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# Unleashing lactoferrin's antidepressant potential through the PI3K/Akt/mTOR pathway in chronic restraint stress rats†

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Depression is a widespread neuropsychiatric illness whose etiology is yet mysterious. Lactoferrin (LF), an iron-binding glycoprotein, is reported to promote neuroprotection through its role in the modulation of oxidative stress and inflammation. The objective of the present research was to evaluate the efficacy of LF against chronic restraint stress (CRS)-induced depressive behavior in rats. Depression was evidenced by a reduced grooming time in the splash test and an increased immobility time in the tail suspension test (TST) and forced swimming test (FST). This effect was also accompanied by reduced GSH and serotonin levels and elevated lipid peroxidation and corticosterone levels in the hippocampus. Additionally, an exaggerated hippocampal inflammatory response was also shown by a rise in NF-κB (p65) and TNF-α levels and a reduced IL-10 level. Moreover, CRS substantially reduced the BDNF content as well as the protein levels of PI3K, Akt, and mTOR while boosting the GSK3β content. Interestingly, LF therapy significantly improved CRS-induced behavioral and biochemical aberrations, an effect which was suppressed upon pretreatment with LY294002 (PI3K inhibitor). This suggests that the antidepressant potential of LF may be mediated through the modulation of the PI3K/Akt/mTOR signaling pathway. Furthermore, LF succeeded in restoring 5-HT and corticosterone levels, diminishing oxidative stress and ameliorating the inflammatory cascades. Therefore, and for the first time, LF might serve as a promising antidepressant drug through targeting the PI3K/Akt/mTOR pathway.

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## 1. Introduction

Depression is a mental health disorder characterized by persistent low mood, sadness, and lack of interest or pleasure in previously enjoyable activities. Other associated symptoms may include difficulty sleeping, changes in appetite, difficulty concentrating, fatigue, and feelings of worthlessness or guilt.[1] According to the World Health Organization, depression affects 264 million people of all ages worldwide. It is expected to become the second leading cause of disability globally, posing a considerable social and economic burden.[2]

Depression is thought to result from genetic and environmental influences.[3] Neuronal pathways may be impaired in the case of major depression.[4] One of the key neuronal pathways involved in depression is the phosphatidylinositol-3 kinase (PI3K) pathway, which regulates cell survival, differentiation, and metabolism. This pathway also mediates the effects of antidepressant drugs and neurotrophic factors on neuroplasticity and neuroinflammation.[5,6]

Chronic restraint stress (CRS) is a widely used animal model which involves restraining rats in a confined space for several hours per day for a prolonged time to induce depressive-like behaviors and neurobiological changes in rodents.[7] CRS has been shown to impair neuronal survival, plasticity, and function in various brain regions, including the hippocampus, prefrontal cortex, and amygdala.[8,9] Past studies have revealed that CRS can impair cognitive functions dependent on the hippocampus, particularly learning and memory.[10] Moreover, CRS has been reported to modulate the PI3K pathway in different ways, depending on the duration and intensity of stress exposure and the brain region and cell type involved.[6]

Traditional antidepressant medications that modulate the levels of neurotransmitters in the brain such as tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs), and monoamine oxidase inhibitors (MAOIs) have

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shown efficacy in a percentage of patients suffering from depression. However, these drugs have several limitations ranging from a delayed onset of action and low remission rates to intolerable side effects. Among the primary side effects of pharmacological therapy is lethargy, sexual dysfunction, constipation, and weight gain.[11–14] More than half of the patients with severe depression do not achieve adequate relief from their symptoms after receiving the first antidepressant medication.[15] Therefore, there is a need for more personalized and precise approaches to treat depression which paved the way for searching for novel therapeutic agents which could offer beneficial alternatives for resistant cases.

One of the natural medicines that has been proposed as a potential treatment for depression is lactoferrin (LF), which is an iron-binding glycoprotein found in human milk and other secretions.[16] LF has various biological functions such as antibacterial, antifungal, antiviral, antioxidant, and anti-inflammatory activities.[16] LF has been suggested as a possible treatment for coronavirus disease 2019 (COVID-19).[17,18] LF has also been detected in the human brain, which may play a role in modulating neuronal activity and protecting against oxidative stress and neuroinflammation. These factors are implicated in the pathophysiology of depression where impaired neuroplasticity and neurogenesis, as well as increased oxidative stress and neuro-inflammation occur in various brain regions.[19] In addition, LF has been documented to enhance PI3K/Akt signaling in human dermal fibroblasts.[20]

Therefore, understanding the molecular mechanisms underlying the PI3K pathway in depression may help in designing new therapeutic strategies with improved efficacy and safety. Thus, for the first time, this study aimed to investigate the effect of LF against CRS-induced depressive-like symptoms in rats and whether the PI3K/Akt/GSK-3β pathway is implicated in these symptoms.

