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

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Type

Research paper

Keywords

neurodegeneration, lipopolysaccharide, neuroinflammation, antioxidant, Acetyl L Carnitine

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 MDD, 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 (PPAR γ) 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 PPAR γ dependent downstream antioxidant and anti-inflammatory pathways such as NOD and pyrin domain-containing protein 3 (NLRP3) linked nuclear factor kappa B (NF- κ B) phosphorylation. Moreover, co-administering NF- κ B inhibitor caffeic acid phenethyl ester (CAPE) with ALC also increased PPAR γ expression significantly and decreased NF- κ B 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 PPAR γ upregulations.

1 **Acetyl-L-Carnitine protects against LPS induced depression via PPAR- γ induced**
2 **inhibition of NF- κ B/NLRP3 pathway**

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42 **Abstract:**

43 **Introduction**

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45 swings and high suicidal attempts. Several studies have reported the role of neuroinflammation
46 in MDD, yet the efficacy of natural drug substances on neuroinflammation-associated
47 depression needs to be further investigated. The present study demonstrated the neuroprotective
48 effects of Acetyl-L- carnitine (ALC) alone or in combination with caffeic acid phenethyl ester
49 (CAPE) on lipopolysaccharide (LPS) induced neuro-inflammation, depression, and anxiety-like
50 behavior.

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54 mechanistic interplay of the peroxisome proliferator-activated receptors (PPAR γ) in depression.
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56 such as a decreased grooming tendency, diminished locomotive activity, and increased
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64 significantly reverted these changes by positively modulating the PPAR γ dependent downstream
65 antioxidant and anti-inflammatory pathways such as NOD and pyrin domain-containing protein
66 3 (NLRP3) linked nuclear factor kappa B (NF- κ B) phosphorylation. Moreover, co-administering
67 NF- κ B inhibitor caffeic acid phenethyl ester (CAPE) with ALC also increased PPAR γ
68 expression significantly and decreased NF- κ B and NLRP3 inflammasome.

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

74
75 **Key Words:** Acetyl-L- carnitine, lipopolysaccharide, antioxidant, neurodegeneration,
76 neuroinflammation.

84

1. INTRODUCTION:

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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.

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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].

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Peroxisome proliferator-activated receptors (PPARs) are transcription factors that belong to the nuclear receptor superfamily [8]. Recently, PPAR γ agonists have revealed antidepressant effects and which could be a plausible target [9]. Several *in vitro* and *in vivo* experiments have shown that PPAR γ inhibits the production of pro-inflammatory cytokines and oxidative stress by inhibiting the NF- κ B nuclear transcription factor [10]. In this regard, pharmacological modulation of PPAR γ 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-1 β [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- κ B) is a vital transcription factor that modulates inflammation and multiple autoimmune diseases.

122

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- κ B inhibitor and has shown protective effects in various experimental models [23].

127

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

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

138 **2. Methodology:**

139

140 **2.1 Animals:**

141 Sprague Dawley male rats weighing 180-220 g were housed three per cage under a 12-hour
142 light/dark cycle with free access to water and food at the animal house of Riphah Institute of
143 Pharmaceutical Sciences (RIPS) under standard laboratory protocols (temp: 22 \pm 1 $^{\circ}$ C; humidity:
144 50% \pm 10%). All experimental procedures were carried out as per the guidelines of the Institute
145 of Laboratory Animal Resources, Commission on Life Sciences University, National Research
146 Council (1996), approved by the RIPS Ethical Committee (Ref. No. REC/RIPS/2019/28).

147

148 **2.2 Experimental design:**

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

164

165

166 **2.3 Behavioral testing:**

167

168 **2.3.1 Sucrose Splash Test (SST):**

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

173 10% sucrose solution on the dorsal surface of the rodent's body and grooming behavior was
174 measured in terms of licking, biting, or scratching the fur to clean. Grooming time was
175 videotaped for five minutes.

176 **2.3.2 Elevated plus Maze Test (EPM):**

177 An elevated plus maze test was conducted to measure the LPS mediated anxiety-like behavior.
178 Briefly, the maze equipment consisted of two oppositely faced open arms and two oppositely
179 faced closed arms (OA, 50 × 10 cm; CA, 50 × 10 cm) in the form of a cross-shaped Plexiglas
180 platform having 40 cm walls and height 50 cm above the floor in a soundproof room with a
181 dimmed light. Each rat was placed at the central point of the Plexiglas platform with its face
182 heading towards any open arm. The time spent in each arm was recorded for all the animals for
183 5 minutes.

184 **2.3.3 Light-dark box test (LDB):**

185 DB test was performed to assess the LPS mediated anxiety-like behavior. LDB equipment is
186 comprised of a light and a dark compartment separated by a partition containing a small gap
187 [28]. Each animal was sited in a dark compartment of a light-dark box and was set free to move
188 around in the box for 5 min. The total time spent in each compartment was videotaped. The
189 olfactory cues were minimized by ensuring thorough cleaning of the light-dark box with alcohol.

190 **2.3.4 Forced swim test:**

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

202 **2.4 Hematoxylin Eosin (H&E) Staining:**

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

216 **2.5 Immuno-histochemical Staining and Analysis:**

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

219 method for antigen retrieval, then washed with PBS consecutively three times for 5 minutes. The
220 slides were immersed in a 3% H₂O₂ to quench the endogenous peroxidase activity followed by
221 washing with PBS. 5% normal goat serum, used as a blocking serum, and slides were incubated
222 for 2 hr. Later, the slides were incubated overnight after applying primary antibodies. The next
223 morning slides were washed with PBS and incubated for 90 min with the secondary antibody,
224 and later with ABC kit (Santa Cruz) in a humidified box for 60 min. Slides were then washed
225 with PBS solution and stained with DAB, followed by dehydration with ethanol (70%, 80%,
226 90%, and 100%). After dehydration, the slides were fixed with xylene and then coverslipped
227 with mounting media. Five immunohistochemical TIF images were captured per slide with a
228 light microscope. ImageJ software was used to quantitatively determine hyperactivated COX2,
229 TNF- α , p-NFKB, in cortex/total area and hippocampus/total area by optimizing the background
230 of images, according to the threshold intensity and analyzing the nuclear p-NFKB and
231 cytoplasmic COX2, TNF- α positive cells at the same threshold intensity for all groups and was
232 expressed as the relative activated positive cells relative to the control.

233 **2.6 Lipid Peroxidation Determination (LPO) in Tissue:**

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

242 **2.7 Reduced glutathione (GSH) Activity:**

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

250 **2.8 Glutathione-S-Transferase (GST)**

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

258 **2.9 Catalase Activity:**

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

$$262 \text{CAT} = \delta\text{O.D} \div E \times \text{Volume of sample (mL)} \times \text{protein (mg)}$$

263 where $\delta\text{O.D}$ represents the change in absorbance per minute and E represents the extinction
264 coefficient of H₂O₂ with a value of 0.071 mmol cm⁻¹.51. The Lowery method was used to
265 measure protein levels. Catalase activity was expressed as μmol of H₂O₂/min/mg of protein.

266 **2.10 Immunosorbent Assay (ELISA) linked with enzymes:**

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

275 **2.11 Western blot:**

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

285

286 **2.12 Statistical Analysis:**

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

289

290 **3. RESULTS:**

291 **3.1 ALC attenuated LPS induced depressive-like anxiety behaviors**

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

304

305 **Figure 2.** ALC attenuated LPS induced depressive-like anxiety behaviors. Effect of ALC and LPS on EPM test (A, B),
306 FST (C, D), LDB test (E, F), and SST (G). Data are expressed as means ± SEM and analyzed by one-way ANOVA
307 followed by post hoc Bonferroni test using GraphPad Prism 6 software. Saline, LPS, LPS+ALC 30mg, LPS+ALC
308 60mg and LPS+FLU groups represent the first cohort (n=10/group), ## $p < 0.01$ compared to the saline group while
309 * $p < 0.05$ compared to the LPS group. ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; FLU: fluoxetine; EPM:
310 elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test.

311

312

313 3.2 ALC elevated PPAR γ level coupled to NLRP3 downregulation

314 To elucidate the underlying mechanism of ALC and its relative role on mitochondrial biogenesis
315 and neuroinflammation, we demonstrated various analyses to determine the level of these
316 associated proteins in the cortical region of the brain. Ligand bound activation of PPAR γ can
317 attenuate dysfunctional mitochondria, neuronal death, oxidative stress, and inflammation, as
318 reported previously. To determine the effect of ALC on PPAR γ level and inflammation, initially,
319 we performed ELISA analysis (Figure 3A). PPAR γ level in the LPS group was decreased (Figure
320 3A, $p < 0.05$) however, this level remained high in ALC 30mg group (Figure 3A, $p < 0.05$).
321 Consistent studies suggested the implication of NF- κ B signaling pathways in depression that can
322 trigger NLRP3 and downstream transcriptional induction of many pro-inflammatory mediators
323 including COX-2 and TNF- α that could exacerbate the symptoms. We studied the expression of
324 p-Nf- κ B, COX2, and TNF- α , either in the cortex or in both cortex and hippocampus by
325 immunohistochemical analysis (Figure 3B-D). All these proteins showed elevated expression in
326 the LPS group ($p < 0.001$), but treatment with ALC at doses 30 and 60 mg equivalently diminished
327 the triggered overexpression of p-NF κ B ($p < 0.05$, $p < 0.05$), COX2 ($p < 0.05$), and TNF- α ($p < 0.05$,
328 $p < 0.05$) (Figure 3).

