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Acetyl-L-Carnitine protects against LPS induced depression via PPAR-γ induced inhibition of NF-κB/NLRP3 pathway

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Keywords

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Abstract

Introduction
Major depressive disorder (MDD) is a debilitating human health status characterized by mood swings and high suicidal attempts. Several studies have reported the role of neuroinflammation in MMD, yet the efficacy of natural drug substances on neuroinflammation-associated depression needs to be further investigated. The present study demonstrated the neuroprotective effects of Acetyl-L-carnitine (ALC) alone or in combination with caffeic acid phenethyl ester (CAPE) on lipopolysaccharide (LPS) induced neuro-inflammation, depression, and anxiety-like behavior.

Material and methods
Male Sprague Dawley (SD) rats were used to explore the relative effects of ALC and the mechanistic interplay of the peroxisome proliferator-activated receptors (PPARγ) in depression. Lipopolysaccharide (LPS) was administered to induce depression and anxiety-like symptoms such as a decreased grooming tendency, diminished locomotive activity, and increased immobility period.

Results
We found marked neuronal alterations in the cortex and hippocampus of LPS intoxicated animals associated with higher inflammatory cytokines expression cyclooxygenase (COX2), tumor necrotic factor-alpha (TNF-α). These detrimental effects exacerbate oxidative stress as documented by a compromised antioxidant system due to high lipid peroxidase (LPO). ALC significantly reverted these changes by positively modulating the PPARγ dependent downstream antioxidant and anti-inflammatory pathways such as NOD and pyrin domain-containing protein 3 (NLRP3) linked nuclear factor kappa B (NF-κB) phosphorylation. Moreover, co-administering NF-κB inhibitor caffeic acid phenethyl ester (CAPE) with ALC also increased PPARγ expression significantly and decreased NF-κB and NLRP3 inflammasome.

Conclusions
These findings indicate that ALC could be a possible depression supplement. The effects are partly mediated by inhibiting neuroinflammation and NLRP3 inflammasome coupled to PPARγ upregulations.

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These findings indicate that ALC could be a possible depression supplement. The effects are partly mediated by inhibiting neuroinflammation and NLRP3 inflammasome coupled to PPARγ upregulations.

Key Words: Acetyl-L- carnitine, lipopolysaccharide, antioxidant, neurodegeneration, neuroinflammation.

1. INTRODUCTION:

Major depressive disorder (MDD) is currently a leading public disorder compounded by heredity factors, environmental problems such as stressful lifestyle, and other predispositions factors [1]. Current drugs used in clinical practice are limited to hydroxytryptamine (5-HT) reuptake inhibitor, selective serotonin reuptake inhibitor (SSRI), suggesting depression is triggered by a deficiency of monoamine neurotransmitters, in particular 5-HT and noradrenaline. Nevertheless, the administration of SSRI / SNRI promptly improves the level of 5-HT or noradrenaline; weeks or even months are typically required for positive results [2]. Moreover, in multiple cases, these drugs are not well tolerated and lead to severe side effects. Furthermore, one-third of MDD patients don't react effectively to the medication [3]. Therefore, research on new alternative pharmacological targets is essential, which in addition to conventional therapy can further supplement the therapeutic approach.

Consistent research work supporting that oxidative stress and the inflammatory process substantiates the pathogenesis of MDD by triggering pro-inflammatory cytokines release [4]. These cytokines are involved in the activation of astrocytes and microglial cells and through a feedback mechanism; there is a more conspicuous surge in inflammatory markers [5][6]. A meta-analysis indicated that patients with MDD are diagnosed with elevated oxidative stress that could cause the death of neuronal cells [7].

Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the nuclear receptor superfamily [8]. Recently, PPARγ agonists have revealed antidepressant effects and which could be a plausible target [9]. Several *in vitro* and *in vivo* experiments have shown that PPARγ inhibits the production of pro-inflammatory cytokines and oxidative stress by inhibiting the NF-κB nuclear transcription factor [10]. In this regard, pharmacological modulation of PPARγ can attenuate different disorders such as neuropathic pain [9], Parkinson's disease [11], and traumatic brain injury [12] due to its anti-inflammatory characteristics. Moreover, mitochondria are not only involved in the generation of energy but also a rich source of reactive oxygen (ROS) species and apoptosis [13]. Several lines of research confirm the critical role of mitochondrial dysfunction in the pathophysiology of chronic diseases such as aging, age-related neurodegenerative diseases, and psychiatric disorders [14][15]. Furthermore, consistent studies suggested that mitochondrial dysfunction can lead to schizophrenia, mood, and anxiety disorders[16][17]. Moreover, mitochondrial dysfunction is a significant activator of inflammasome-mediated inflammation [18]. NLRP3 inflammasome, which interacts with the ASC (Apoptosis associated speck-like protein containing a C-terminal caspase recruitment domain) and triggers the release of inflammatory cytokines such as IL-18 and IL-1ß [19]. Expression levels of NLRP3 inflammasome mRNA are substantially increased in the brain of stressed lipopolysaccharide-induced mice (LPS), indicating that NLRP3 inflammasome is the mediator of inflammation during stress and depression [20]. Nuclear factor-kappa B (NF-κB) is a vital transcription factor that modulates inflammation and multiple autoimmune diseases.

Acetyl-L- carnitine (ALC) is a small molecule of increasing importance as it possesses favorable biological and pharmacological properties. A variety of studies have shown that ALC has demonstrated antioxidant, neuromodulator, and neuroprotective effects [21]. The protective effects of ALC on mood disorders, including major depressive disorder and dysthymia, have been confirmed, particularly in elderly people [22]. Caffeic acid phenethyl ester (CAPE) acts as an NF-kB inhibitor and has shown protective effects in various experimental models [23].

Moreover, studies showed that it has anti-inflammatory, antioxidant, immunomodulatory, and anti-cancer properties.

Lipopolysaccharide (LPS) is a part of gram-negative bacterial cell walls that induces a pro-inflammatory reaction [24] and is widely employed for research purposes [25][26]. Moreover, LPS can also trigger depressive-like symptoms and neuroinflammation in laboratory animals because inflammation is an integral disease mediating component of depression [27]. Furthermore, LPS administration compromises the endogenous antioxidant enzymes and prone neural cells to oxidative stress. So in this study, we investigated neuroprotective effects of ALC and CAPE via activating PPARγ linked downregulation of NF-kB and NLRP3 neuroinflammation.

2. Methodology:

2.1 Animals:

Sprague Dawley male rats weighing 180-220 g were housed three per cage under a 12-hour light/dark cycle with free access to water and food at the animal house of Riphah Institute of Pharmaceutical Sciences (RIPS) under standard laboratory protocols (temp: 22±1℃; humidity: 50%±10%). All experimental procedures were carried out as per the guidelines of the Institute of Laboratory Animal Resources, Commission on Life Sciences University, National Research Council (1996), approved by the RIPS Ethical Committee (Ref. No. REC/RIPS/2019/28).

2.2 Experimental design:

Rats were randomly divided into five groups and two cohorts, each containing n = 10 animals/group. First cohort includes 1) Saline (10 mL/kg with 5% DMSO), 2) LPS (500 μg/kg), 3) LPS + ALC30 (ALC 30 mg/kg), 4) LPS + ALC 60 (ALC 60 mg/kg), and 5) LPS + FLU (fluoxetine 20 mg/kg). The dose of LPS and FLU were selected as previously described and were dissolved in saline (with 5% DMSO). Moreover, LPS was administered for 14 alternate days, while ALC and FLU were administered continuously for 14 days, each time 1 h before LPS administration. The second cohort included three animal groups: LPS+CAPE (CAPE 10 mg/kg), LPS+CAPE+ALC (ALC 30 mg/kg), LPS+CAPE+FLU. CAPE was dissolved in normal saline (containing 5% DMSO) and administered once daily (i.p.) for 14 consecutive days 1 h before LPS administration. 24 h after (15^{th} day) the last LPS injection animals were analyzed for depressive/anxiety-like behavior (despair) by carrying out sucrose splash test (SST), elevated plus maze test (EPM), light-dark box test (LDB), and forced swim test (FST). Following the behavioral tests, animals were terminally anesthetized with sodium pentobarbital (60 mg/kg, i.p.). Brain tissues were extracted from the prefrontal cortex and hippocampus and either preserved in 4% formaldehyde or were snap-frozen and stored at –80°C.

2.3 Behavioral testing:

2.3.1 Sucrose Splash Test (SST):

The sucrose splash test was conducted per the previously reported study to evaluate the depressive behavior of rats [28]. The sucrose splash test was conducted per the previously reported study to evaluate the depressive behavior of rats. The shorter the time of grooming behavior, the higher will be the incidence of depression. The test was performed by spraying a

10% sucrose solution on the dorsal surface of the rodent's body and grooming behavior was measured in terms of licking, biting, or scratching the fur to clean. Grooming time was videotaped for five minutes.

2.3.2 Elevated plus Maze Test (EPM):

An elevated plus maze test was conducted to measure the LPS mediated anxiety-like behavior. Briefly, the maze equipment consisted of two oppositely faced open arms and two oppositely faced closed arms (OA, 50 × 10 cm; CA, 50 × 10 cm) in the form of a cross-shaped Plexiglas platform having 40 cm walls and height 50 cm above the floor in a soundproof room with a dimmed light. Each rat was placed at the central point of the Plexiglas platform with its face heading towards any open arm. The time spent in each arm was recorded for all the animals for 5 minutes.

2.3.3 Light-dark box test (LDB):

DB test was performed to assess the LPS mediated anxiety-like behavior. LDB equipment is comprised of a light and a dark compartment separated by a partition containing a small gap [28]. Each animal was sited in a dark compartment of a light-dark box and was set free to move around in the box for 5 min. The total time spent in each compartment was videotaped. The olfactory cues were minimized by ensuring thorough cleaning of the light-dark box with alcohol.

2.3.4 Forced swim test:

The rat was placed in a Plexiglas cylinder which was 70 cm in height and 30 cm in diameter, at a specific temperature of 23±1℃. A preswim exposure test was performed 24 h before the test to delineate the antidepressant-like activity. The use of a preswim ensures that the rats quickly adopt an immobile posture on the test day, which enables the effect of the tested compounds to be easily observed. The test was videotaped for seven minutes, and the last four minutes were randomly assessed for every 5-sec interval. The previously described Porsolt swim test has been employed in this study in its modified version. Climbing, swimming, and/ floating were the predominant behaviors observed during each 5-sec interval. Rats were categorized as immobile when they kept on floating in a motionless manner while showing only movements that were necessary for keeping their heads above the surface of the water. Horizontal movements were considered as swimming whereas vertical movements were taken as climbing.

2.4 Hematoxylin Eosin (H&E) Staining:

Absolute xylene was used to de-paraffinize the tissue-coated slides followed by rehydration with gradient ethanol concentration (100% to 70%). Afterward, slides were washed with distilled water and rinsed in hematoxylin stain for 10 min to localize the nucleus. The slides were then kept under running water in a glass jar for 10 min, treated with 1% HCl and 1% ammonia water as reported previously. Eosin solution was used afterward for 5 to 10 min for cytoplasmic staining. After the specified time, slides were rinsed with water and air-dried for a short interval. Graded concentrations (70%, 95%, and 100%) of ethanol were used for slides rehydration and cleared with xylene. The slides were then coverslipped by using mounting media. A light microscope (Olympus, Japan) was used to take the images of slides. Further slides were analyzed using ImageJ (ImageJ 1.3; https://imagej.nih.gov/ij/). Five images per slide per group were analyzed with a specific focus on cellular infiltrations, the formation of vacuoles, and neuronal karyolysis. For all the groups, images of the same threshold intensity were optimized in the TIFF format.

2.5 Immuno-histochemical Staining and Analysis:

We employed the previously described procedure with slight modifications for immune-histochemical analysis. After the de-paraffinization step, slides were processed by the enzymatic

method for antigen retrieval, then washed with PBS consecutively three times for 5 minutes. The slides were immersed in a 3% H2O2 to quench the endogenous peroxidase activity followed by washing with PBS. 5% normal goat serum, used as a blocking serum, and slides were incubated for 2 hr. Later, the slides were incubated overnight after applying primary antibodies. The next morning slides were washed with PBS and incubated for 90 min with the secondary antibody, and later with ABC kit (Santa Cruz) in a humidified box for 60 min. Slides were then washed with PBS solution and stained with DAB, followed by dehydration with ethanol (70%, 80%, 90%, and 100%). After dehydration, the slides were fixed with xylene and then coverslipped with mounting media. Five immunohistochemical TIF images were captured per slide with a light microscope. ImageJ software was used to quantitatively determine hyperactivated COX2, TNF-α, p-NFKB, in cortex/total area and hippocampus/total area by optimizing the background of images, according to the threshold intensity and analyzing the nuclear p-NFKB and cytoplasmic COX2, TNF-α positive cells at the same threshold intensity for all groups and was expressed as the relative activated positive cells relative to the control.