## 2. Materials and methods

### 2.1. Animals

Male Wistar rats weighing 180–200 g at 7–8 weeks of age were purchased from the Modern Veterinary Office for Laboratory Animals, Giza, Egypt. The animals were housed in plastic cages and left to acclimatize for one week at the animal facility of the Faculty of Pharmacy, Cairo University (Egypt). Rats were kept under a constant temperature (23 ± 2 °C), a 12-hour light/dark cycle, and constant relative humidity throughout the experimental period. All animals were allowed free access to a standard diet and water *ad libitum* during the investigation period. The experiment complied with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 2011) and the ARRIVE guidelines. It was approved by the Ethics Committee for Animal Experimentation at the Faculty of Pharmacy, Cairo University, Serial No: PT (3046). All efforts were made to minimize animal suffering and limit the number of animals used.

### 2.2. Drugs and chemicals

Lactoferrin (LF) from bovine milk and LY294002 (a PI3K inhibitor) were purchased from Sigma (St. Louis, MO, USA). LF was dissolved in distilled water and given orally using a round-tipped stainless steel bent gavage-feeding needle (16-gauge; tip diameter, 3 mm; length, 75 mm), and LY294002 (Specific PI3K inhibitor) was dissolved in 1% dimethylsulfoxide (DMSO) which was administered intraperitoneally (i.p.) using a 23-gauge sized needle before LF administration.

### 2.3. CRS induction

Each rat was placed in plastic tubes (height: 5 cm, width: 6 cm, length: 22 cm) without access to food or water for 3 hours every day (10 a.m. to 1 p.m.) for 5 weeks.[21] At the end of the 3 hours, the rats were returned to their cages for immediate water and food consumption.

### 2.4. Experimental design

Fifty rats were randomly assigned to five groups ($n$ = 10 per group): Group I (control group: CTRL) received normal saline (0.5 ml) orally for 5 weeks; Group II (LF control group: LF CTRL) received LF (300 mg/kg per day)[22] orally for 5 weeks;[23] Group III (CRS group) was exposed to restraint stress for 3 hours per day for 5 weeks; Group IV (CRS + LF group) was exposed to restraint stress for 5 weeks and on day 15, the rats were treated with LF (300 mg/kg per day) orally for 21 days; and Group V (CRS + LF + LY294002 group) was exposed to restraint stress and on the 15$^{th}$ day, the rats were treated with LY294002 (1.2 mg/kg per day) intraperitoneally[24] 30 min before administration of LF (300 mg/kg per day) orally for 21 days. After the experimental period, the rats underwent three behavioral tests before being euthanized (Fig. 1).

### 2.5. Behavioral tests

**2.5.1. Splash test (ST).** This test evaluates anhedonia by measuring the grooming time.[25] The rats in their cages were sprayed with 10% sucrose solution on their dorsal coats. The graph was drawn after the results were computed based on the time spent on the animal grooming.

**2.5.2. Tail suspension test (TST).** The TST was used to assess the impact of LF on despair-like behavior.[26] Movements were recorded while the animals were suspended upside down by their tails on a horizontal bar using adhesive tape. Then the time spent immobile was measured for 5 min.

**2.5.3. Forced swimming test (FST).** In rats, the FST was used to identify "behavioral despair".[27] A vertical Plexiglas cylinder (diameter 22.5 cm, height 50 cm) was used to hold 35 cm of fresh water (approximately 25 °C). On the day before the FST, all rats were individually forced to swim for 15 minutes, then dried with towels and returned to their home cage. The rats were re-subjected to the FST for 5 minutes after 24 hours. The immobility time was recorded (tiny movements are required to maintain the head above water).