329

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

339

340 3.3 Effect of ALC on enzymatic and non-enzymatic anti-oxidants

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

350

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

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

356 **Experimental results of Cohort 2**

357 **3.4 Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral** 358 **outcomes.**

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

372

373 **Figure 5.** Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes. Effect
374 of CAPE+ALC and CAPE+LPS on EPM (A, B), FST (C, D), LDB test (E, F), and SST (G). Data are expressed as
375 means \pm SEM and analyzed by one-way ANOVA followed by a post hoc Bonferroni test using GraphPad Prism 6
376 software. # $p<0.05$, ### $p<0.001$ compared to the saline group while * $p<0.05$, ** $p<0.01$ compared to the LPS group.
377 ALC: Acetyl-L- carnitine; LPS: lipopolysaccharide; CAPE: caeffic acid phenethyl ester; FLU: fluoxetine; EPM:
378 elevated plus maze; FST: forced swim test; LDB: light-dark box; SST: sucrose splash test. H&E staining shows the
379 extent of surviving neurons in the cortex and hippocampus (Corno amonus, CA; dentate gyrus, DG). Bar 50 μ m,
380 magnification 40 \times . Surviving neurons were characterized by swollen cytoplasm, vacuolization, scalloped
381 morphology with intense cytoplasmic eosinophilia, and nuclear basophilia. The Saline, LPS, ALC30mg+LPS, ALC
382 60mg+LPS, and FLU+LPS groups were taken first cohort (n=5/group), while CAPE+LPS, and CAPE+ALC30 mg
383 +LPS, were from the second cohort (n=5/group).

384 **3.5 Effect of co-administration on mitochondrial biogenesis and neuroinflammatory** 385 **markers.**

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

396

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

408 **3.6 Co-administration reversed the downregulation of antioxidant enzymes.**

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

420

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

426

427 **4. Discussion:**

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

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

442 and is involved in many inflammatory processes [37]. It is reported that PPAR γ agonist could
443 ameliorate depression-like disorders, possibly by modulating the inflammatory cascade,
444 oxidative stress mechanism, and synaptic modulation. Moreover, pioglitazone which is a
445 reference PPAR γ agonist exhibited antidepressant-like results by modulating NF- κ B/IL-
446 6/STAT3 and CREB/BDNF pathway [39]. Furthermore, pioglitazone, either alone or as an add-
447 on therapy to conventional treatments was found to induce remission of depression [40]. Another
448 PPAR γ agonist NP031115 demonstrated antidepressant-like effects by inhibiting GSK-3 β [41],
449 while PPAR γ antagonist reversed these anti-depressive like effects [42]. In all these studies,
450 PPAR γ activation, ameliorated the classical behavioral deficits with improved therapeutic
451 response. Therefore, stimulating PPAR γ could be an appropriate therapeutic target to uncover
452 new medicines for mood disorders. The results of the present study was also in line with studies
453 where ALC and CAPE improved the depression and anxiety-like behaviors in LPS rats presented
454 by an increase in the time spent in the open arm while a decrease in the time spent in the closed
455 arm of the elevated plus-maze. Additionally, in the forced swimming test, these compounds
456 increased struggling while decreasing immobility time, and the results coincide with light-dark
457 box and grooming behavior. PPAR γ is a widely distributed cell transcription factor that executes
458 vital functions in redox homeostasis, neurogenesis, diabetes, and other vascular disorders. The
459 protective effects of PPAR γ are also investigated in other neurodegenerative disorders such as
460 Parkinson's, Alzheimer's, Huntington's, and stroke [43]. We used a combination of ALC and
461 CAPE here as previously a combination of a low dose of PPAR γ agonist with other inflammatory
462 and glutamatergic antagonists substantiated the antidepressive effects of PPAR γ [44]. In this
463 context, ALC demonstrated antioxidant, neuromodulatory, and neuroprotective effects including
464 MDD in elderly patients [22]. Likely, CAPE has been reported to have anti-inflammatory,
465 antioxidant, and immunomodulatory properties.

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

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

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

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

495

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

498

499 **5. Conclusion:**

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

507

508

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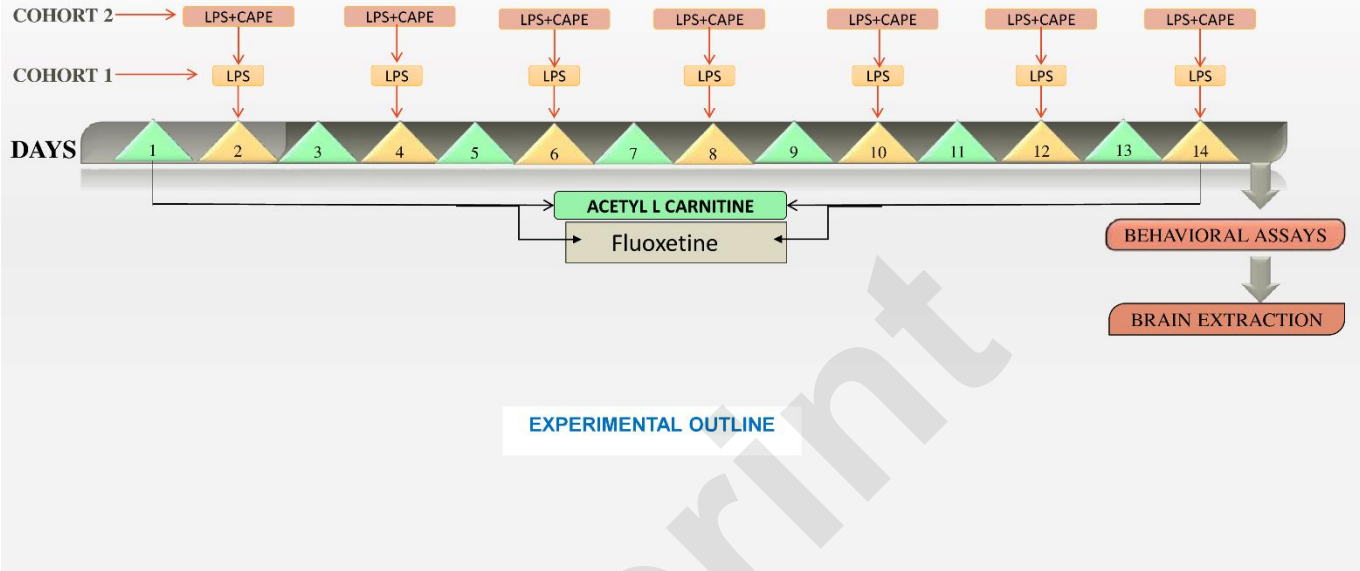
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Figures