2.6 Lipid Peroxidation Determination (LPO) in Tissue:

Lipid peroxidation (LPO) in the brain of rats was studied as previously shown [29]. Homogenization of rat brain tissues was conducted at 20 mM Tris-HCl, pH 7.4 (10 ml) at 4C utilizing a polytron homogenizer. After centrifugation of the homogenate, the supernatant was collected at 1000 g for 10 min at 4C. Freshly formulated ferric or ferrous ammonium sulphate was applied to the brain homogenate tissues (40 ml) for lipid peroxidation and incubated at 37C for 30 min. Subsequently, 75 ml of 2-thiobarbituric acid (TBA; 0.8%) was applied, which was formulated by dissolving TBA (400 mg) in distilled water (50 ml). The absorbance was calculated at 532 nm with a plate reader.

2.7 Reduced glutathione (GSH) Activity:

Like previously published data with minor modifications, a reduced amount of glutathione (GSH) was determined [30]. We mixed 0.2 mL of the tissue supernatant with 2 mL of DTNB mixture, followed by the addition of 0.2 M phosphate buffer to yield a final volume of 3 mL. The absorbance was measured after 10 min using a spectrophotometer at 412 nm, where phosphate buffer and DTNB solution were used as a blank and control, respectively. The real absorbance value was obtained by subtracting the absorbance of the control from that of the tissue lysate. The final GSH activity was expressed in units of μmol/mg of protein.

2.8 Glutathione-S-Transferase (GST)

To calculate GST activity, we freshly prepared 1 mM CDNB and 5 mM GSH solutions in 0.1 M phosphate buffer. Three replicates of the 1.2 mL reaction mixture, kept in glass vials, followed by the addition of 60 μL of tissue homogenated to each of these mixtures. The blank contained water rather than tissue lysate. Next, 210 μL aliquots from the reaction mixture were pipetted out in a microtiter plate; further, absorbance was measured at 340 nm for 5 min at 23 °C using an ELISA plate reader (BioTek ELx808, Winooski, VT, USA). GST activity was expressed in units of μmol of CDNB conjugate/min/mg of protein [31].

2.9 Catalase Activity:

We added 0.05 mL of tissue homogenate to 1.95 mL of phosphate buffer (50 mM, pH 7) and 1 mL of H2O2 solution (30 mM). The absorbance of the final mixture was measured at a wavelength of 240 nm. The catalase activity was calculated using the following formula:

$CAT = \delta O.D \div E \times \text{Volume of sample (mL)} \times \text{protein (mg)}$

where δO.D represents the change in absorbance per minute and E represents the extinction coefficient of H2O2 with a value of 0.071 mmol cm−1.51. The Lowery method was used to measure protein levels. Catalase activity was expressed as μmol of H2O2/min/mg of protein**.**

2.10 Immunosorbent Assay (ELISA) linked with enzymes:

Approximately 70 g of tissue was homogenized in PBS, containing PMSF as protease inhibitor using a Silent Crusher M (Heidolph). The resultant homogenate was then centrifuged at 15000 RPM at 4oC for 20 min and the supernatant was carefully collected from the top avoiding pallet at the bottom. The protein concentration was then determined for each group using a BCA kit (Thermo Fisher), and the concentration for each protein was determined using respective 96 well ELISA plates according to the protocols provided by the manufacturer. The readings were taken using an ELISA microplate reader (BioTek ELx808) and, the concentration (pg/mL) was then normalized to total protein content (pg/mg total protein).

2.11 Western blot:

Western blotting was also performed according to the standard protocol. Briefly, the protein samples were denatured by boiling at 100 °C for 5 min and separated on SDS PAGE [32]. The separated protein was then transferred onto a nitrocellulose membrane. The membrane was blocked with nonfat milk in TBST (Tris-buffered saline, 0.1% Tween 20) and then incubated with primary antibody (1:500) overnight at 4 °C. The next day, the membrane was washed and incubated with the secondary antibody (1:1000) for 1 h at 4 °C. Detection was performed using the ECL Super signal chemiluminescence kit following the manufacturer's protocol. Blots were developed using the ChemiDoc MP imaging system (Bio-Rad). Densitometry analysis of the bands was performed using the Image Lab software.

2.12 Statistical Analysis:

Data were expressed as mean ± SEM. One-way analysis of variance (ANOVA) was analyzed, followed by a post hoc Bonferroni test. The value $p<0.05$ was found to be statistically significant.

3. RESULTS:

3.1 ALC attenuated LPS induced depressive-like anxiety behaviors

To evaluate whether ALC at different doses can attenuate anxiety-like behavior, we performed a series of behavioral tests. In the EPM, the LPS injection rats were more inclined to the closed arm, indicating depressive-like behavior relative to the control group (Figure 2B, $p<0.05$). In comparison, ALC pretreatment (30 mg/kg) has reversed this tendency (Figure 2A, $p<0.05$). Similarly, rats behaved in an increased immobility period in the FST (Figure 2D, $p<0.05$), whereas 30 mg/kg dose of ALC improved the struggling behavior in the FST relative to the LPS group (Figure 2C, $p<0.05$). In the LDB test, rats injected with the LPS spent more time in the dark compartment (Figure 2E, $p<0.05$) than in the light chamber. On the other side, ALC increased the probing power in this behavioral test. Consistently, ALC (30 mg/kg) pretreatment increased the grooming time in the SST (Figure 2G, $p<0.05$) group. We demonstrated no significant effect at 60mg/Kg of dose in these batteries of the test except in SST, where we observed an increase in grooming time (Figure 2G, $p<0.05$).

Figure 2. ALC attenuated LPS induced depressive-like anxiety behaviors. Effect of ALC and LPS on EPM test (**A, B**), FST (**C, D**), LDB test (**E, F**), and SST (**G**). Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by post hoc Bonferroni test using GraphPad Prism 6 software. Saline, LPS, LPS+ALC 30mg, LPS+ALC 60mg and LPS+FLU groups represent the first cohort (n=10/group), ##p<0.01 compared to the saline group while *p<0.05 compared to the LPS group. ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; FLU: fluoxetine; EPM: elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test.

3.2 ALC elevated PPARγ level coupled to NRLP3 downregulation

To elucidate the underlying mechanism of ALC and its relative role on mitochondrial biogenesis and neuroinflammation, we demonstrated various analyses to determine the level of these associated proteins in the cortical region of the brain. Ligand bound activation of PPARγ can attenuate dysfunctional mitochondria, neuronal death, oxidative stress, and inflammation, as reported previously. To determine the effect of ALC on PPARγ level and inflammation, initially, we performed ELISA analysis (Figure 3A). PPARγ level in the LPS group was decreased (Figure 3A, $p<0.05$) however, this level remained high in ALC 30mg group (Figure 3A, $p<0.05$). Consistent studies suggested the implication of NF-kB signaling pathways in depression that can trigger NLRP3 and downstream transcriptional induction of many pro-inflammatory mediators including COX-2 and TNF-α that could exacerbate the symptoms. We studied the expression of *p*-Nf-kB, COX2, and TNF-α, either in the cortex or in both cortex and hippocampus by immunohistochemical analysis (Figure 3B-D). All these proteins showed elevated expression in the LPS group ($p<0.001$), but treatment with ALC at doses 30 and 60 mg equivalently diminished the triggered overexpression of p-NFkB ($p<0.05$, $p<0.05$), COX2 ($p<0.05$), and TNF-α ($p<0.05$, $p<0.05$) (Figure 3).

Figure 3. Effect of ALC on mitochondrial biogenesis implicated neuroinflammation. **(A)** PPAR γ levels were measured by ELISA, Data are expressed as means ± SEM. $^{\#}p<0.05$ compared to the saline group while $^{*}p<0.05$ and compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC 60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine; PPAR γ: peroxisome proliferator-activated receptor. **(B)** Immunohistochemistry results for NF-κB in the cortex and hippocampus. **(C)** Immunohistochemistry results for COX-2 in the cortex and hippocampus. **(D)** Immunohistochemistry results for TNF-α in the cortex and hippocampus. Bar 50 μm, magnification 40×. Data are expressed as means ± SEM, $^{\#\#\#}p<0.001$ compared to the saline group while $^{*}p<0.05$, $^{*}p<0.05$ compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine;

3.3 Effect of ALC on enzymatic and non-enzymatic anti-oxidants

The antioxidant activity of ALC (30mg, 60 mg/kg) was also studied using GST, GSH, and catalase levels in both cortex and hippocampus (Figure 4A-D). Non-enzymatic antioxidant GSH and enzymatic antioxidant catalase and GST substantially improved in the ALC treated groups relative to the LPS group. The ALC 30mg/ kg group reported a marked improvement in GSH (Figure 4A-B, $p<0.05$) and GST and catalase levels in cortex and hippocampus, respectively (Figure 4C-F, $p<0.05$, $p<0.01$). A TBARS test was also conducted, and peroxides displayed a dramatic rise in the LPS-induced population, an outcome that could be recovered by ALC (30mg and 60 mg/kg) therapy. The LPO content in the cortex of the LPS group was increased compared to the control group (Figure 4G, $p<0.001$).

Figure 4. Effect of ALC on oxidative stress-related antioxidant enzymes. Effects of LPS and ALC on levels of GSH (**A, B**); GST (**C, D**); CAT (**E, F**), and TBARS (**G, H**). Data are expressed as means ± SEM. Where $^{\#\#}p<0.01$, $^{\#\#\#}p<0.001$ compared to the saline group while $^{*}p<0.05$, $^{**}p<0.01$ compared to LPS group. ALC: Acetyl-L-

carnitine L Carnitine; LPS: lipopolysaccharide; FLU: fluoxetine; CAT: catalase; GSH: reduced glutathione; GST: Glutathione-S-transferase; TBARS: thiobarbituric acid reactive substances.

Experimental results of Cohort 2

3.4 Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes.

Co-treatment of ALC and CAPE significantly cope with the anxiety-like behavioral deficits (Figure 5), while the effects were not significant in many behavioral tests in the LPS+CAPE group (second cohort, data not shown). Moreover, to evaluate the rate and extent of neuronal survival, we performed H & E staining. Histological variations in the hippocampal and cortical regions of rats have been observed in both cohorts. In the control group, the hippocampal neurons were round, with an intact structure (Figure 5H). Compared to the control group, the hippocampal neurons of the LPS group displayed karyopyknosis, deepened staining of irregular, polygonal, and spindle forms (Figure 5, $p<0.01$). The cortical neurons in the LPS group were poorly organized due to a decrease in the number of neurons. Relative to the LPS group, most of the neurons in the CAPE+LPS and CAPE+ALC 30 mg/kg groups were preserved, and a few of them displayed karyopyknosis (Figure 5, $p<0.05$). The CAPE+ALC30 mg/kg group showed circular and transparent neurons relative to the CAPE+LPS group in the hippocampus region (Figure 5, $p<0.05$).

Figure 5. Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes. Effect of CAPE+ALC and CAPE+LPS on EPM (**A, B**), FST (**C, D**), LDB test (**E, F**), and SST (**G**). Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by a post hoc Bonferroni test using GraphPad Prism 6 software. $^{\#}p<0.05$, $^{\#\#\#}p<0.001$ compared to the saline group while $^{*}p<0.05$, $^{**}p<0.01$ compared to the LPS group. ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; CAPE: caeffic acid phenethyl ester; FLU: fluoxetine; EPM: elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test. H&E staining shows the extent of surviving neurons in the cortex and hippocampus (Corno amonus, CA; dentate gyrus, DG**).** Bar 50 μm, magnification 40×. Surviving neurons were characterized by swollen cytoplasm, vacuolization, scalloped morphology with intense cytoplasmic eosinophilia, and nuclear basophilia. The Saline, LPS, ALC30mg+LPS, ALC 60mg+LPS, and FLU+LPS groups were taken first cohort (n=5/group), while CAPE+LPS, and CAPE+ALC30 mg +LPS, were from the second cohort (n=5/group).

3.5 Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers.

