production as observed herein and previously [82]. The modulatory effect of LNG on both C/
Fig. 6. COX-2-immune-stained photomicrograph of (I) Liver and (II) hippocampal sections. I: (a) liver of control rat showing negative expression, (b) liver of TAA rat showing marked expression, (c) liver of TAA + LNG-10 and (d) TAA + LNG-20 treated rats showing marked dose-related decreased expression, (e) The area percent of the positive brown color of COX-2 expression presented as optical density. II: (a) the hippocampus of a control rat showing negative expression (b) TAA group showing marked expression of COX-2, (c) TAA + LNG-10, and (d) TAA + LNG-20 treated rats showing dose-related decreased expression. (e) The area percent of the positive brown color of COX-2 expression is presented as optical density. Results are presented as mean ± SD. One-way ANOVA was performed for the statistical analysis, followed by Tukey's Multiple Comparison test, where ****p < 0.0001 vs. control, @@@@@p < 0.0001 vs. TAA-treated group, #p < 0.05 vs. LNG-10, ##p < 0.01 vs. LNG-10. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 7. TNF-α-immune-stained photomicrograph of (I) Liver and (II) hippocampal sections. I: (a) liver of control rat showing negative expression, (b) liver of TAA rat showing marked TNF-α expression, (c) liver of TAA + LNG-10 and (d) TAA + LNG-20 treated rats showing marked dose-related decreased expression, (e) The area percent of the positive brown color of COX-2 expression presented as optical density. II: (a) hippocampus of a control rat showing negative expression (b) TAA group showing marked expression of TNF-α, (c) TAA + LNG-10, and (d) TAA + LNG-20 treated rats showing dose-related decreased expression. (e) The area percent of the positive brown color of TNF-α expression presented as optical density. Results are presented as mean ± SD. One-way ANOVA was performed for the statistical analysis, followed by Tukey's Multiple Comparison test, where ****p < 0.0001 vs. control, @@@@@p < 0.0001 vs. TAA-treated group, #p < 0.05 vs. LNG-10, ##p < 0.01 vs. LNG-10. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
EBP-β and Fractalkine/CX3CL1 could be attributed in part to its ability to restore hippocampal BDNF via boosting GLP-1 as reported previously [83]. In addition, Matsuda et al. [84] revealed that a DPP-4 inhibitor reduced the expression of C/EBP-β via induction of activating AMP-activated protein kinase to re induce the function of pancreatic beta cells in mice. In the same context, LNG alleviated pathological aggregations in AD via a mechanism involving fractalkine receptor patrolling of monocytes that can phagocytize endogenous Aβ residues and reduce Aβ concentration in cerebral vascular space [85].

In Conclusion: LNG exhibited a potent hepatoprotective activity along with an effective neuroprotective potential that significantly hampered the progression of HE following TAA administration. Our findings revealed the significant antioxidant and anti-inflammatory properties of LNG. It markedly modulated various inflammatory mediators; COX-2, IL-1β, and TNF-α, together with the transcription factor, C/EBPβ, and the chemokine, Fractalkine/CX3CL1, which modify microglial activation during neuroinflammation. Consequently, LNG ameliorated the hippocampal contents of GABA and glutamate as well as BDNF to guard against neurobehavior deficits of HE.

CRediT authorship contribution statement

Yosra A. Hussien: Methodology, Software, Data curation, Writing – original draft, Formal analysis. Dina F. Mansour: Conceptualization, Visualization, Investigation, Writing – review & editing. Somaia A. Nada: Conceptualization, Supervision, Validation. Sahar A. Abd El-Rahman: Methodology, Software, Data curation, Writing – original draft. Rania M. Abdelsalam: Conceptualization, Visualization, Investigation, Writing – review & editing. Amina S. Attia: Conceptualization, Supervision, Validation. Dalia M. El-Tanbouly: Conceptualization, Visualization, Investigation, Formal analysis, Writing – review & editing.

Declaration of competing interest

None declared.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.lfs.2022.120378.

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[4] O. Cauli, R. Rodrigo, B. Piedrafita, M. Llansola, M.T. Mansouri, V. Felipo, Neuroinflammation could be attributed in part to its ability to restore hippocampal BDNF via boosting GLP-1 as reported previously [83]. In addition, Matsuda et al. [84] revealed that a DPP-4 inhibitor reduced the expression of C/EBP-β via induction of activating AMP-activated protein kinase to re induce the function of pancreatic beta cells in mice. In the same context, LNG alleviated pathological aggregations in AD via a mechanism involving fractalkine receptor patrolling of monocytes that can phagocytize endogenous Aβ residues and reduce Aβ concentration in cerebral vascular space [85].

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