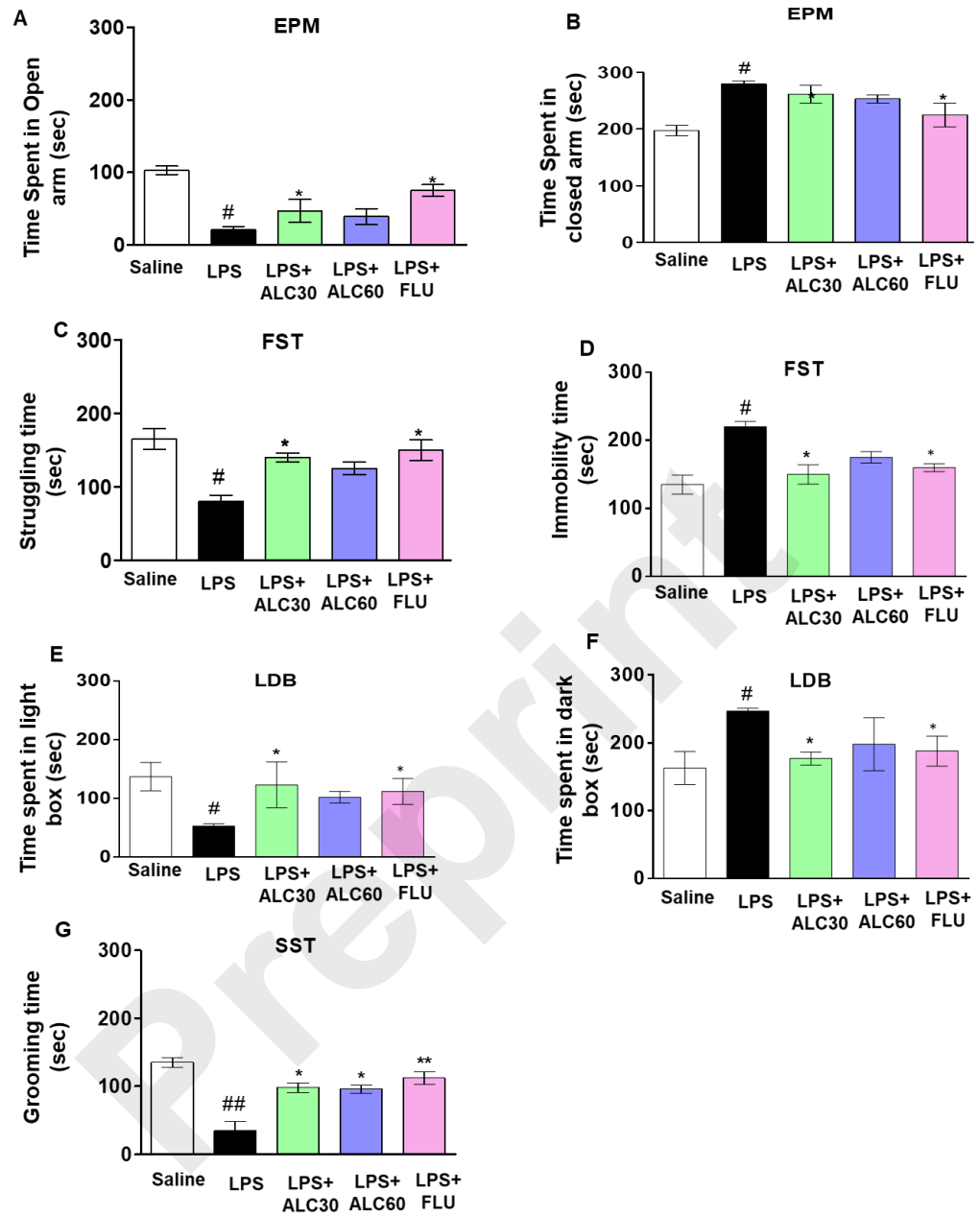
Figure 1



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Figure 2



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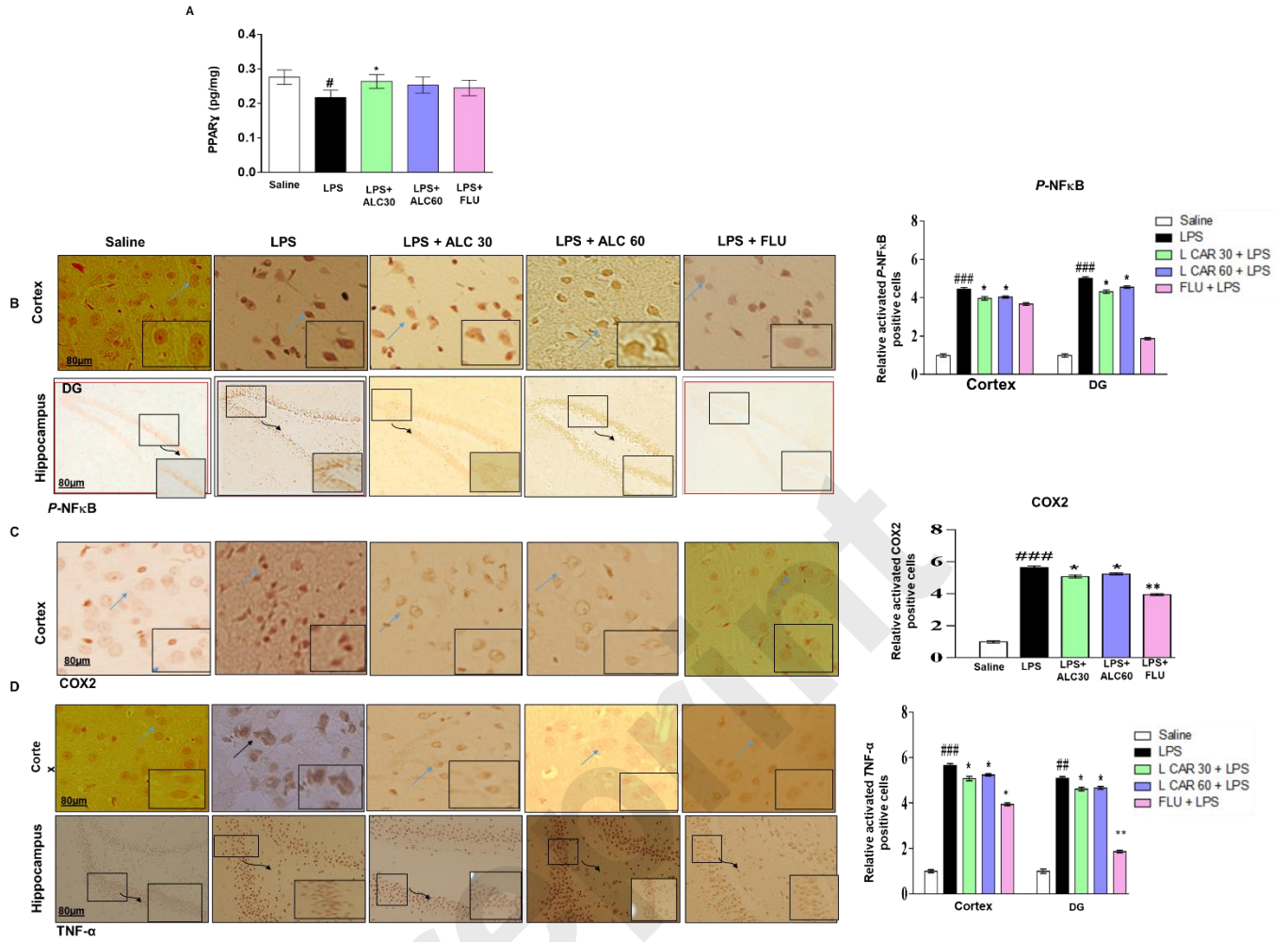
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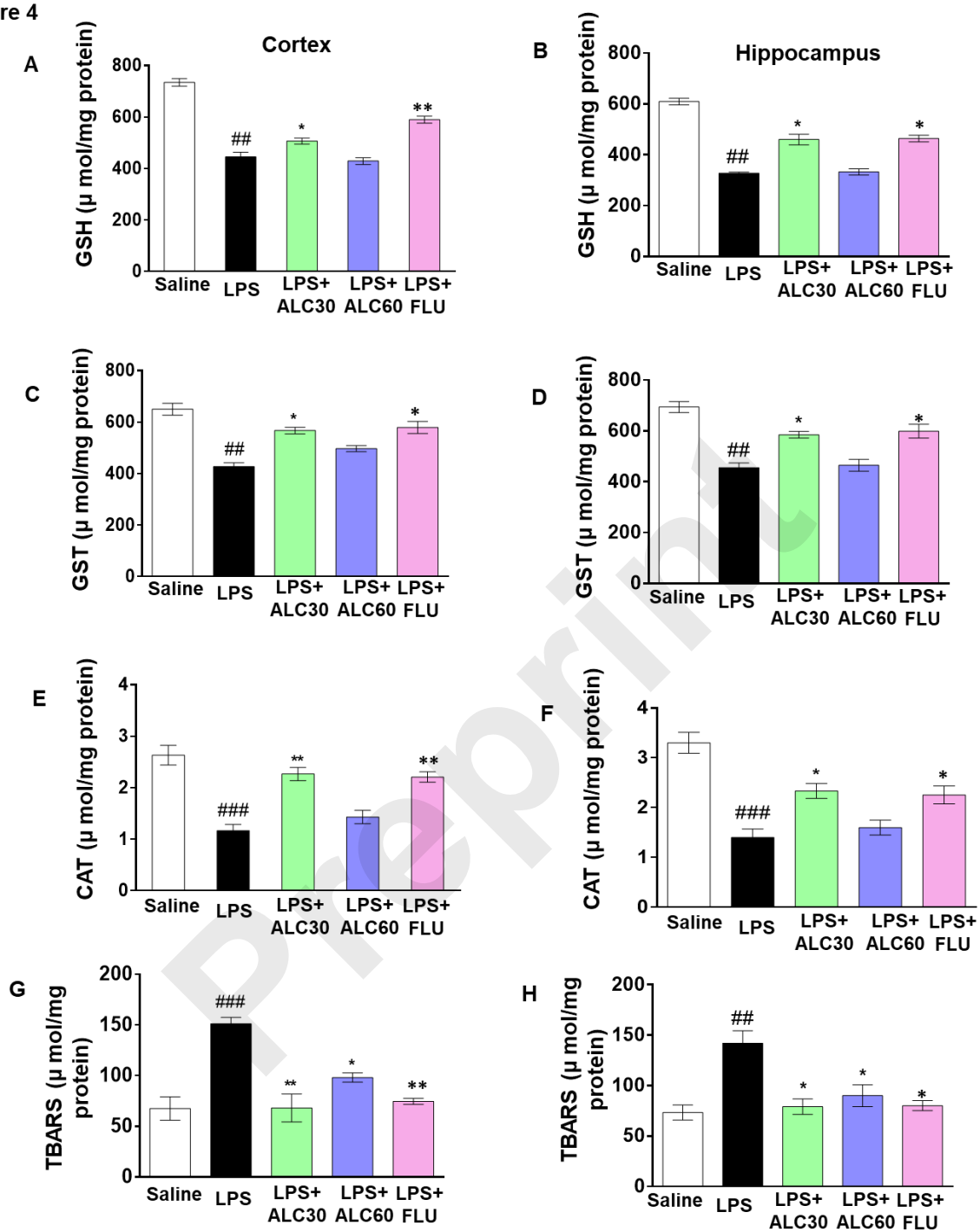
Figure 3