To determine the co-effect of ALC and CAPE on the PPARγ, NLRP3, and p-NFkB level in the cortex, we performed western blot analysis (Figure 6A). PPARγ level in the LPS group was decreased; however, this level remains elevated in the co-administered group ($p<0.05$). Furthermore, NLRP3 and p-NFkB levels were elevated in the LPS group ($P<0.001$, $p<0.01$), significantly attenuated by co-administration. To further validate, we performed ELISA analysis, and similar results were demonstrated for PPARγ (Figure 6B). Next, we performed immune histochemical analysis for COX-2, p-NFkB, and TNF-α that could exacerbate the symptoms (Figure 6C-E). All these proteins showed elevated expression in the LPS group ($p<0.001$), but co-treatment diminished the triggered overexpression of p-NFkB ($p<0.01$, $p<0.05$), COX2 ($p<0.05$, $p<0.05$), and TNF-α ($p<0.05$, $p<0.05$) (Figure 6).

Figure 6. Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers **(A)** Representative bar graphs and western blots indicating the protein levels in the cortex (n = 5). Image J software was used for quantifying the western blots and graphs were generated using GraphPad Prism. Data are expressed as mean ± SEM, and results were analyzed using one-way ANOVA followed by post-hoc analysis. $p < 0.05$ was considered statistically significant**. (B)** PPARγ levels were measured by ELISA in the cortex. Data are expressed as means ± SEM. **(C)** Immunohistochemistry results for NF-κB in the cortex and hippocampus tissues of the brain. **(D)** Immunohistochemistry of COX2 in the cortex and **(E)** Immunohistochemistry of TNF-α in the cortex and hippocampus tissues. Data are expressed as means ± SEM. $^{###}p<0.001$ compared to the saline group while $*p<0.05$ compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC 60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine; PPAR γ: peroxisome proliferator-activated receptor. CAPE: caeffic acid phenethyl ester;

3.6 Co-administration reversed the downregulation of antioxidant enzymes.

The antioxidant effect of the NF-κB inhibitor (CAPE) with LPS and ALC (30mg, 60mg/kg) was also studied using GST, GSH, and catalase levels in both cortex and hippocampus (Figure 7A-D). The non-enzymatic antioxidant GSH and enzymatic antioxidant catalase and GST were substantially improved by CAPE+ ALC 30mg/kg in the cortical region relative to the CAPE+ LPS group. The CAPE+ALC 30mg/kg group reported a marked improvement in GST, catalase, and GSH ($p<0.05$, $p<0.05$) levels in the cortex rather than hippocampus (Figure 7). The TBARS test was conducted, and the peroxides displayed a sharp increase in the LPS-induced group, an outcome that could be retrieved by the CAPE+LPS group. However, CAPE+ ALC (30 mg/kg) treatment is explored to improve the outcome ($p<0.01$). The LPO content in the cortex of the LPS group was increased relative to the control group ($p <0.01$). CAPE+ALC 30mg/kg attenuated this increased TBRAS content in cortex and hippocampus ($p <0.01$, $p<0.01$).

Figure 7. Co-administration reversed the downregulation of antioxidant enzymes. Effects of CAPE, LPS, and ALC on levels of GSH (**A, B**); GST (**C, D**); CAT (**E, F**), and TBARS (**G, H**). Data are expressed as means ± SEM. Where $###p<0.001$ or $##p<0.01$ compared to the saline group while $*p<0.05$ or $**p<0.01$ compared to LPS group. ALC: Acetyl-L- carnitine LPS: lipopolysaccharide; CAPE: caeffic acid phenethyl ester; FLU: fluoxetine; GSH: reduced glutathione; GST: Glutathione-S-transferase; CAT: catalase; TBARS: thiobarbituric acid reactive substances

4. Discussion:

Natural drug compounds are significantly researched for various therapeutic potentials, including their role in different neurodegenerative models, not only to unveil the pathophysiology but also to track more suitable therapeutic alternatives. In this research, we aim to examine the neuroprotective effects of the naturally-derived substance ALC against depression, oxidative stress-mediated anxiety and neuroinflammation. ALC is a dietary and nutritional supplement, which could reverse the antidepressant-like symptoms by modulating synaptic plasticity-associated neuronal functions. The potential benefits of ALC in depression were tested both in preclinical trials and in several randomized clinical trials [33][34]. Furthermore, consistent studies suggested the linage of ALC's antidepressant effect with its interaction with epigenetic pathways [35]. Moreover, ALC is marketed in several countries for neuropathic pain [36].

Here we examined the neuroprotective potential of ALC in the LPS-induced depressive model by targeting mitochondrial biogenesis-associated neuroinflammatory pathways. PPARγ is a ligand-dependent transcription factor belonging to the superfamily of nuclear hormone receptors

and is involved in many inflammatory processes [37]. It is reported that PPARγ agonist could ameliorate depression-like disorders, possibly by modulating the inflammatory cascade, oxidative stress mechanism, and synaptic modulation. Moreover, pioglitazone which is a reference PPARγ agonist exhibited antidepressant-like results by modulating NF-κB/IL-6/STAT3 and CREB/BDNF pathway [39]. Furthermore, pioglitazone, either alone or as an add-on therapy to conventional treatments was found to induce remission of depression [40]. Another PPARγ agonist NP031115 demonstrated antidepressant-like effects by inhibiting GSK-3β [41], while PPARγ antagonist reversed these anti-depressive like effects [42]. In all these studies, PPARγ activation, ameliorated the classical behavioral deficits with improved therapeutic response. Therefore, stimulating PPARγ could be an appropriate therapeutic target to uncover new medicines for mood disorders. The results of the present study was also in line with studies where ALC and CAPE improved the depression and anxiety-like behaviors in LPS rats presented by an increase in the time spent in the open arm while a decrease in the time spent in the closed arm of the elevated plus-maze. Additionally, in the forced swimming test, these compounds increased struggling while decreasing immobility time, and the results coincide with light-dark box and grooming behavior. PPARγ is a widely distributed cell transcription factor that executes vital functions in redox homeostasis, neurogenesis, diabetes, and other vascular disorders. The protective effects of PPARγ are also investigated in other neurodegenerative disorders such as Parkinson's, Alzheimer's, Huntington's, and stroke [43]. We used a combination of ALC and CAPE here as previously a combination of a low dose of PPARγ agonist with other inflammatory and glutamatergic antagonists substantiated the antidepressive effects of PPARγ [44]. In this context, ALC demonstrated antioxidant, neuromodulatory, and neuroprotective effects including MDD in elderly patients [22]. Likely, CAPE has been reported to have anti-inflammatory, antioxidant, and immunomodulatory properties.

Inflammasome (NLRP3) activation can significantly enhance the release of inflammatory cytokines and can augment oxidative stress. Moreover, the NLRP3 dependent inflammatory cascade can be inhibited by the NF-κB inhibitor [45]. Furthermore, p-NFkB pathway activation plays an important role in pro-inflammatory gene expressions such as COX2 and iNOS. Herein, we administered CAPE to antagonize the p-NFkB, and we investigated in parallel the potential effects of ALC on PPARγ activation. These effects were translated into a reduction in immobility time in our behavior tests. It has been demonstrated previously, that PPARγ activation can downregulate p-NFkB and COX-2 activation [46]. In the current study, we demonstrated that ALC could mediate the PPARγ-dependent signaling system, as our results showed a rise in PPARγ level and decreased level of p-NF-kB and NLRP3 inflammasome, which possibly could be responsible mechanism to mitigate the depressive-like symptoms. The present results indicate that the anti-inflammatory effects of ALC are correlated with PPAR-mediated inhibition of the NF-kB signaling pathway, which results in suppressing inflammatory cytokines. LPS causes an up-regulation of NF-kB-dependent NLRP3 expression, which further induces activation of other inflammatory markers such as TNF-α, and COX2. Taken together, our findings have shown the ALC therapy relieved the depressive-like behavior found in LPS-induced rats.

LPS is a well-established entity to elicit depressive-like behavior in animal model by stimulating the immune system which in response provoke an inflammatory cascade. Intraperitoneal administered of LPS also challenges the immune response, resulting in oxidative stress. In the present study, we demonstrated that oxidative stress was ameliorated by ALC and CAPE administration significantly increased CAT, GST, and GSH activities and levels respectively. Furthermore, several studies reported that PPARγ agonists modulate anti-oxidative enzymes

such as CAT and SOD [47]. Parallel to these previous findings, the results of the current study demonstrate that both CAPE and ALC may possess free radical scavenging properties which modulated the behavioral dysfunction induced by LPS.

Hence, findings of the current study showed that ALC demonstrated neuroprotective effects possibly by modulating the PPARγ/NF-κB/NLRP3 axis. Furthermore, treatment with ALC decreased the TNF-α production and suppressed the release of COX-2 inflammatory mediators by downregulating p-NFkB in the animal model of depression (Figure 8).

Figure 8. The suggested mechanism for ALC pre-treatment effects on LPS mediated oxidative stress, neuroinflammation, and neurodegeneration

5. Conclusion:

In conclusion, our in vivo findings showed that ALC is a powerful anti-oxidant and anti-neuro inflammatory agent with significant neuroprotective properties in LPS-induced depression/anxiety model. Furthermore, our hypothesized neuroprotective mechanism indicates that ALC could stimulate the PPARγ which may be associated with negative regulation of the NF-κB and other neuroinflammatory mediators. Therefore, current study suggest potential new therapeutic choice for preventing and controlling oxidative stress and neuroinflammation in neurodegenerative disorders such as depression and anxiety.

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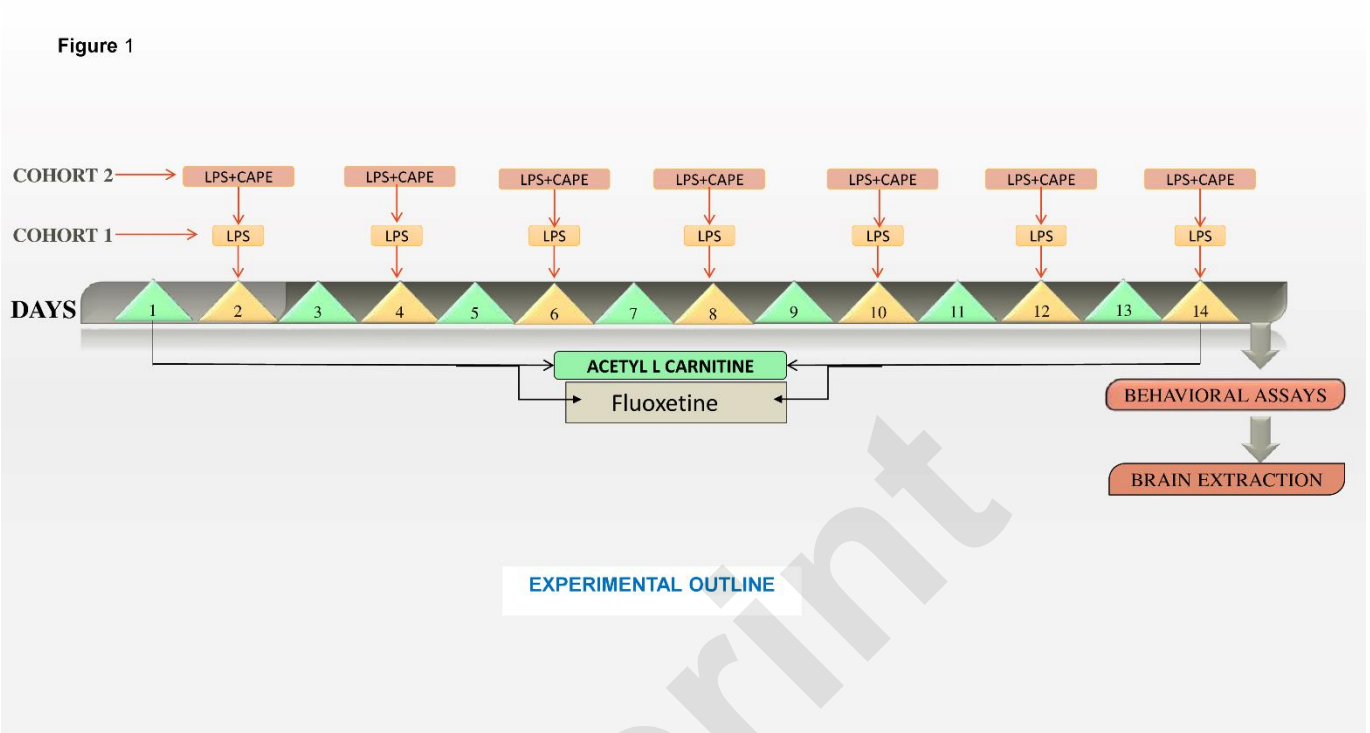
Figures