 

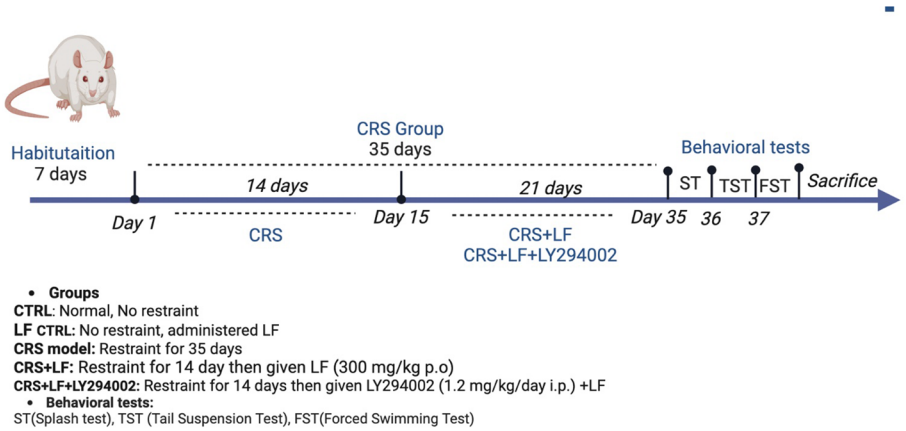


**Fig. 1** Experimental design procedure.

### 2.6. Biochemical measurements

**2.6.1. Brain sample preparation.** After the last behavioral test, the rats were kept to dry and rest for one hour, and then blood samples were collected from the retro-orbital sinus of the rats, and the brains were rapidly removed after the rats were euthanized under anesthesia with thiopental sodium (50 mg $kg^{-1}$, i.p.).[28] Three brains were fixed in 10% formalin for histological and immunohistochemical assessments. On ice, the rest of the brains were used to isolate the hippocampi and frozen at −80 °C for further biochemical analysis. The protein content was measured according to the method of Lowry *et al.*, (1951).[29]

**2.6.2. Measurement of corticosterone and serotonin (5-HT) levels.** The levels of corticosterone and serotonin (5-HT) in the hippocampus were determined using rat enzyme-linked immunosorbent assay (ELISA) kits (MyBioSource, San Diego, CA, USA; Cat. # MBS727040 and MBS9362408, respectively). Procedures were performed according to the manufacturer's instructions.

**2.6.3. Measurement of oxidative stress biomarkers.** The contents of thiobarbituric acid reactive substance (TBARS), as a measure of lipid peroxidation, and reduced glutathione (GSH) in the hippocampus were assessed using rat-specific ELISA kits purchased from AFG Bioscience LLC., USA (Cat. # EK720510), and Shang Hai Blue Gene Biotech CO., LTD (Cat. # E02G0367), respectively. The procedures were carried out following the manufacturer's instructions.

**2.6.4. Measurement of interleukin-10 (IL-10) and brain-derived neurotrophic factor (BDNF).** The anti-inflammatory IL-10 and the neurogenesis factor BDNF were measured in the hippocampus with rat-specific ELISA kits (MyBioSource, San Diego, CA, USA; Cat. # MBS2707969 and MBS355345, respectively). The steps were performed in line with the manufacturer's instructions.

**2.6.5. Measurement of the hippocampal content of PI3K/Akt/mTOR/GSK-3β.** The level of hippocampal PI3K, Akt, mTOR, and GSK-3β were measured with rat-specific ELISA kits (CUSABIO., USA, Cat. # CSB E08418r; Lifespan Biosciences, Washington, USA, Cat. # LS-F49321; Lifespan Biosciences, Washington, USA, Cat. # LS-F17553; MyBioSource, San Diego, CA, USA; Cat. # MBS909078, respectively).

### 2.7. Histopathological investigation

Three brain samples were collected from all groups and then fixed in 10% neutral buffered formalin. Paraffin sections of 5μm thickness were prepared and stained with hematoxylin and eosin (H&E)[30] for histopathological examination using a light microscope (Olympus BX50, Tokyo, Japan). An experienced pathologist blinded to treatments performed the histopathological investigation. Neuropathologic damage in the hippocampus was graded from (0–4) through the determination of the percentage of the lesions in five randomly non-overlapped examined microscopic fields per animal as follows: (0) indicated no changes; (1) indicated the percentage area affected (<10%); (2) indicated the percentage area affected (20–30%); (3) indicated the percentage area affected (40–60%) and (4) indicated the percentage area affected (>60%).[31]