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Figure 4



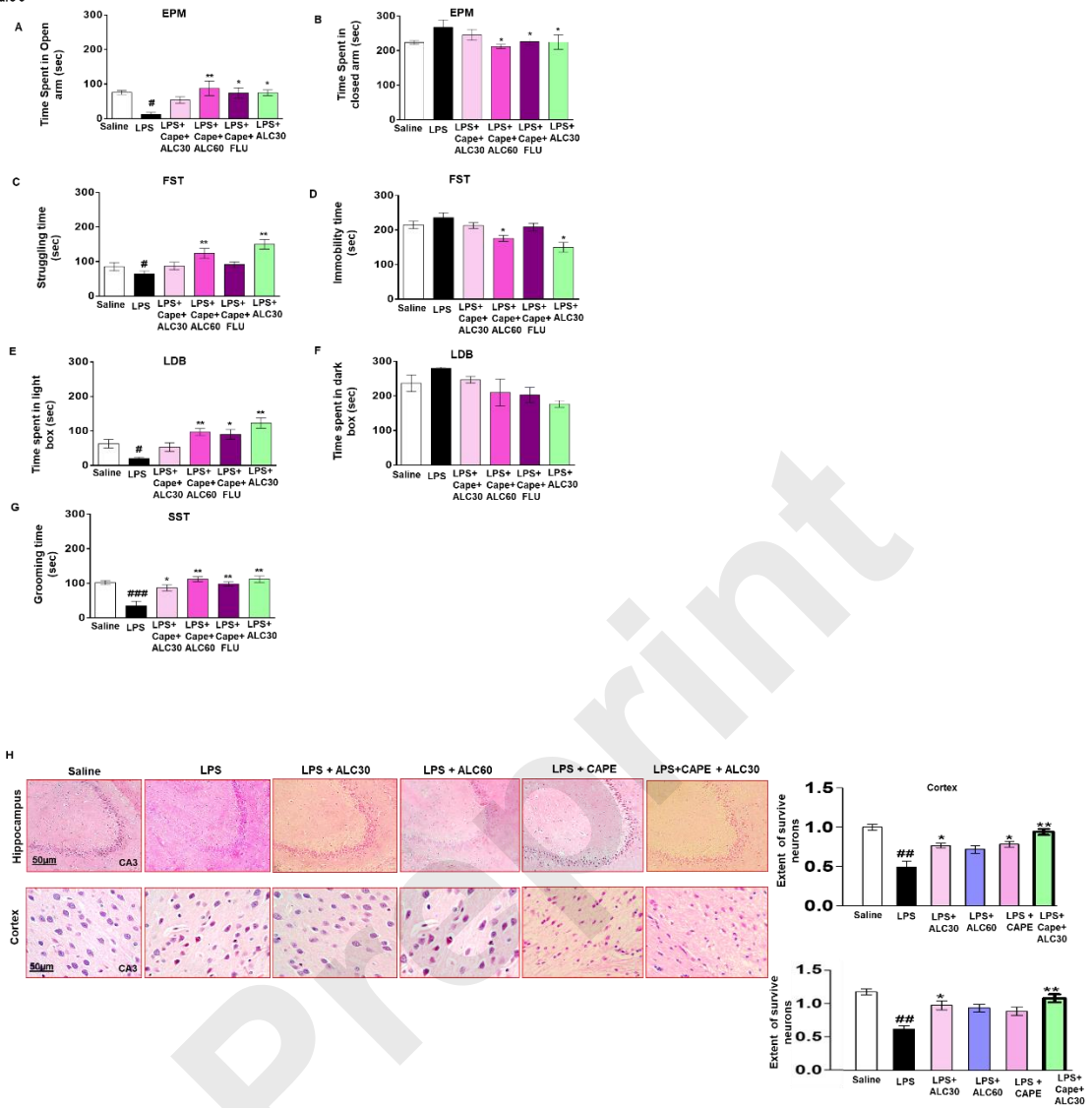
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Figure 5



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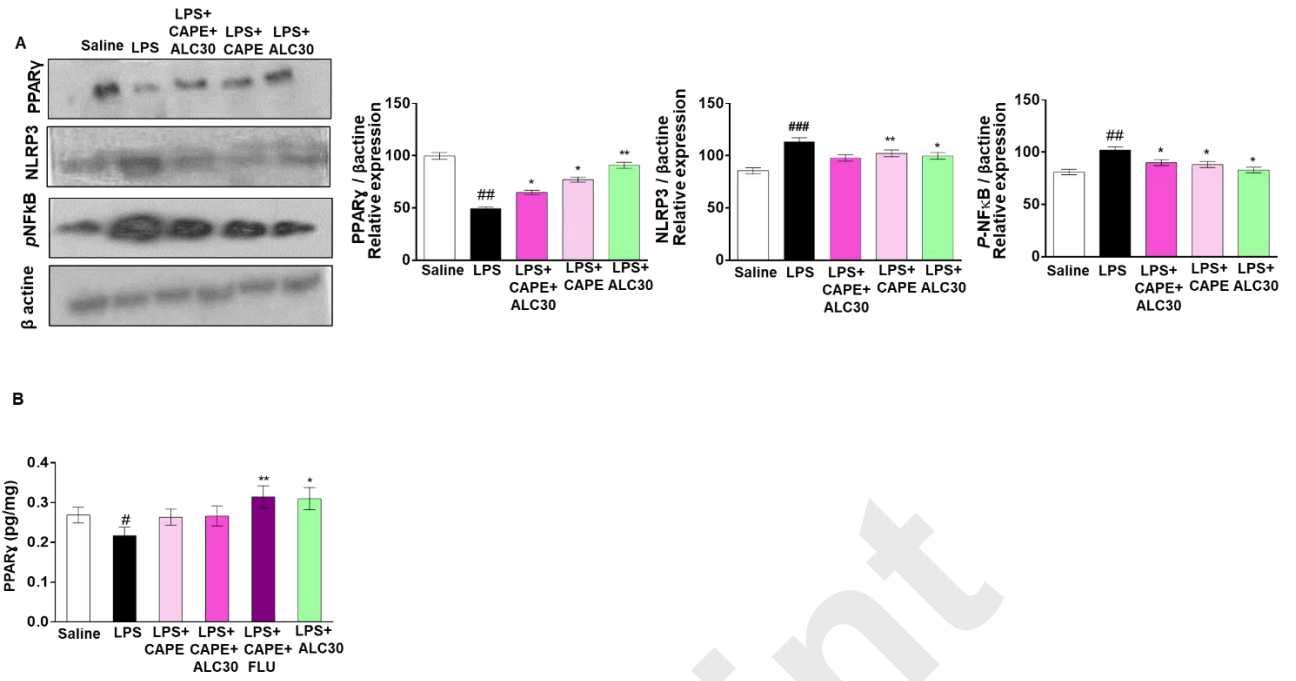
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Figure 6



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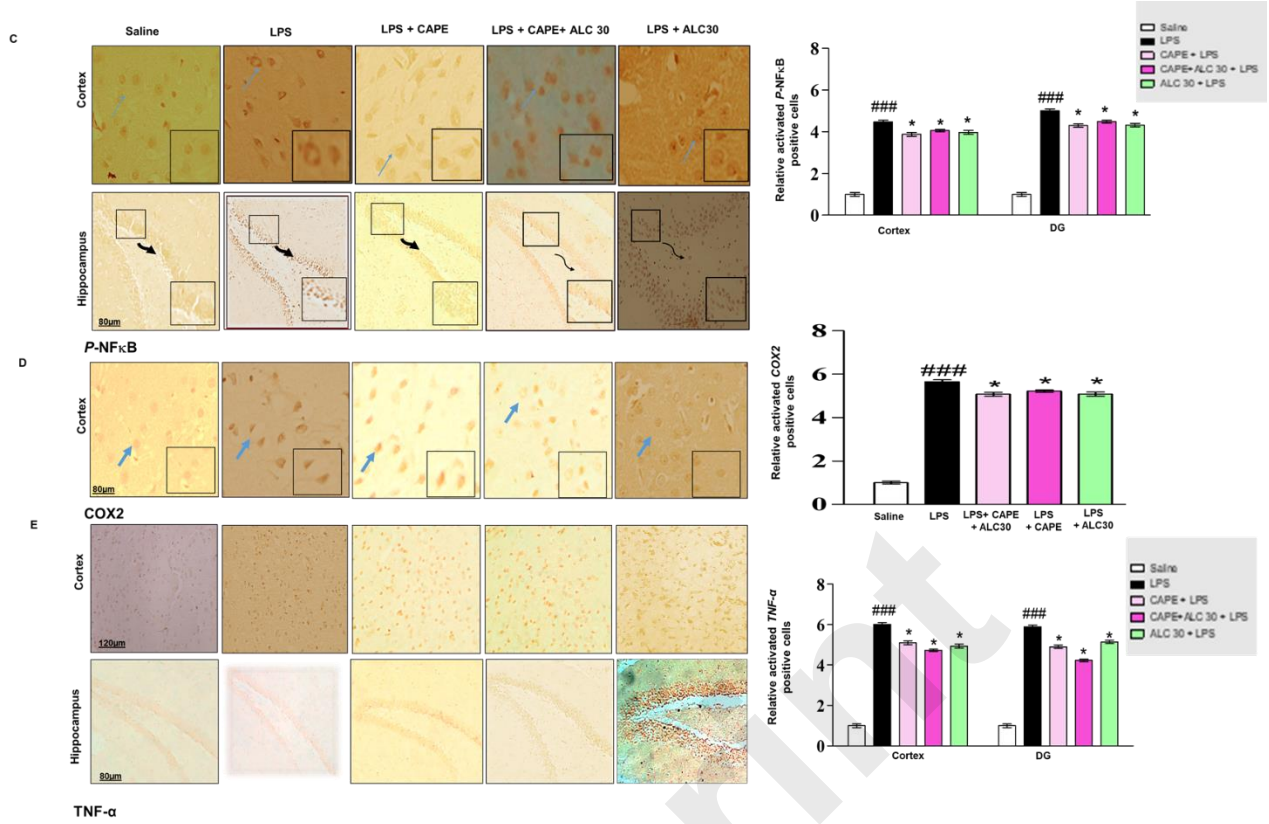
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Figure 7