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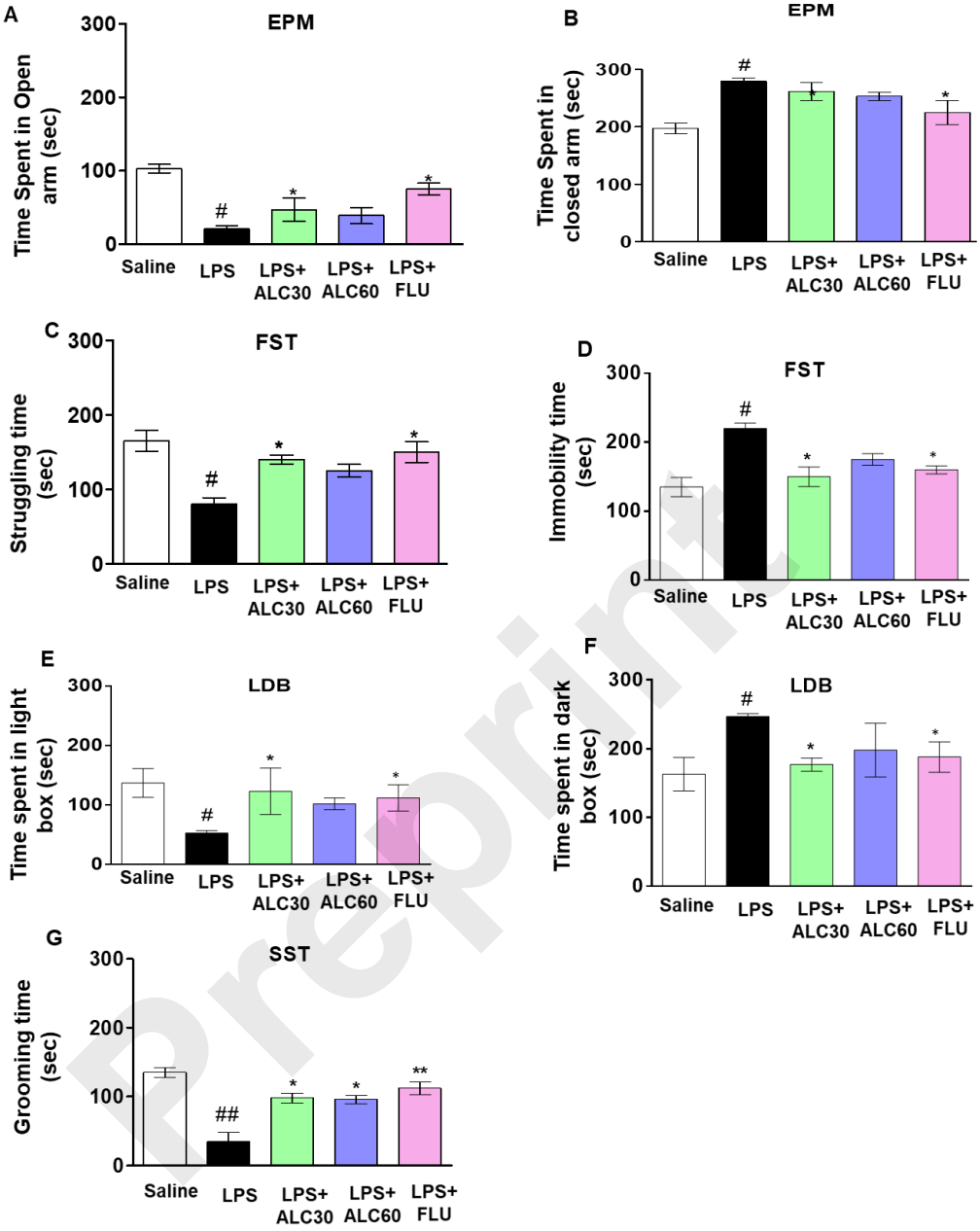