### 2.8. Immunohistology assessment of nuclear factor-kappa B (NF-κB (p65)) and tumor necrosis factor-alpha (TNF-α)

NF-κB (p65) and TNF-α expression levels in the hippocampus were examined. Sections were incubated with monoclonal anti-NF-κB (p65) (sc-8008) and anti-TNF-α (sc-28318) at 1 : 200 dilutions (Santa Cruz Biotechnology Inc., Dallas, TX, USA). The immune reaction was visualized using diaminobenzidine tetrachloride (DAB, Sigma Chemical Co., St. Louis, MO, USA). Positive staining was represented as the area percentage of expression measured by Cell Sens dimensions (Olympus software) by calculating the area % of positive cells in 5 randomly chosen fields in each section.[32] The positive immune reactive cells showed brown-stained cytoplasm and/or nuclei. Staining intensity and distribution were graded as negative (no staining), weak, moderate, or strong. Quantification of NF-κB (p65) and TNF-α was estimated by measuring the % area expression using the image analysis software (Image J, version 1.46a, NIH, Bethesda, MD, USA).

### 2.9. Statistical analysis

Data were expressed as means ± S.D. Statistical comparisons were made using a one-way analysis of variance (ANOVA) followed by the Tukey multiple comparisons test except for total histological scoring analysis using Kruskal–Wallis ANOVA followed by Dunn's *post hoc* test. Statistical analysis was performed using GraphPad Prism 9 (GraphPad Software, San Diego, CA, USA). The signs of significance were denoted as ns: non-significant, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ and $****p < 0.0001$.

## 3. Results

The LF CTRL group revealed no significant difference from the control (CTRL) group in all measured parameters.

### 3.1. Effect of LF on the grooming time in ST as well as immobility time in TST and FST in CRS rats

As illustrated in Fig. 2, behavioral tests were introduced to assess the antidepressant-like potential of LF. The CRS group showed a significant decrease by about 74% in the grooming time when splashed with 10% sucrose solution as compared with the CTRL group. LF treatment showed a significant elevation in the grooming time by 144% compared to the CRS group. In contrast, LY294002 abolished the changes in the grooming behavior that ensued in the treatment group.

CRS rats showed despair behavior as manifested by a marked increase in immobility time of the TST and FST by about 45% and 69%, respectively when compared to controls. The CRS + LF group retaliated the depressive behavior and exhibited a reduction in immobility time by 21% and 14.6% in TST and FST, respectively in contrast to the CRS group (Fig. 2b and c). LY294002 obliterated the positive impact of the LF in modulating the behavioral response in rats exposed to chronic restraint stress.

### 3.2. Effect of LF on 5-HT and corticosterone levels in CRS rats

After 5 weeks of CRS exposure, the 5-HT level was significantly decreased by 78%, while the corticosterone level was augmented by 255% when compared to the CTRL group. On administering LF (300 mg per kg per day), the 5-HT level was significantly elevated by about 263% whilst a significant decline in corticosterone level by 56% was reported compared to the stressed rats. However, pretreatment with LY294002 counteracted the beneficial effect of the LF administration (Fig. 3a and b).

### 3.3. Effect of LF on GSH and TBARS contents in CRS rats

As reported in Fig. 4, CRS provoked a severe oxidative response in the hippocampi of rats as marked by a 325% upsurge in TBARS and halting GSH content by 71% compared to the CTRL group. In contrast, LF administration to CRS-exposed