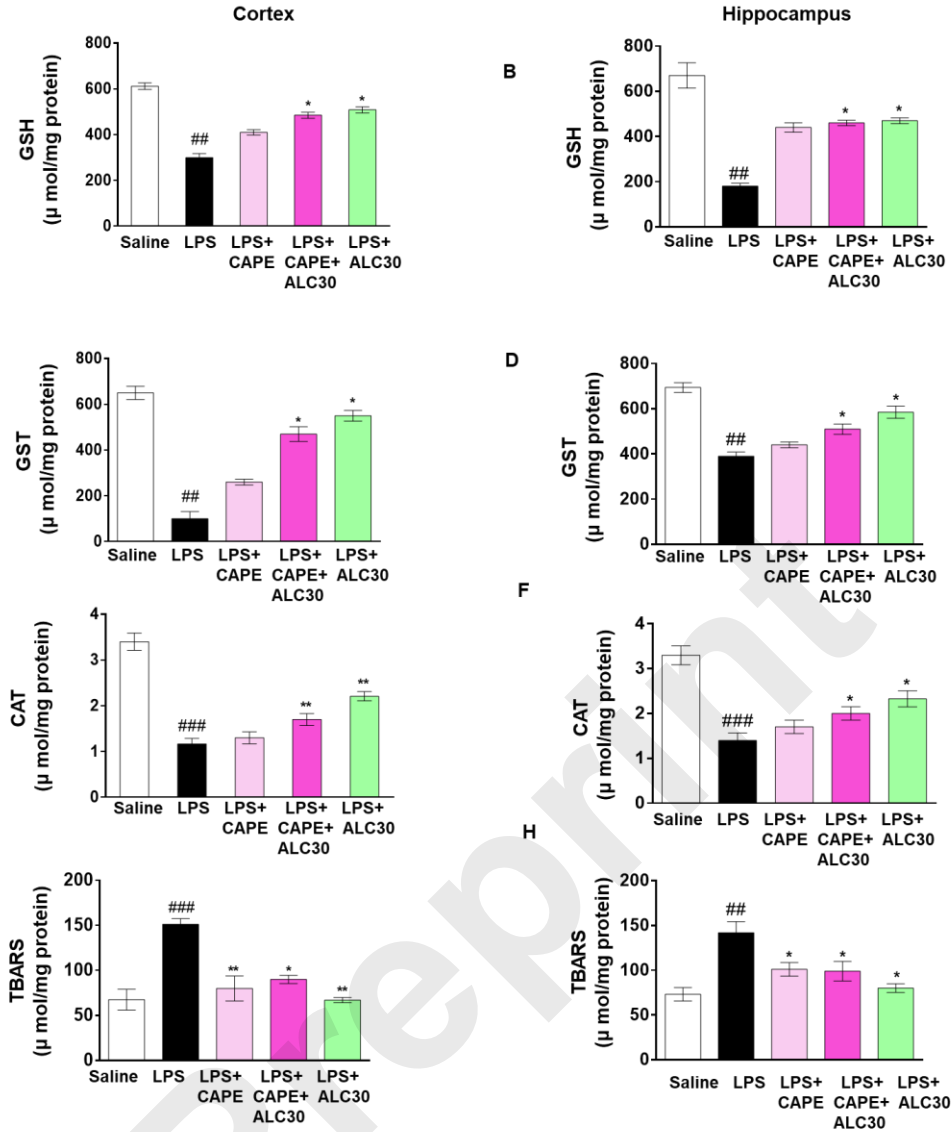
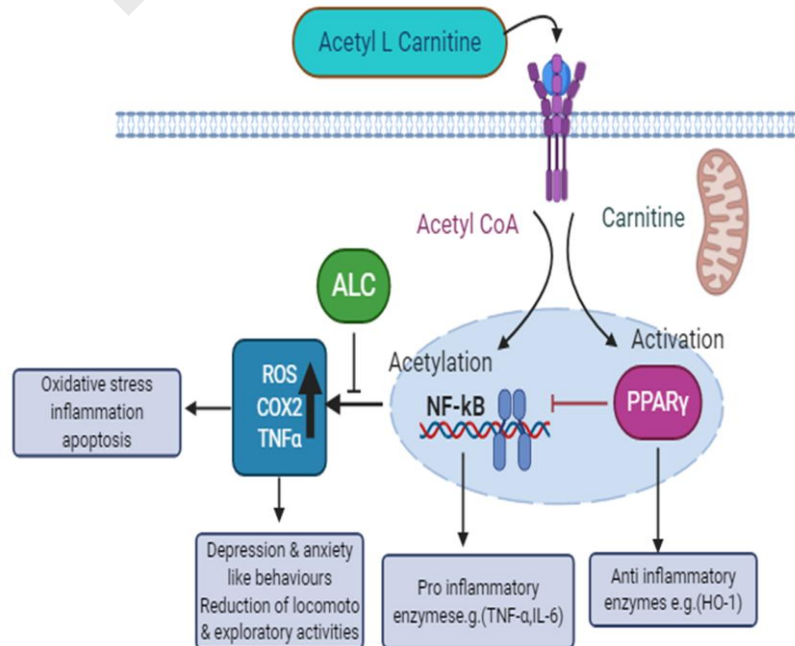


Figure 8. The suggested mechanism for ALC pre-treatment effects on LPS mediated oxidative stress, neuroinflammation, and neurodegeneration



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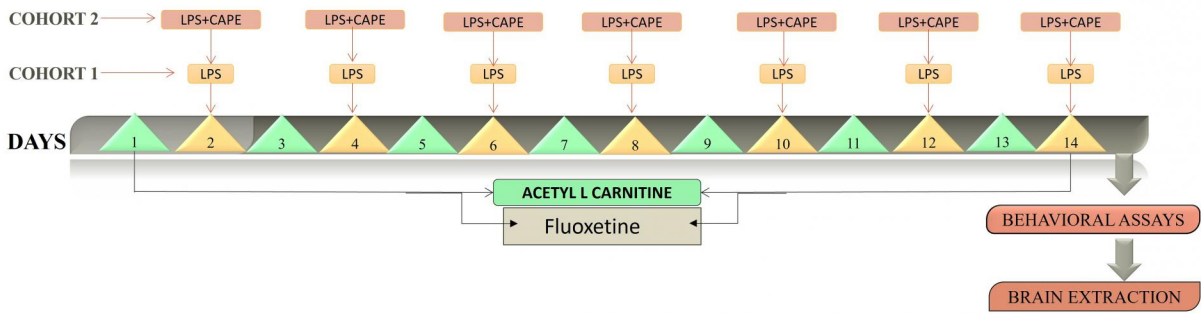
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EXPERIMENTAL OUTLINE