Figure 3

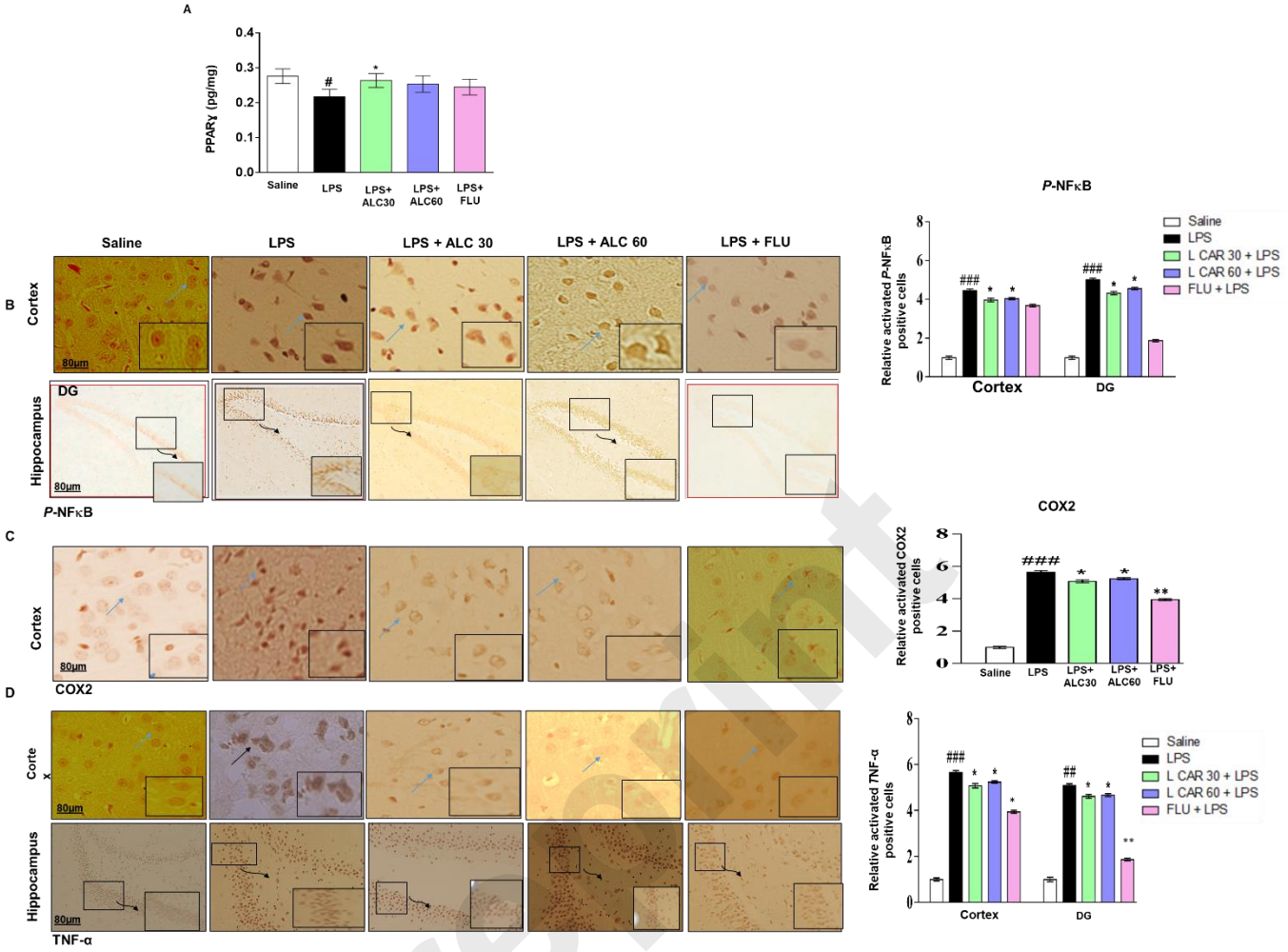

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Figure 4

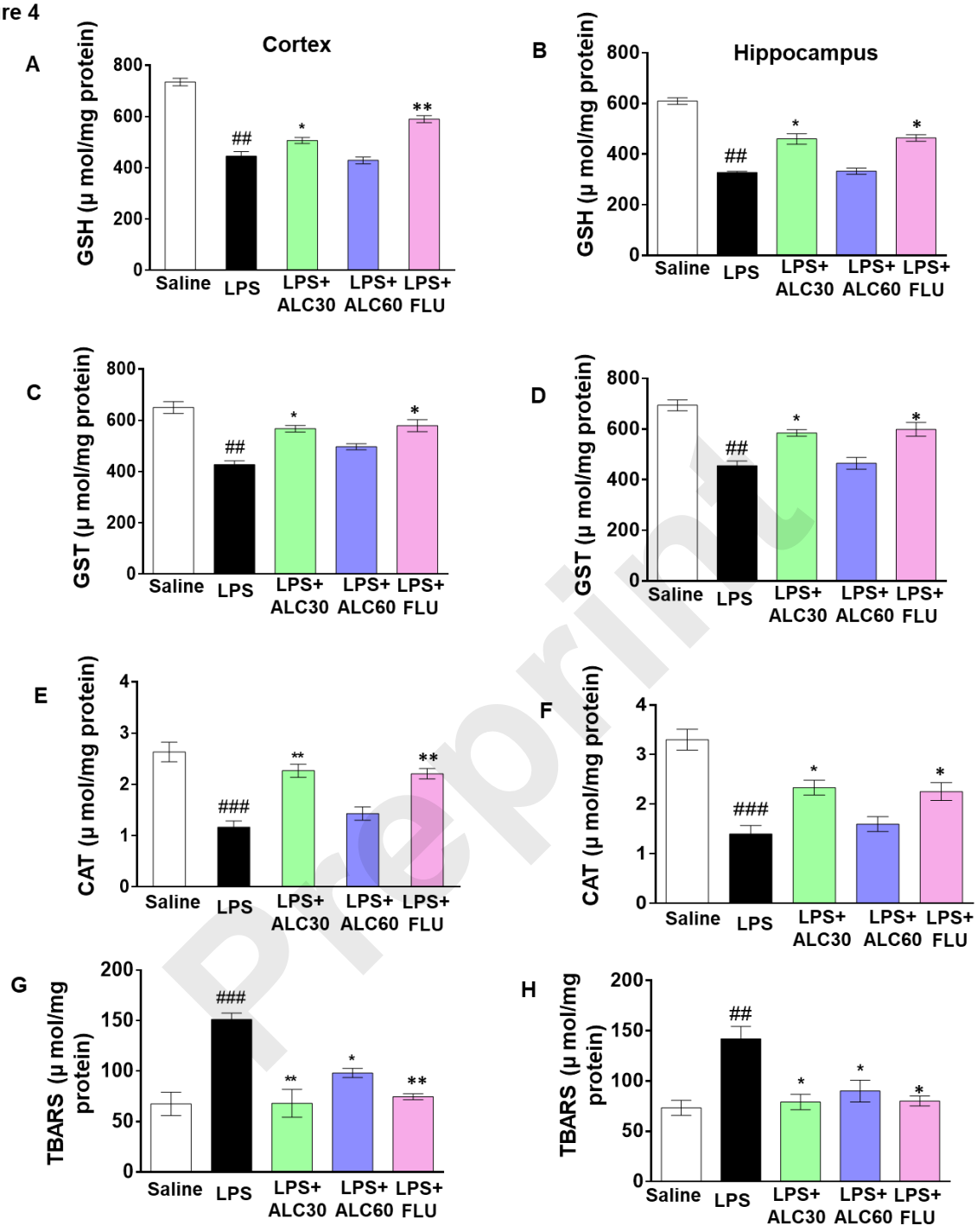


Figure 5

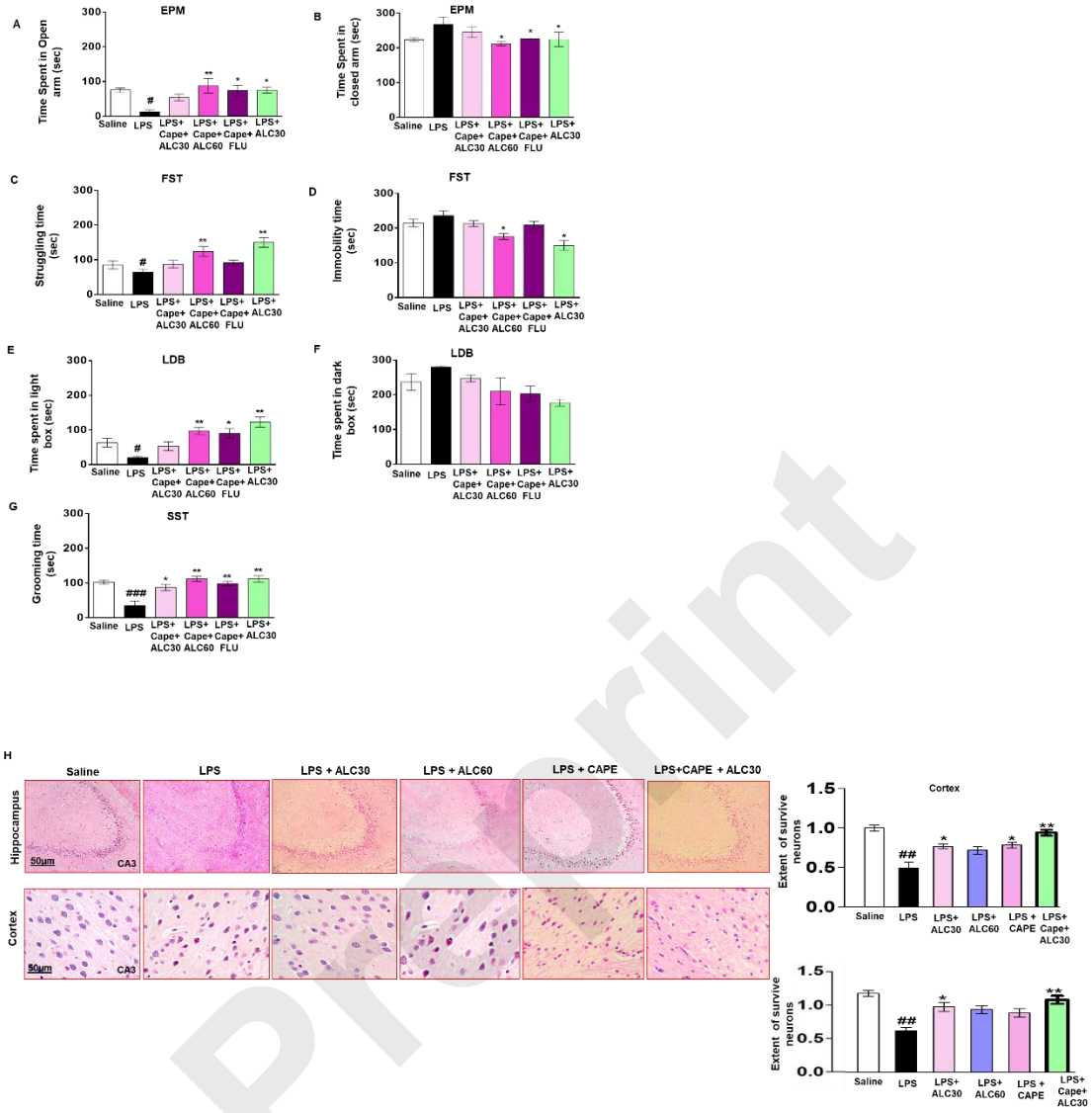
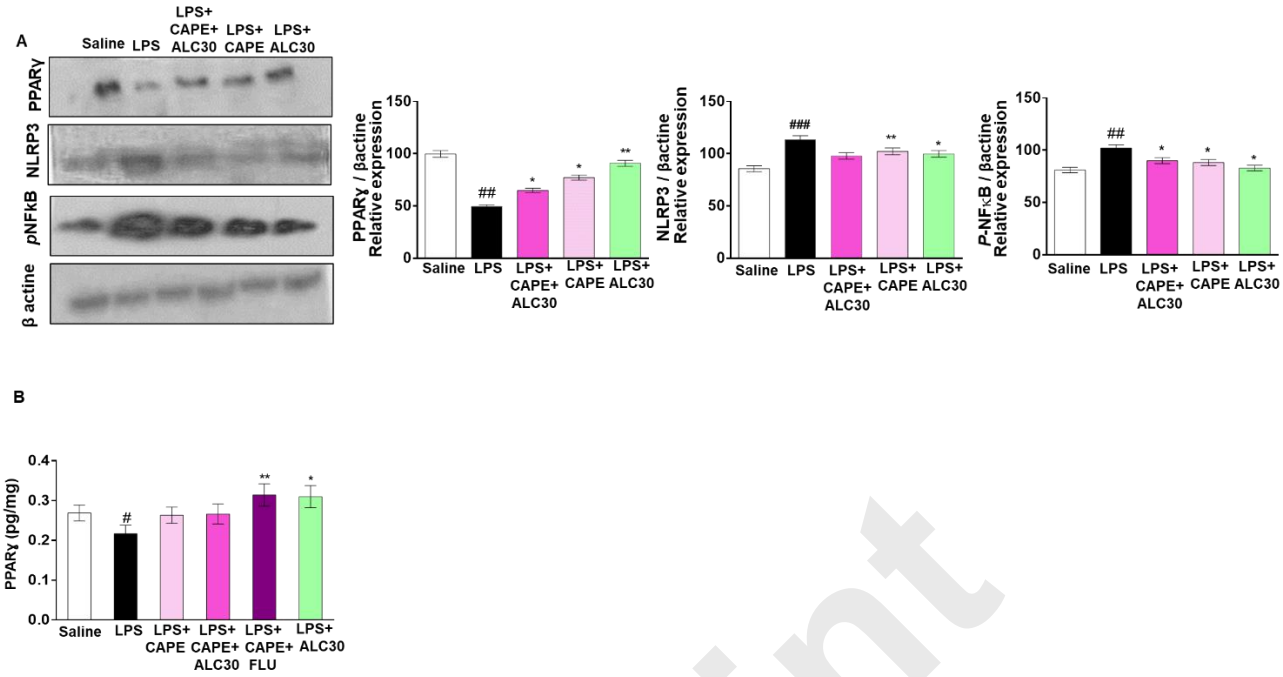


Figure 6



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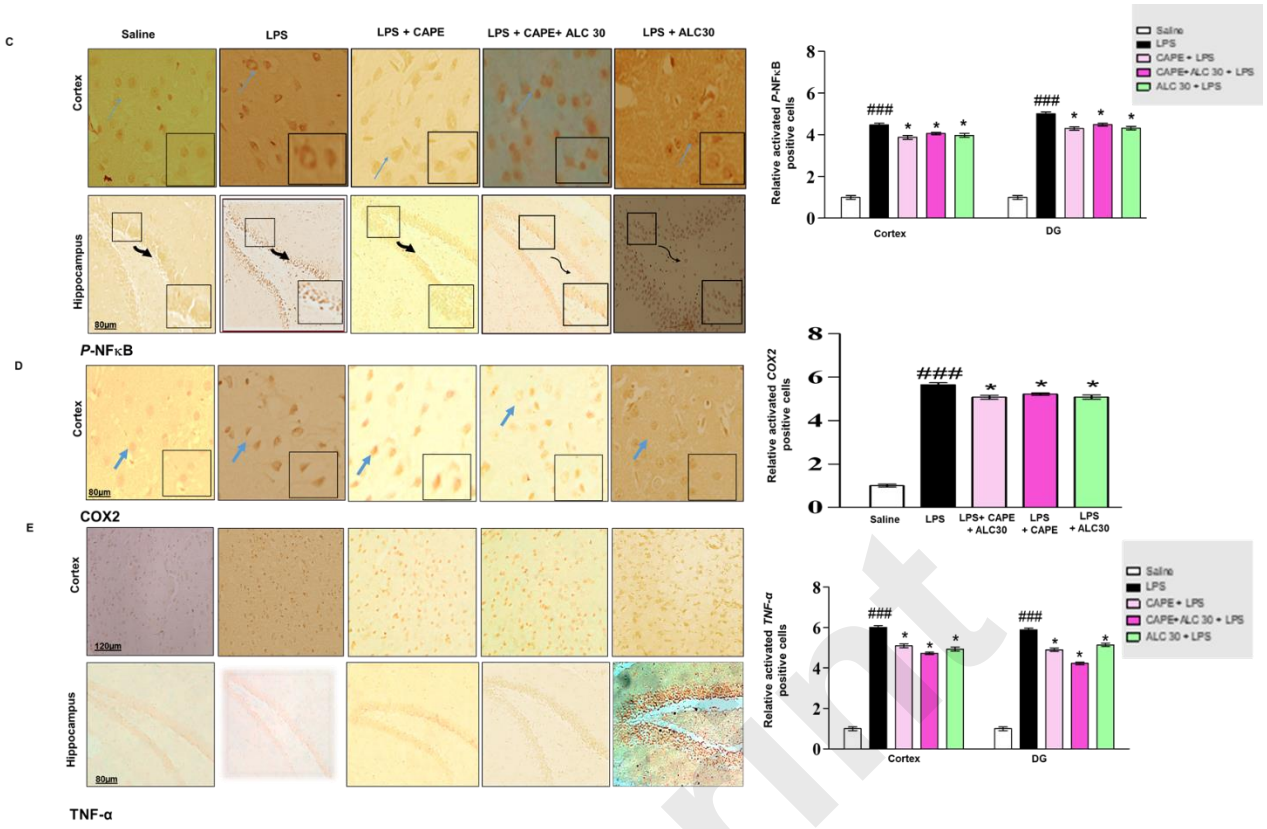

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Figure 7

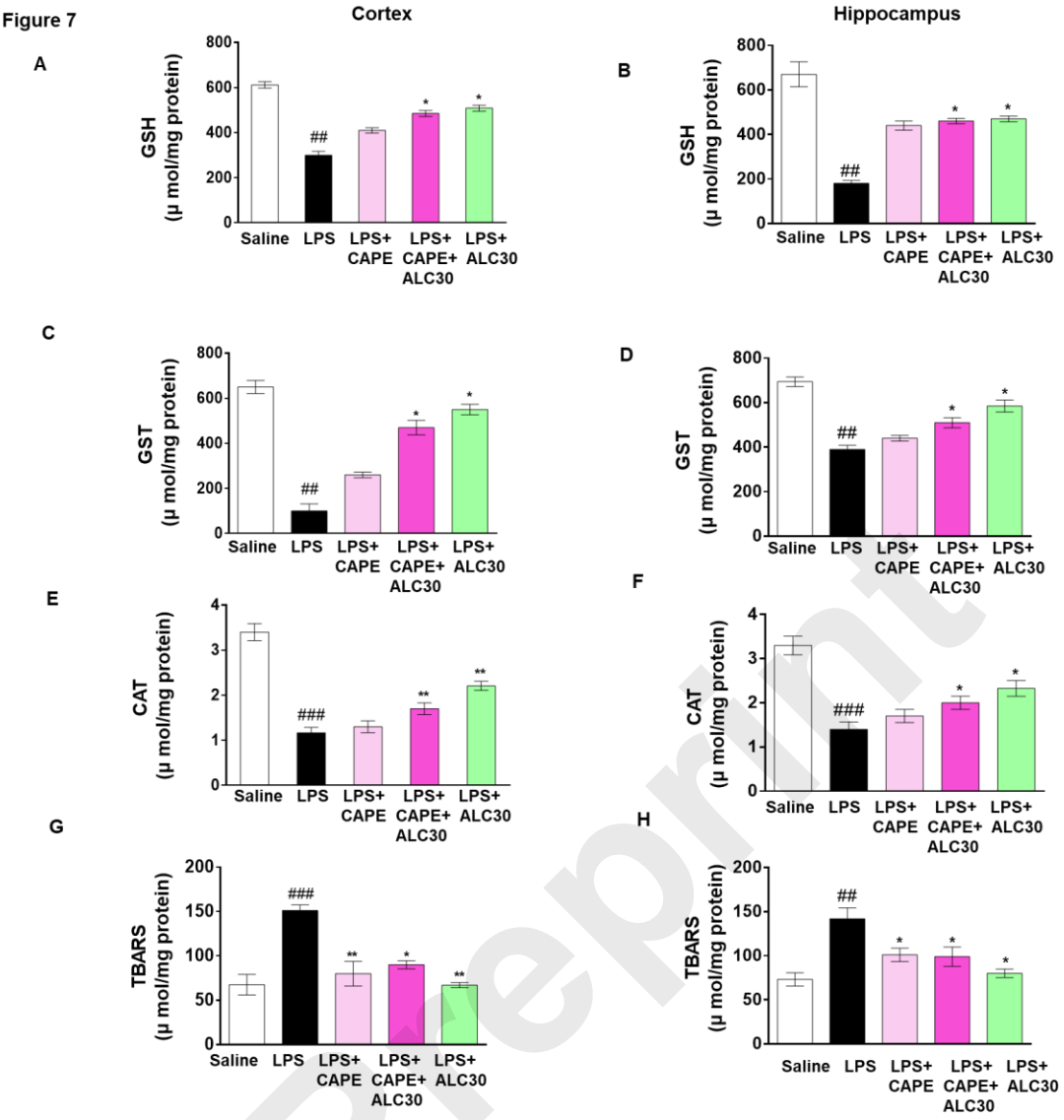


Figure 8. The suggested mechanism for ALC pre-treatment effects on LPS mediated oxidative stress, neuroinflammation, and neurodegeneration