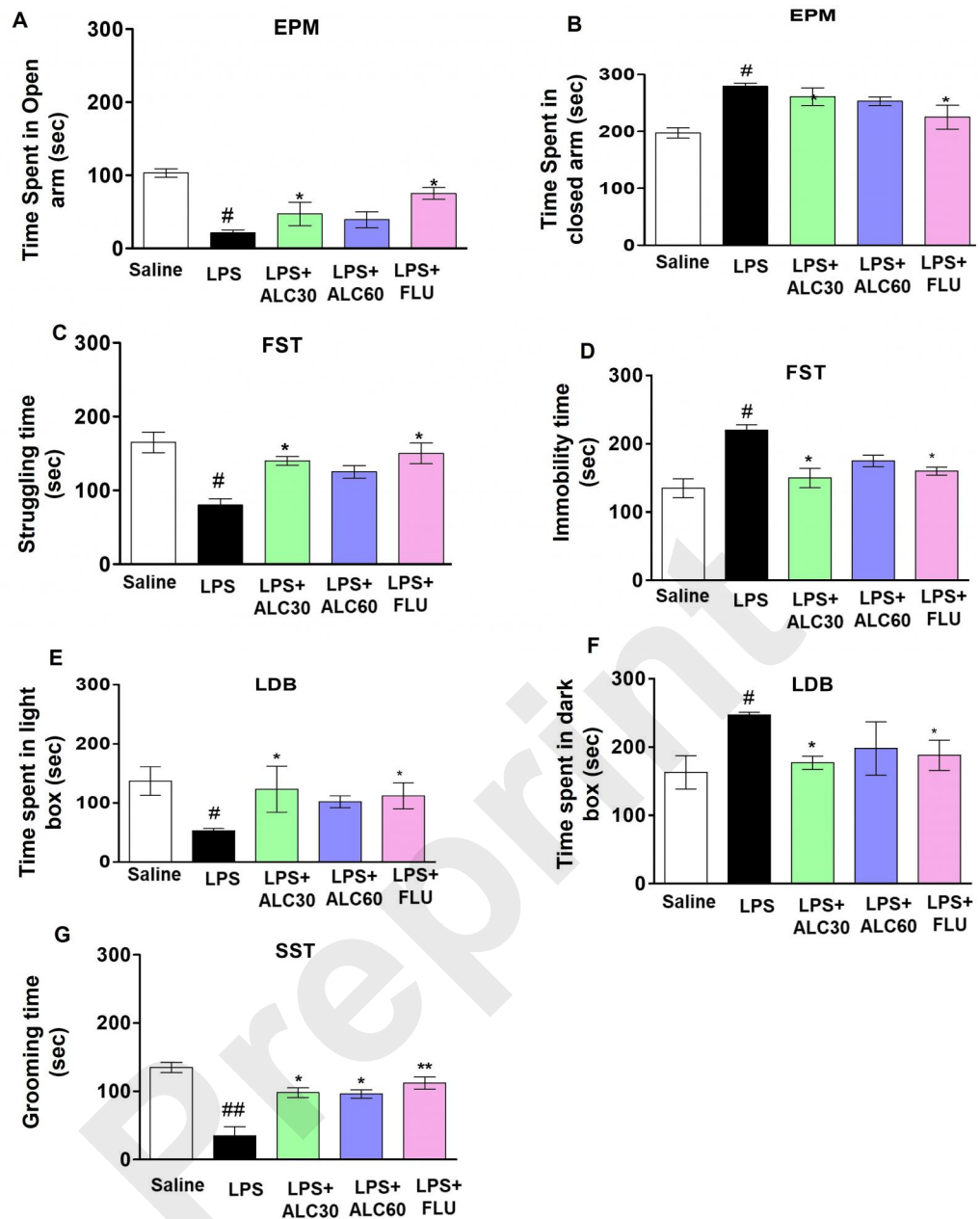
Figure 1



Experimental Outline

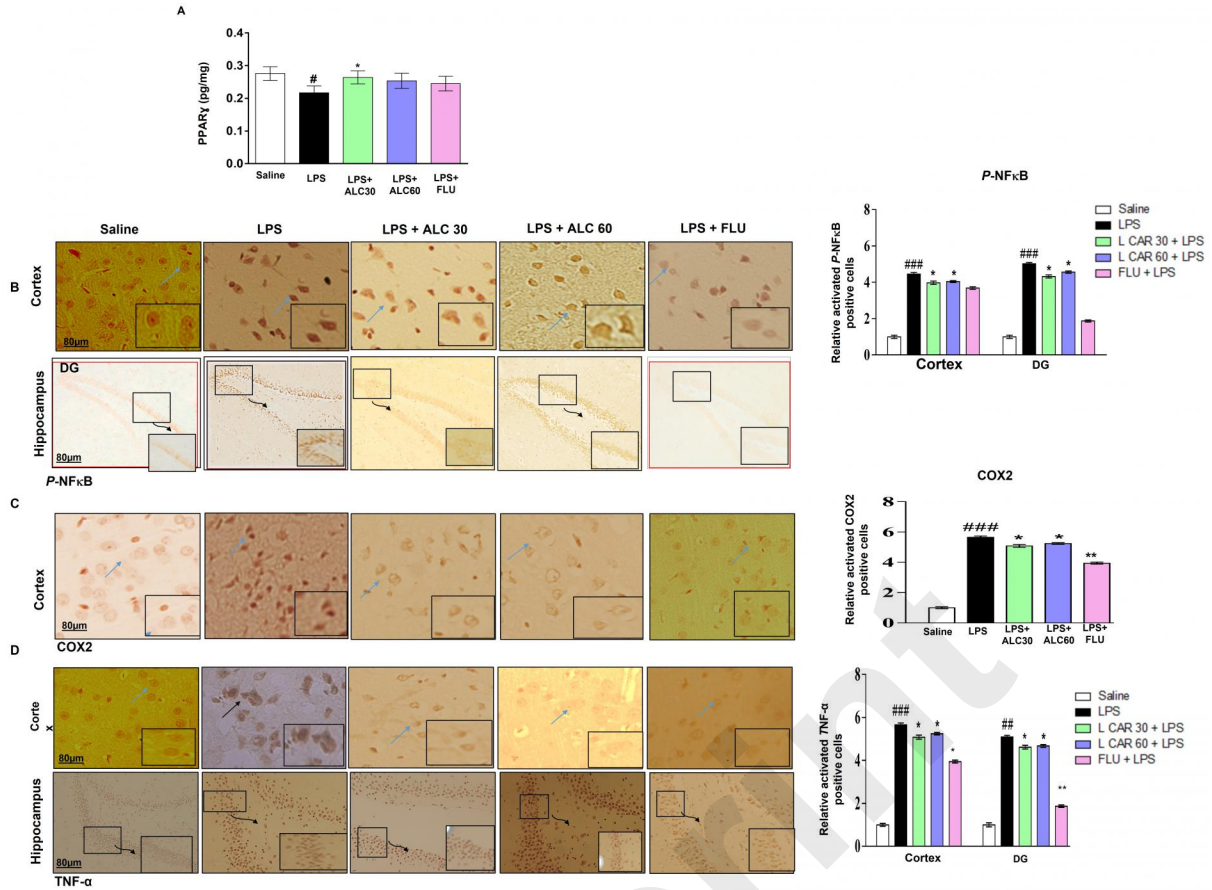
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Figure 2



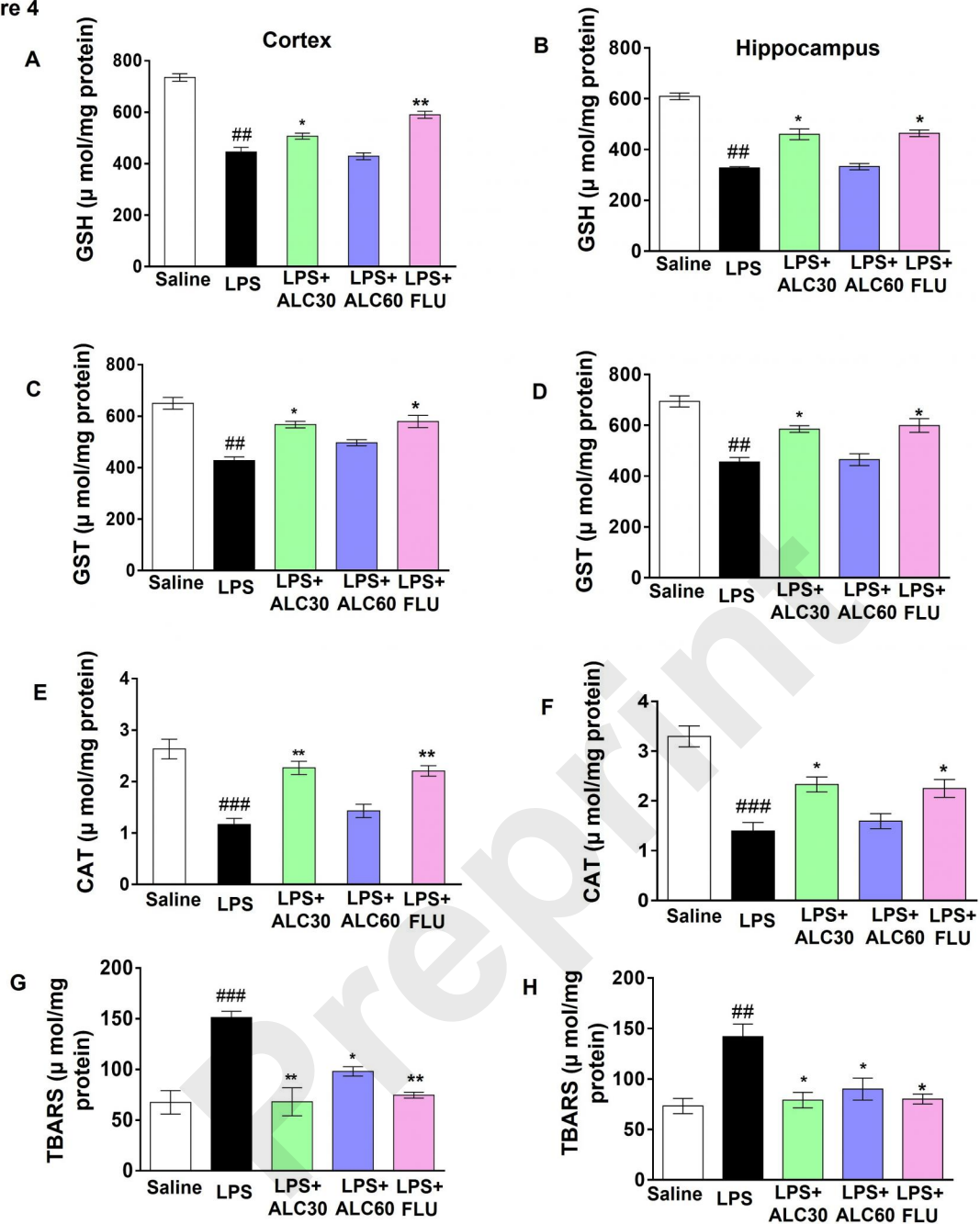
ALC attenuated LPS induced depressive-like anxiety behaviors. Effect of ALC and LPS on EPM test (A, B), FST (C, D), LDB test (E, F), and SST (G). Data are expressed as means \pm SEM and analyzed by one-way ANOVA followed by post hoc Bonferroni test using GraphPad Prism 6 software. Saline, LPS, LPS+ALC 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

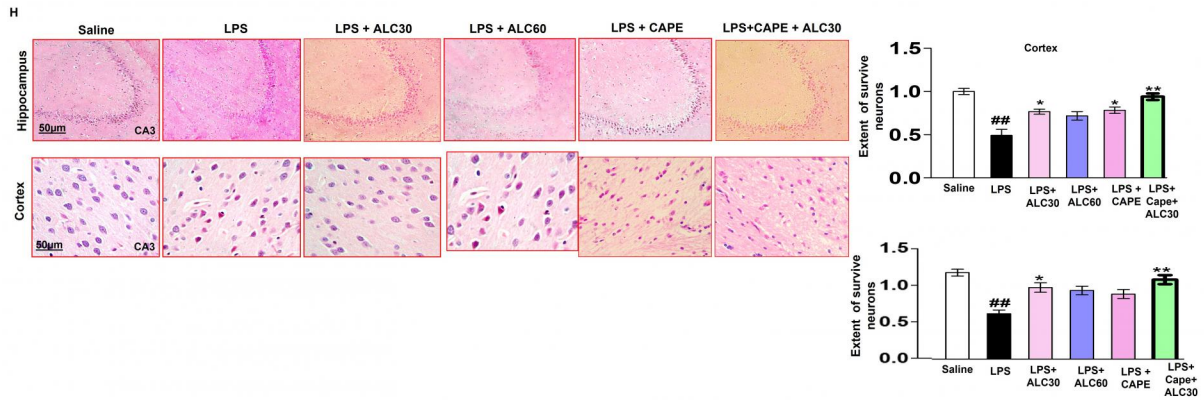


Effect of ALC on mitochondrial biogenesis implicated neuroinflammation. (A) PPAR γ levels were measured by ELISA, Data are expressed as means \pm SEM. $\#p < 0.05$ compared to the saline group while $*p < 0.05$ and compared to the LPS group. ALC 30: Acetyl-L- carnitine (30 mg/kg); ALC 60: Acetyl-L- carnitine (60 mg/kg); LPS: lipopolysaccharide; FLU: fluoxetine; PPAR γ : peroxisome proliferator-activated receptor. (B) Immunohistochemistry results for NF- κ B 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 μ m, magnification 40 \times . Data are expressed as means \pm 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

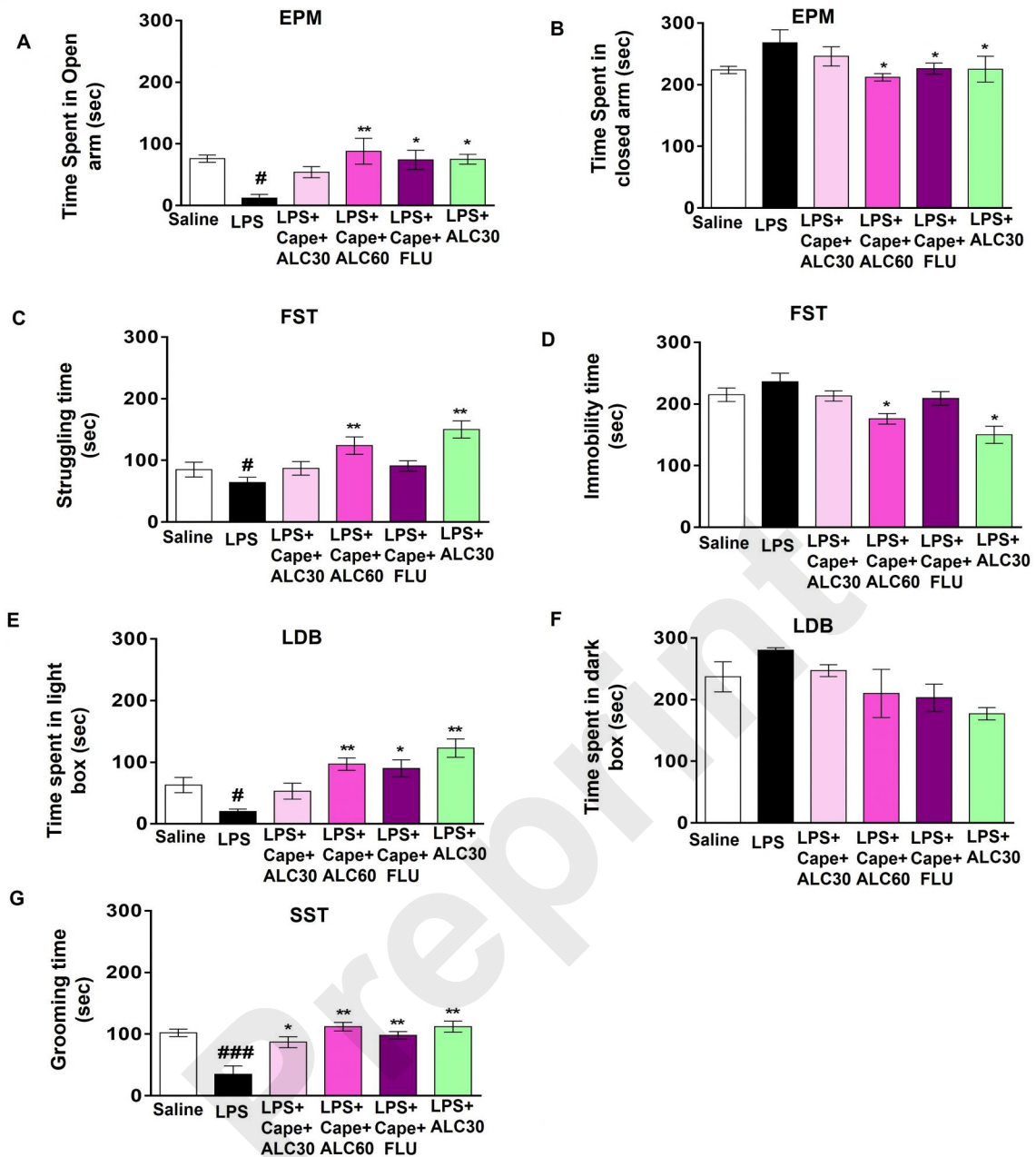


Effect of ALC on oxidative stress-related antioxidant enzymes. Effects of LPS and ALC on levels of GSH (A, B); GST (C, D); CAT (E, F), and TBARS (G, H). Data are expressed as means \pm SEM. Where ## p <0.01, ### p <0.001 compared to the saline group while * p <0.05, ** p <0.01 compared to LPS group. 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 μ m, magnification 40 \times . 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

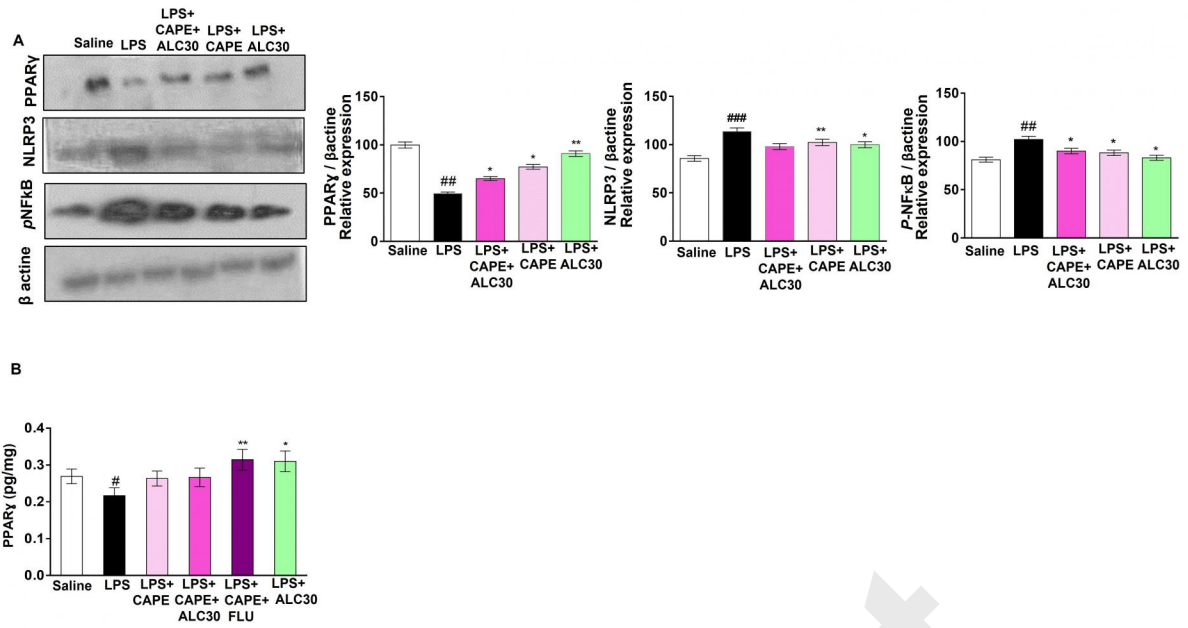


Effect of co-administration of CAPE and ALC on neurodegeneration and behavioral outcomes. Effect of CAPE+ALC and CAPE+LPS on EPM (A, B), FST (C, D), LDB test (E, F), and SST (G). Data are expressed as means \pm SEM and analyzed by one-way ANOVA followed by a post hoc Bonferroni test using GraphPad Prism 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 μ m, magnification 40 \times . 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