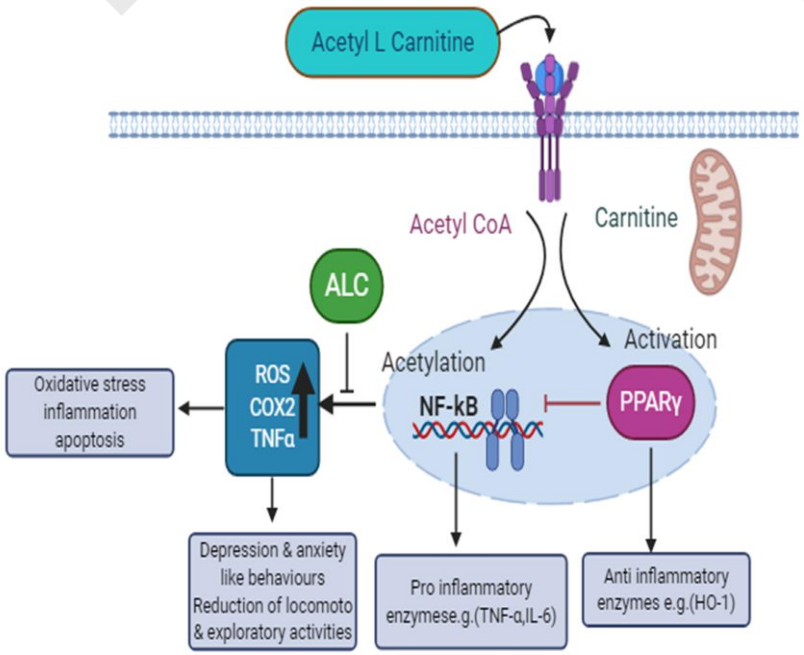
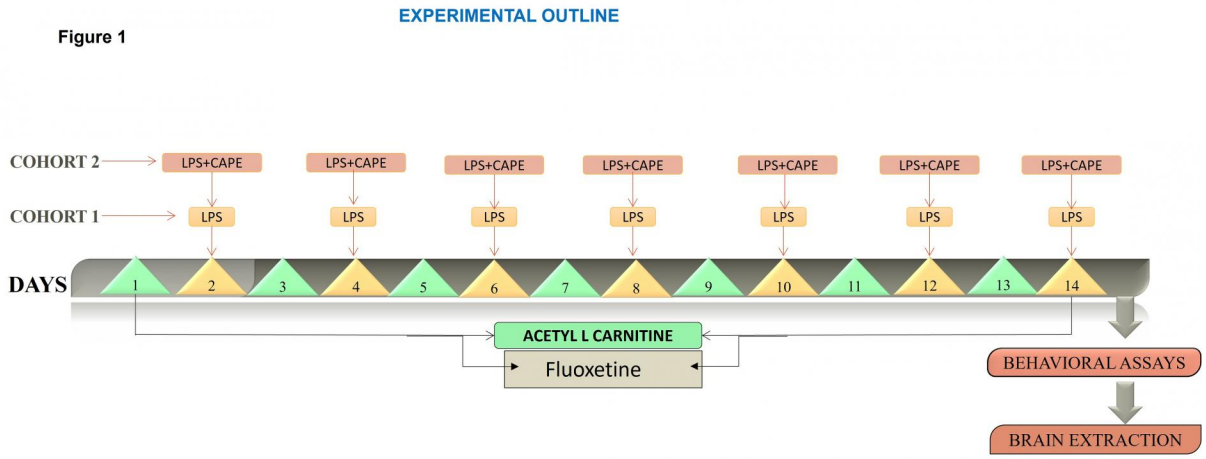
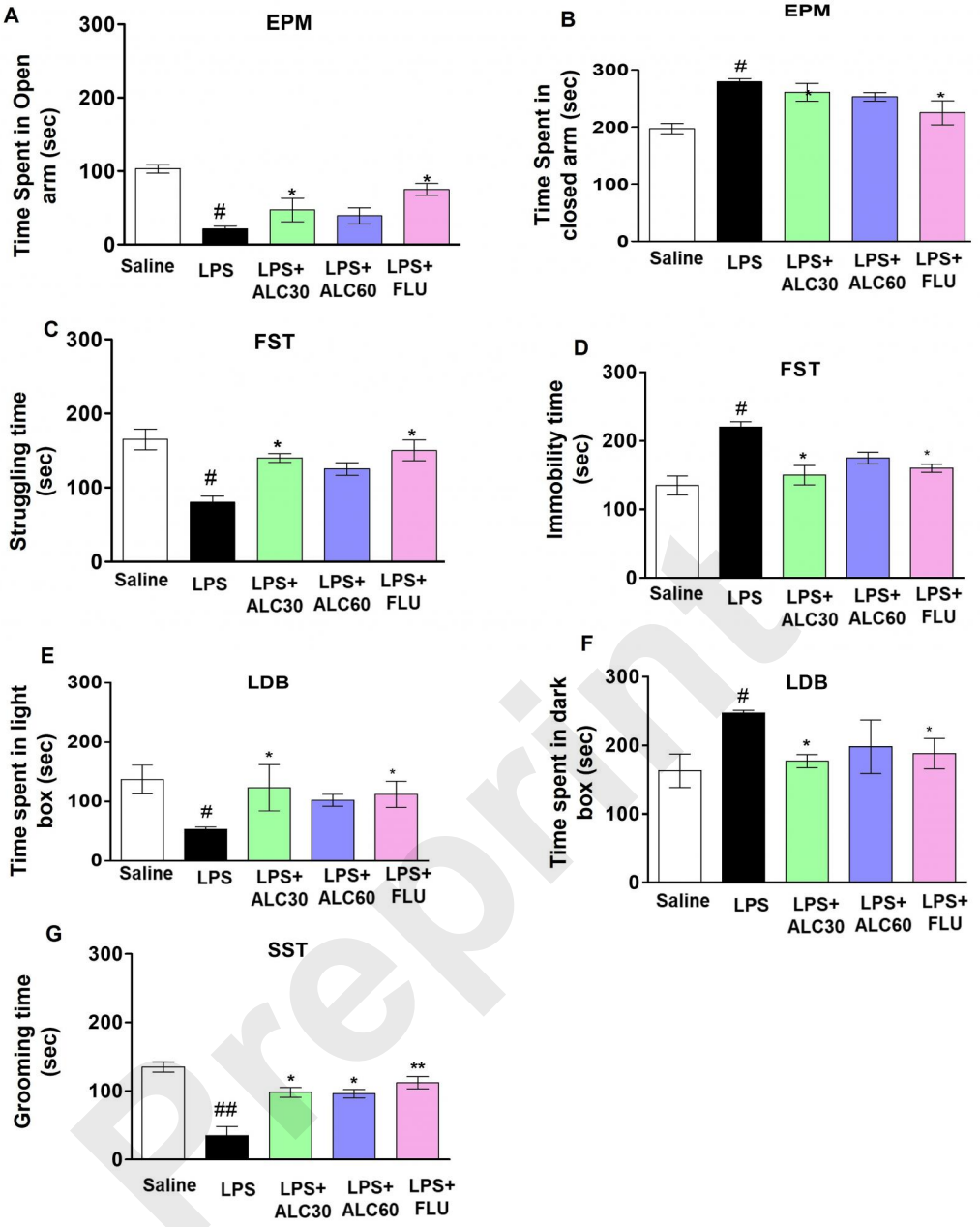


Figure 1



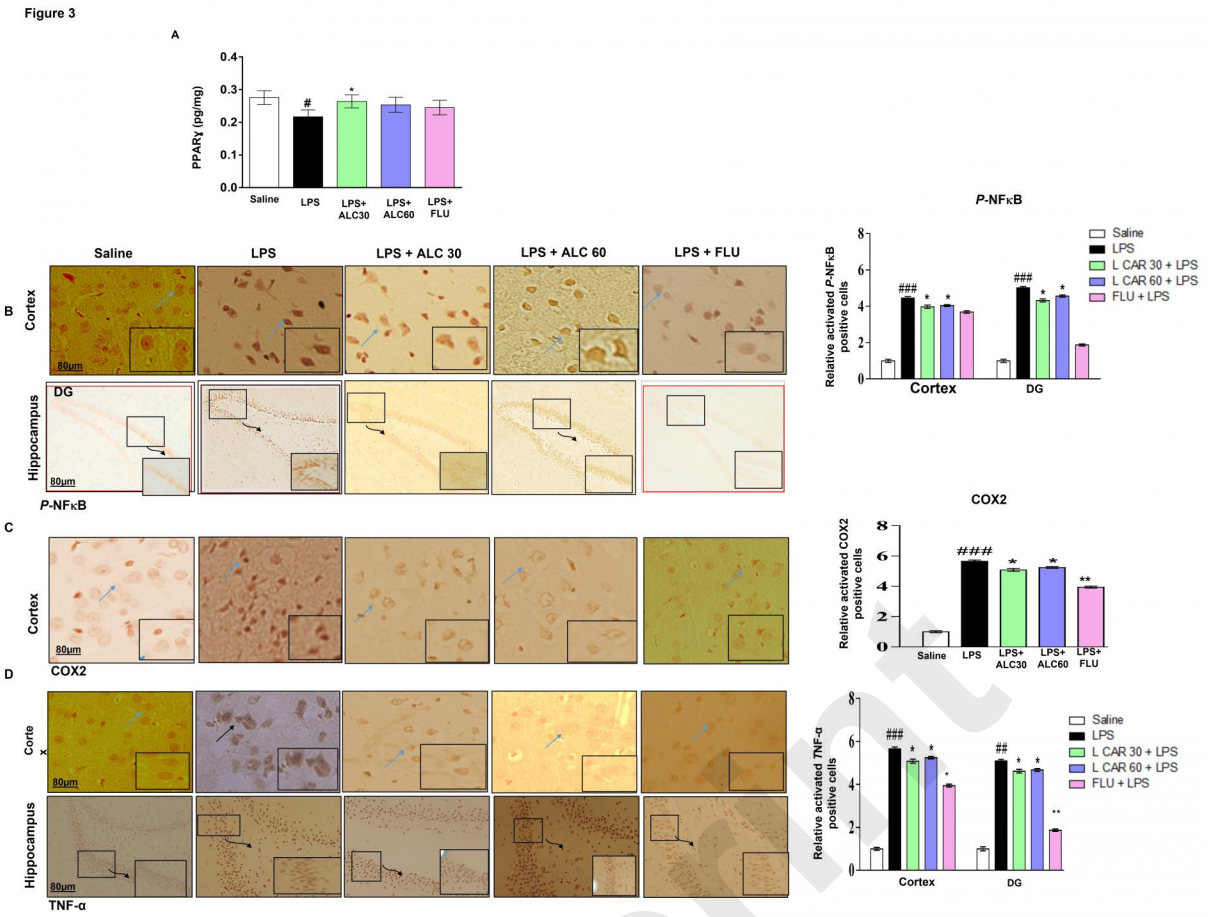
Experimental Outline

Figure 2



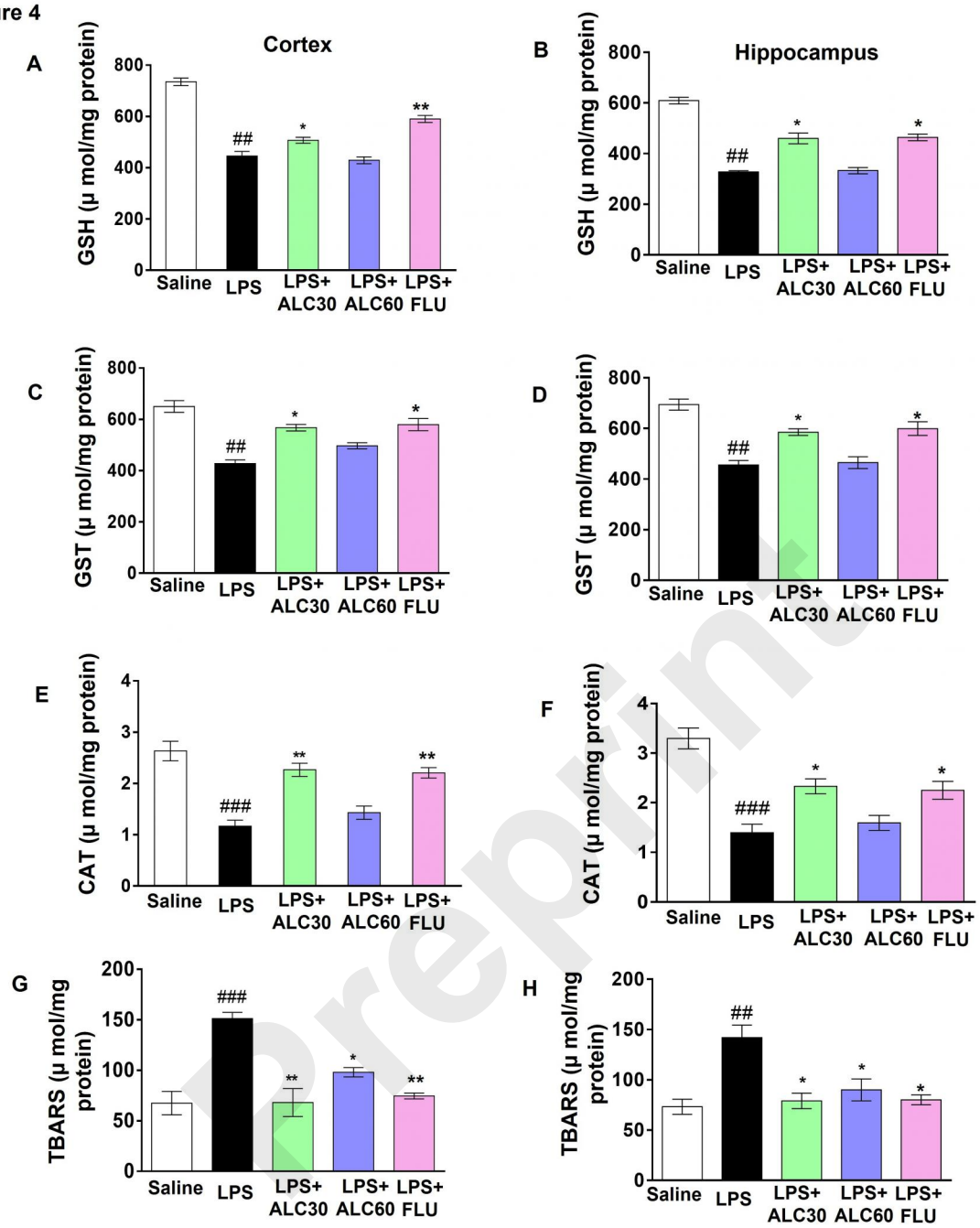
ALC attenuated LPS induced depressive-like anxiety behaviors. Effect of ALC and LPS on EPM test (A, B), FST (C, D), LDB test (E, F), and SST (G). Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by post hoc Bonferroni test using GraphPad Prism 6 software. Saline, LPS, LPS+ALC 30mg, LPS+ALC 60mg and LPS+FLU groups represent the first cohort (n=10/group), ##p<0.01 compared to the saline group while *p<0.05 compared to the LPS group. ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; FLU: fluoxetine; EPM: elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test.

Figure 3

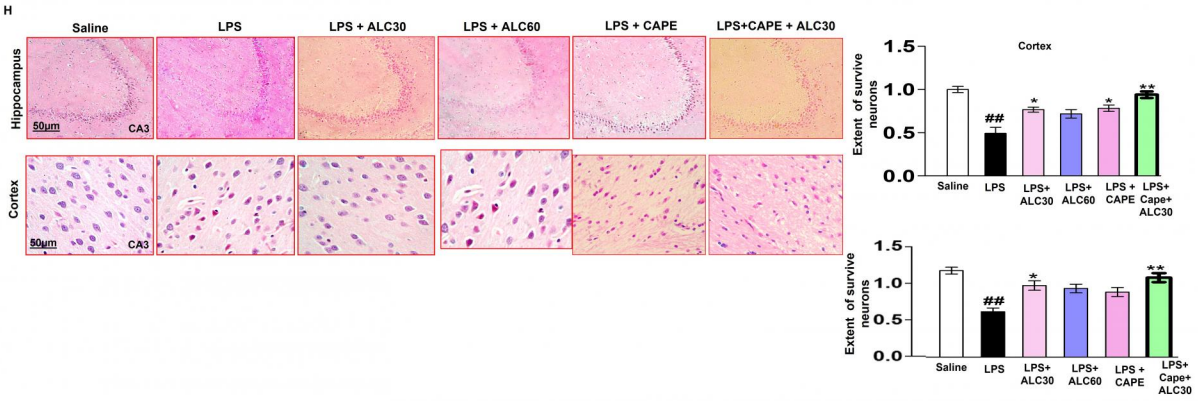


Effect of ALC on mitochondrial biogenesis implicated neuroinflammation. (A) PPAR γ levels were measured by ELISA, Data are expressed as means ± SEM. #p<0.05 compared to the saline group while *p<0.05 and compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC 60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine; PPAR γ: peroxisome proliferator-activated receptor. (B) Immunohistochemistry results for NF-κB in the cortex and hippocampus. (C) Immunohistochemistry results for COX-2 in the cortex and hippocampus. (D) Immunohistochemistry results for TNF-α in the cortex and hippocampus. Bar 50 µm, magnification 40×. Data are expressed as means ± SEM, ###p<0.001 compared to the saline group while *p<0.05, *p<0.05 compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine;

Figure 4

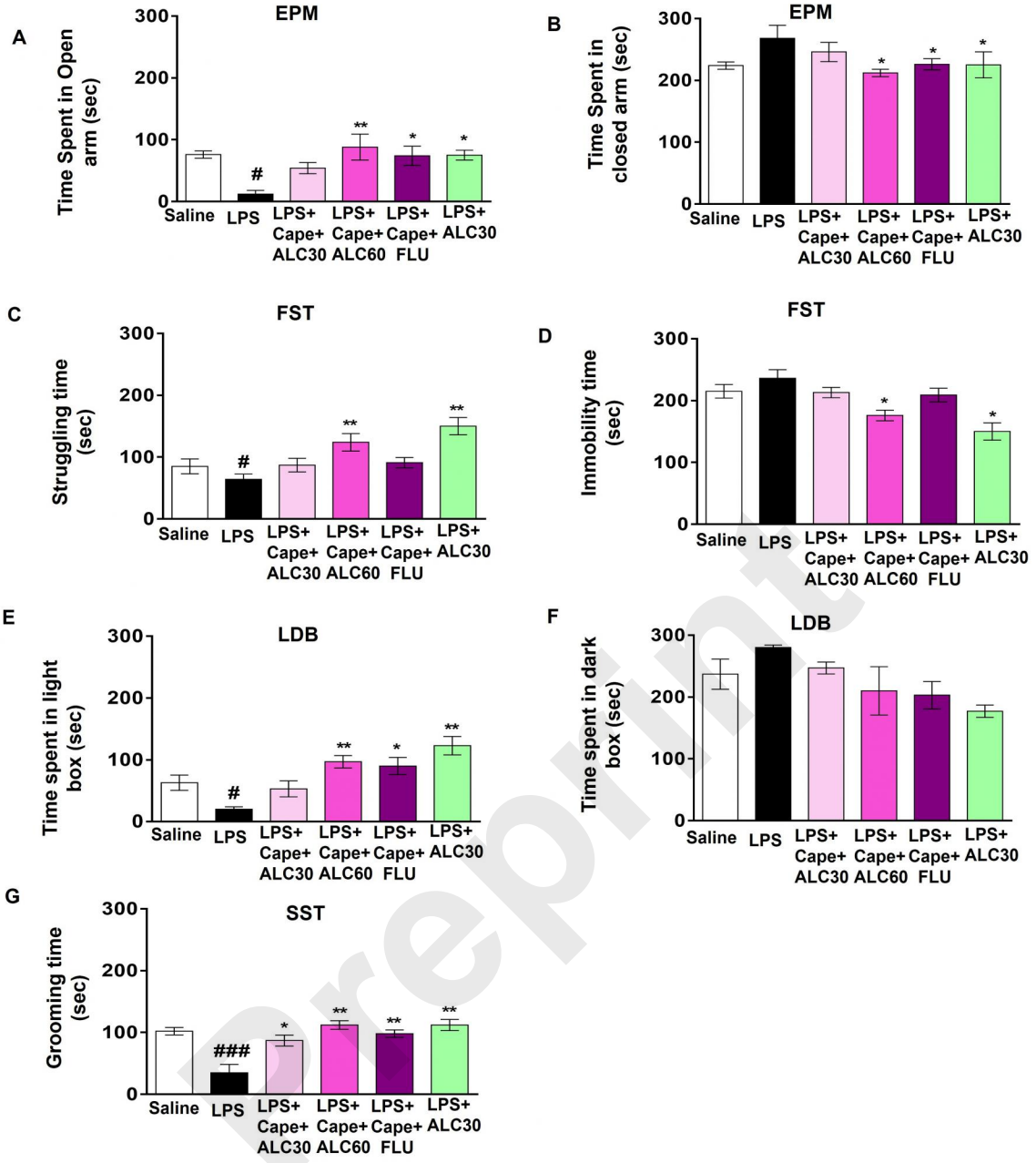


Effect of ALC on oxidative stress-related antioxidant enzymes. Effects of LPS and ALC on levels of GSH (A, B); GST (C, D); CAT (E, F), and TBARS (G, H). Data are expressed as means ± SEM. Where ##p<0.01, ###p<0.001 compared to the saline group while *p<0.05, **p<0.01 compared to LPS group. ALC: Acetyl-L- carnitine L Carnitine; LPS: lipopolysaccharide; FLU: fluoxetine; CAT: catalase; GSH: reduced glutathione; GST: Glutathione-S-transferase; TBARS: thiobarbituric acid reactive substances.



Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes.H&E staining shows the extent of surviving neurons in the cortex and hippocampus (Corno amonus, CA; dentate gyrus, DG). Bar 50 µm, magnification 40×. Surviving neurons were characterized by swollen cytoplasm, vacuolization, scalloped morphology with intense cytoplasmic eosinophilia, and nuclear basophilia. The Saline, LPS, ALC30mg+LPS, ALC 60mg+LPS, and FLU+LPS groups were taken first cohort (n=5/group), while CAPE+LPS, and CAPE+ALC30 mg +LPS, were from the second cohort (n=5/group).