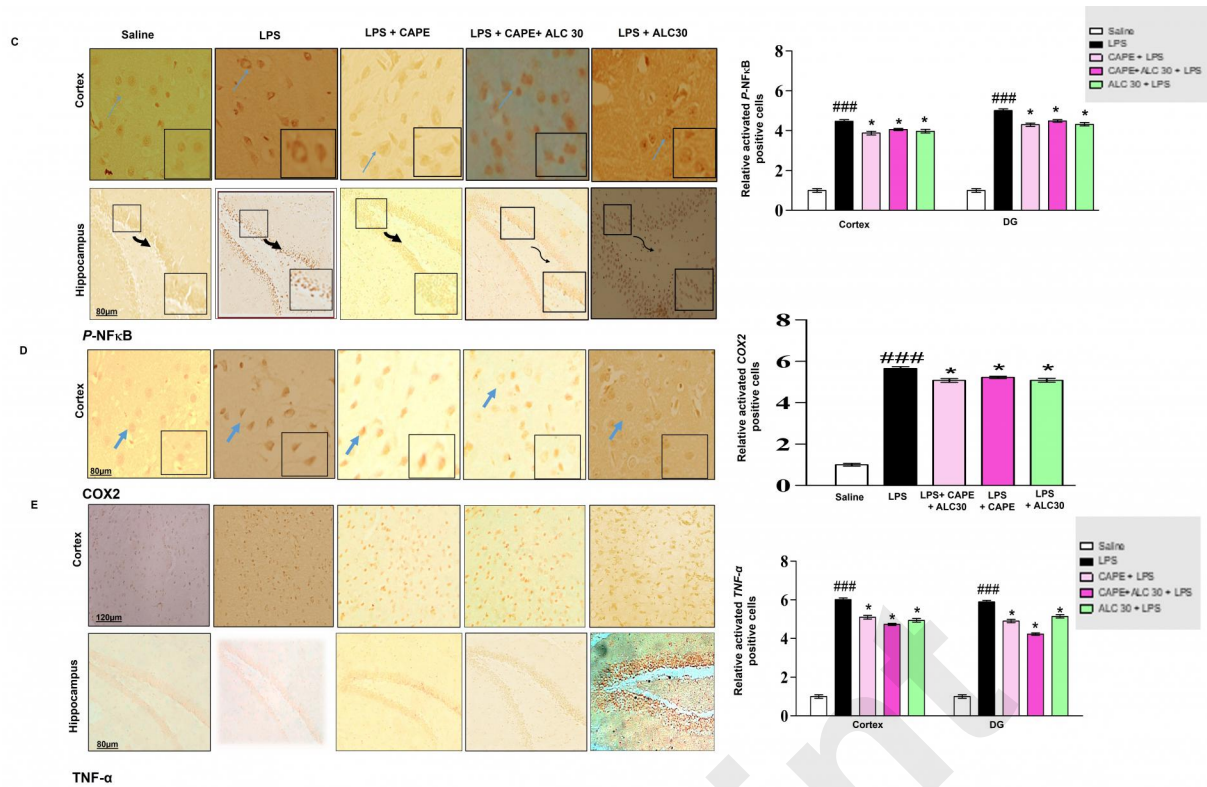
(n=5/group).

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Figure 6

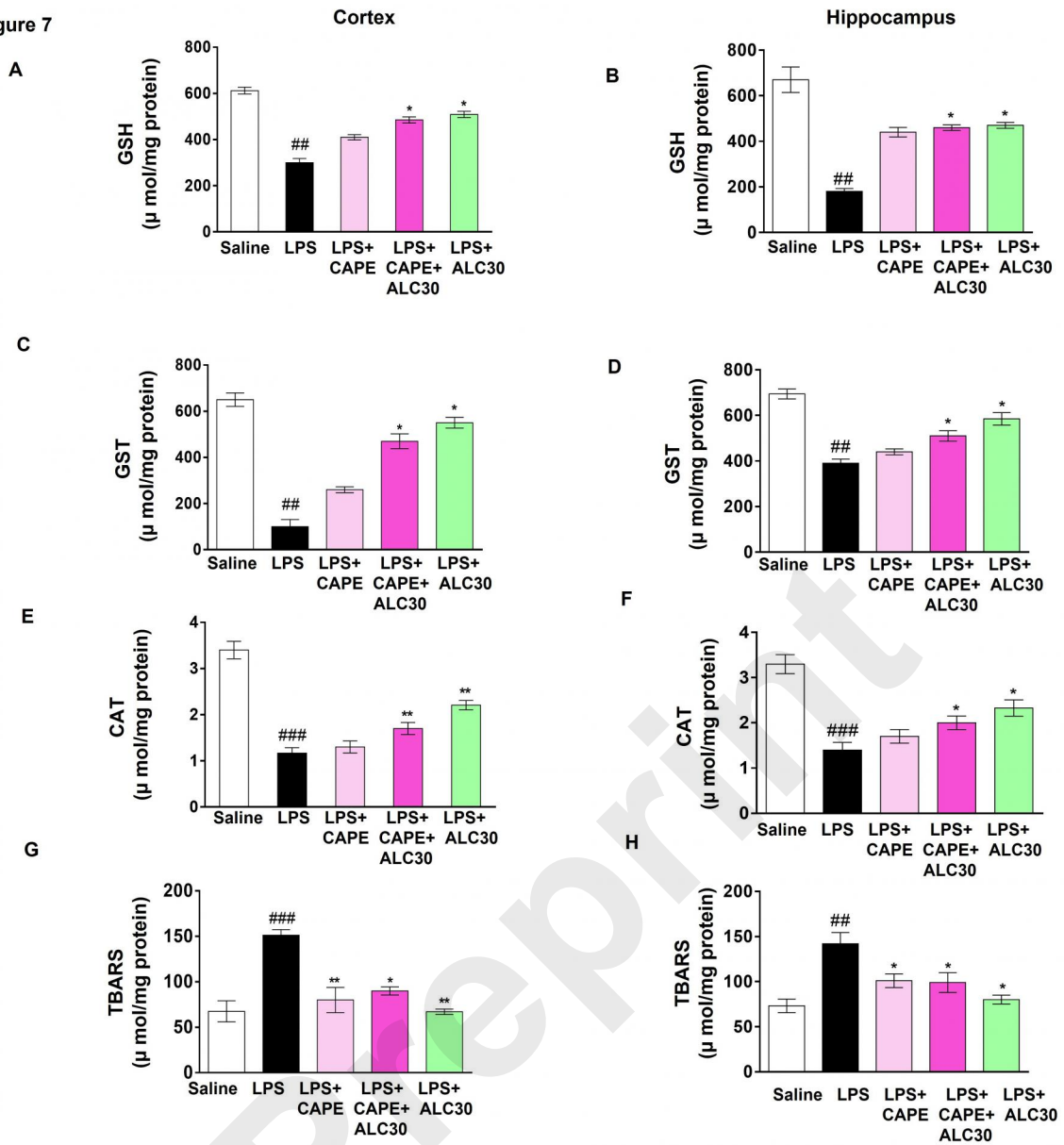


Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers (A) Representative bar graphs and western blots indicating the protein levels in the cortex (n = 5). Image J software was used for quantifying the western blots and graphs were generated using GraphPad Prism. Data are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



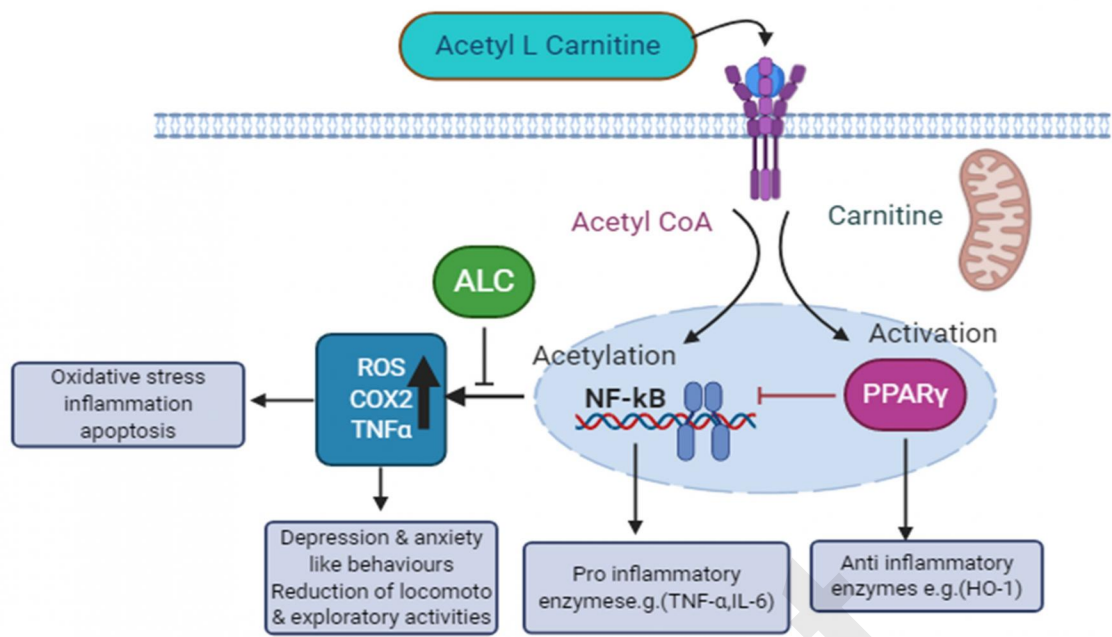
Effect of co-administration on mitochondrial biogenesis and neuroinflammatory markers (C) Immunohistochemistry results for NF-κB in the cortex and hippocampus tissues of the brain. (D) Immunohistochemistry of COX2 in the cortex and (E) Immunohistochemistry of TNF-α in the cortex and hippocampus tissues. 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 \pm 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