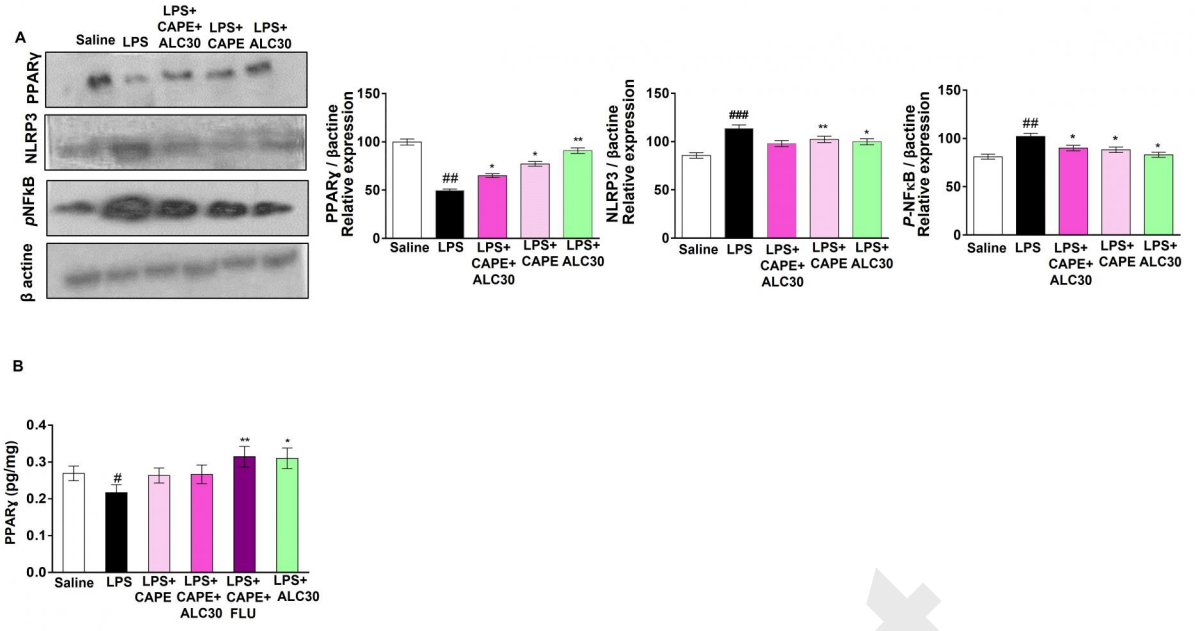
Figure 5



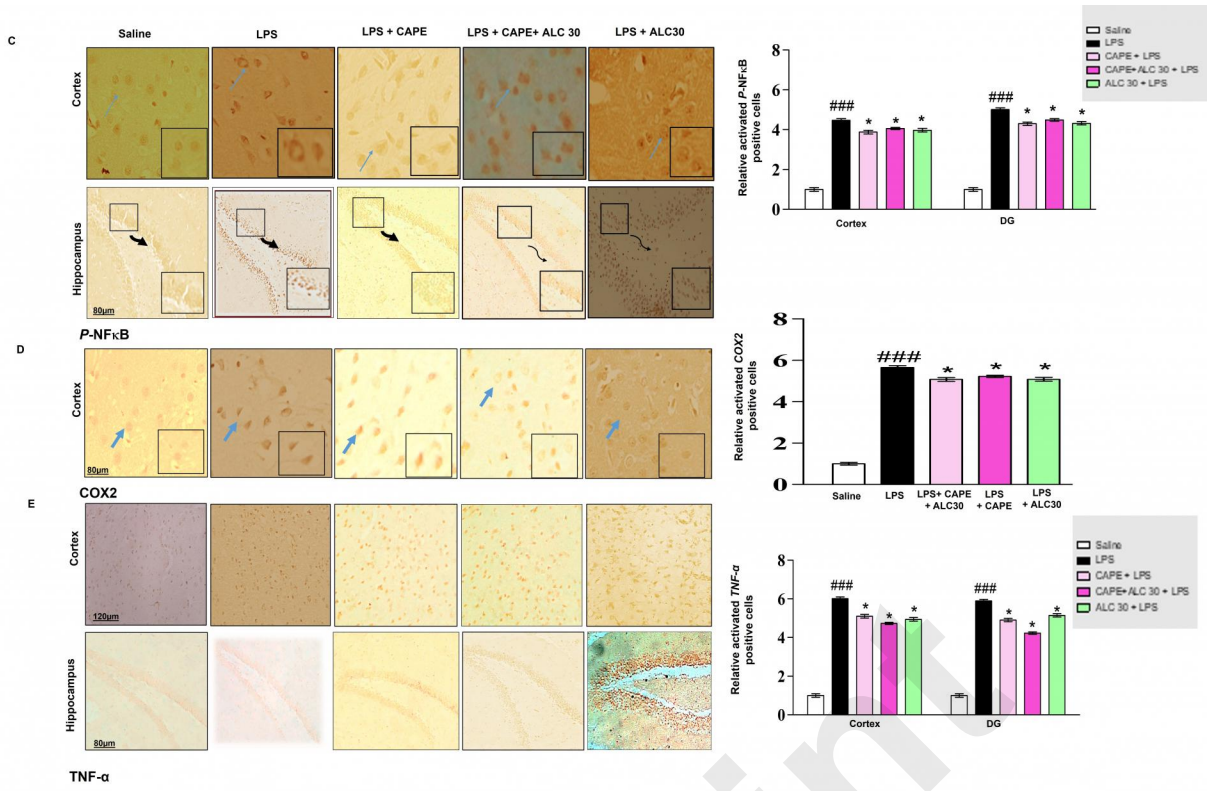
Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes. Effect of CAPE+ALC and CAPE+LPS on EPM (A, B), FST (C, D), LDB test (E, F), and SST (G). Data are expressed as means ± SEM and analyzed by one-way ANOVA followed by a post hoc Bonferroni test using GraphPad Prism 6 software. #p<0.05, ###p<0.001 compared to the saline group while *p<0.05, **p<0.01 compared to the LPS group. ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; CAPE: caeffic acid phenethyl ester; FLU: fluoxetine; EPM: elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test. H&E staining shows the extent of surviving neurons in the cortex and hippocampus (Corno amonus, CA; dentate gyrus, DG). Bar 50 µm, magnification 40×. Surviving neurons were characterized by swollen cytoplasm, vacuolization, scalloped morphology with intense cytoplasmic eosinophilia, and nuclear basophilia. The Saline, LPS, ALC30mg+LPS, ALC 60mg+LPS, and FLU+LPS groups were taken first cohort (n=5/group), while CAPE+LPS, and CAPE+ALC30 mg +LPS, were from the second cohort

(n=5/group).

Figure 6

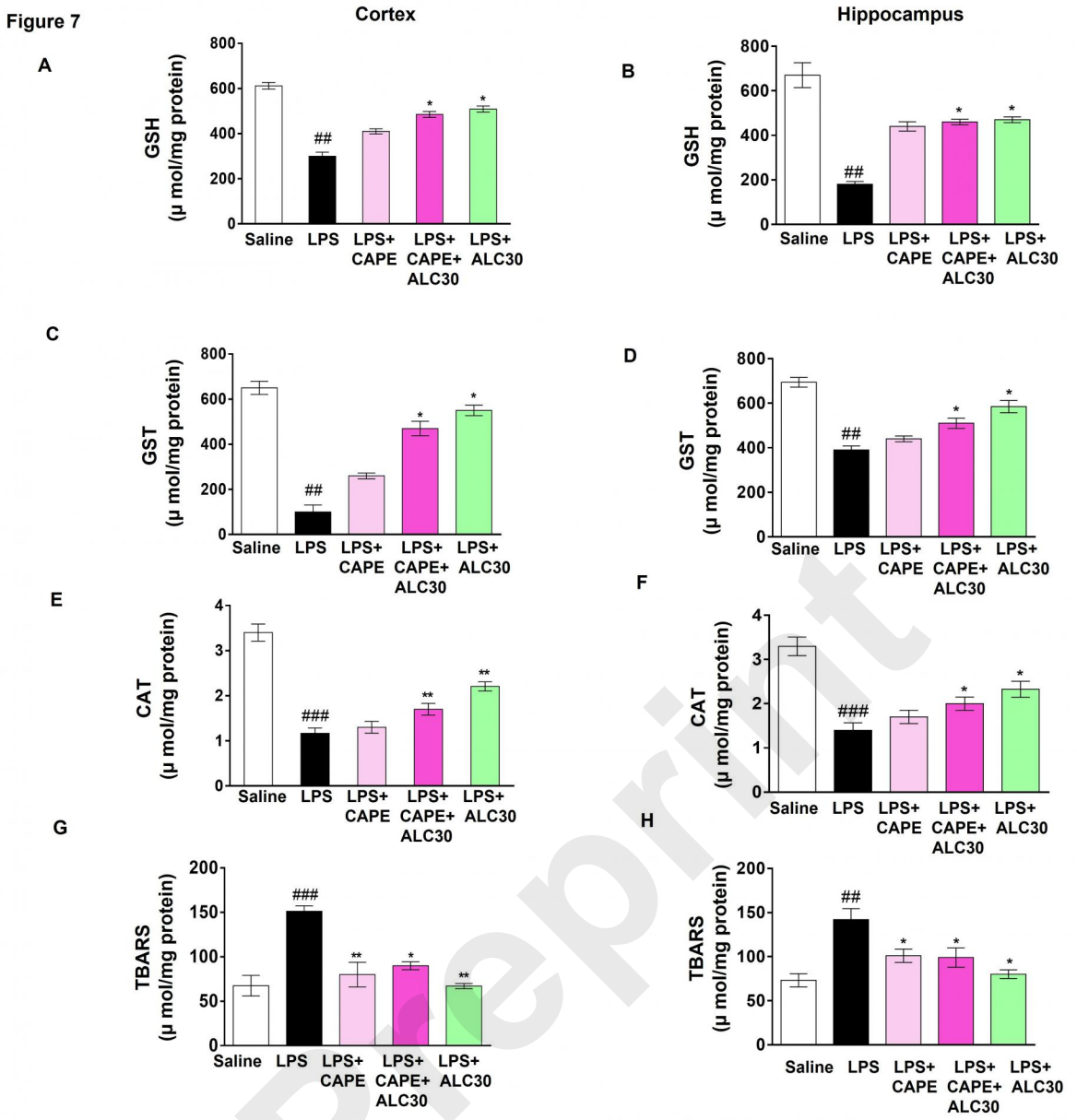


Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers (A) Representative bar graphs and western blots indicating the protein levels in the cortex (n = 5). Image J software was used for quantifying the western blots and graphs were generated using GraphPad Prism. Data are expressed as mean ± SEM, and results were analyzed using one-way ANOVA followed by post-hoc analysis. $p < 0.05$ was considered statistically significant. (B) PPARγ levels were measured by ELISA in the cortex. Data are expressed as means ± SEM.



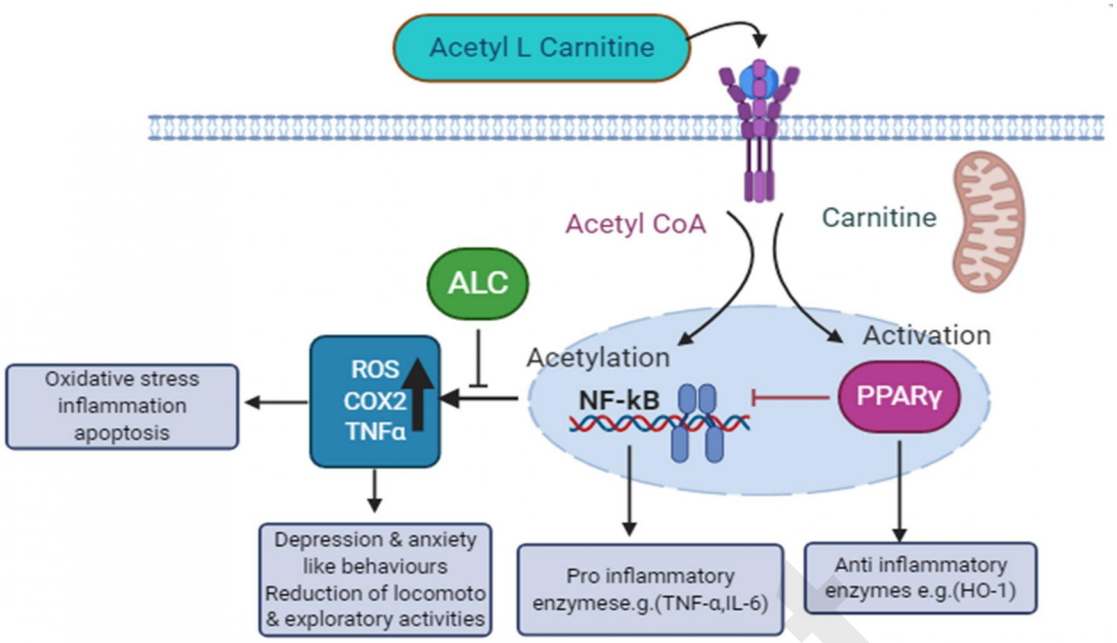
Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers (C) Immunohistochemistry results for NF-κB in the cortex and hippocampus tissues of the brain. (D) Immunohistochemistry of COX2 in the cortex and (E) Immunohistochemistry of TNF-α in the cortex and hippocampus tissues. Data are expressed as means ± SEM. ###p<0.001 compared to the saline group while *p<0.05 compared to the LPS group. ALC 30: Acetyl-L-carnitine (30 mg/kg); ALC 60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine; PPAR γ: peroxisome proliferator-activated receptor. CAPE: caeffic acid phenethyl ester;

Figure 7



Co-administration reversed the downregulation of antioxidant enzymes. Effects of CAPE, LPS, and ALC on levels of GSH (A, B); GST (C, D); CAT (E, F), and TBARS (G, H). Data are expressed as means ± SEM. Where ###p<0.001 or ##p<0.01 compared to the saline group while *p<0.05 or **p<0.01 compared to LPS group. ALC: Acetyl-L- carnitine LPS: lipopolysaccharide; CAPE: caeffic acid phenethyl ester; FLU: fluoxetine; GSH: reduced glutathione; GST: Glutathione-S-transferase; CAT: catalase; TBARS: thiobarbituric acid reactive substances

Figure 9



The suggested mechanism for ALC pre-treatment effects on LPS mediated oxidative stress, neuroinflammation, and neurodegeneration