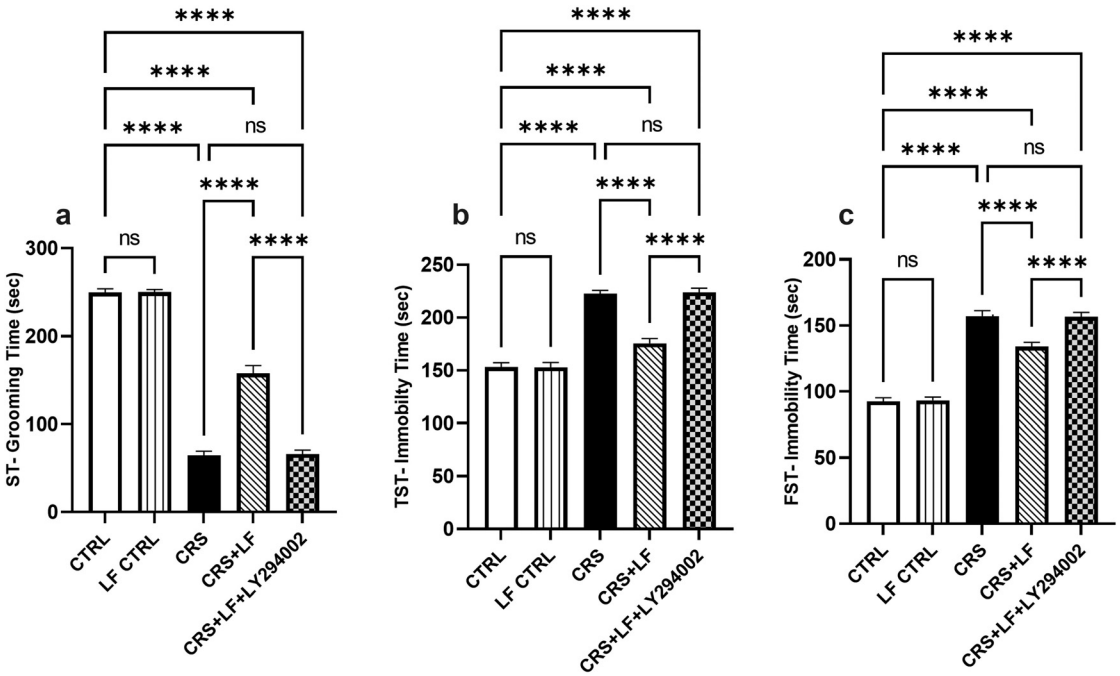


**Fig. 2** Effect of lactoferrin (LF) on the depression-like behavioral deficits triggered by chronic restraint stress (CRS) rats. (a) Grooming time in the splash test (ST), (b) immobility time in the tail suspension test (TST), and (c) immobility time in the forced swimming test (FST). Values are expressed as mean ± SD ($n$ = 10). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant and $****P < 0.0001$.

 

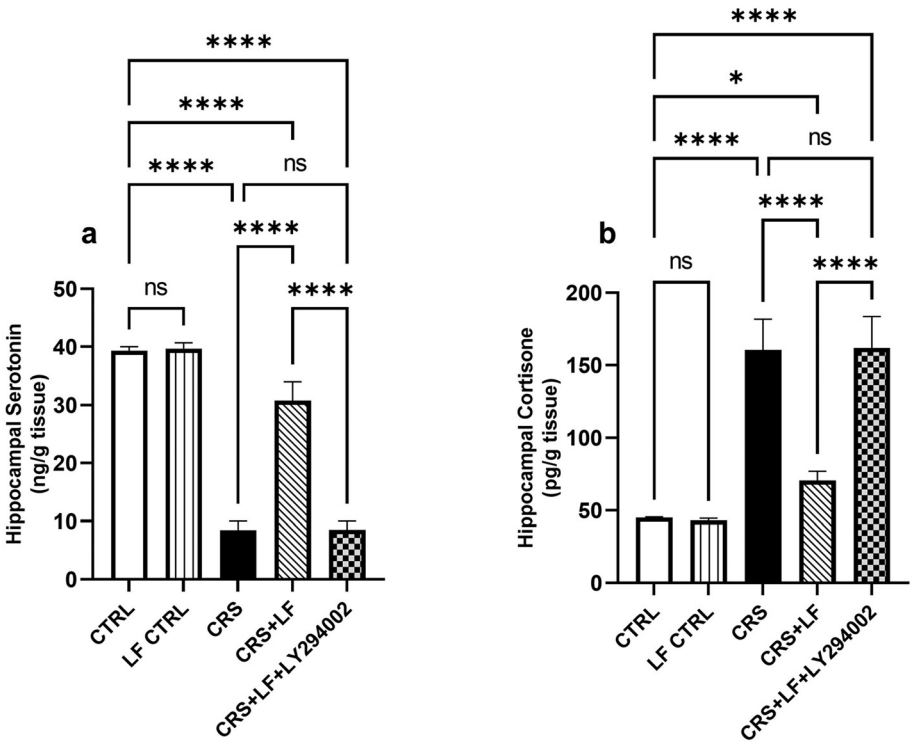


**Fig. 3** Effect of lactoferrin (LF) on hippocampal (a) serotonin (5-HT) and (b) corticosterone levels in chronic restraint stressed (CRS) rats. Values are expressed as mean ± SD ($n$ = 6). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant, *$P$ < 0.05, and ****$P$ < 0.0001.

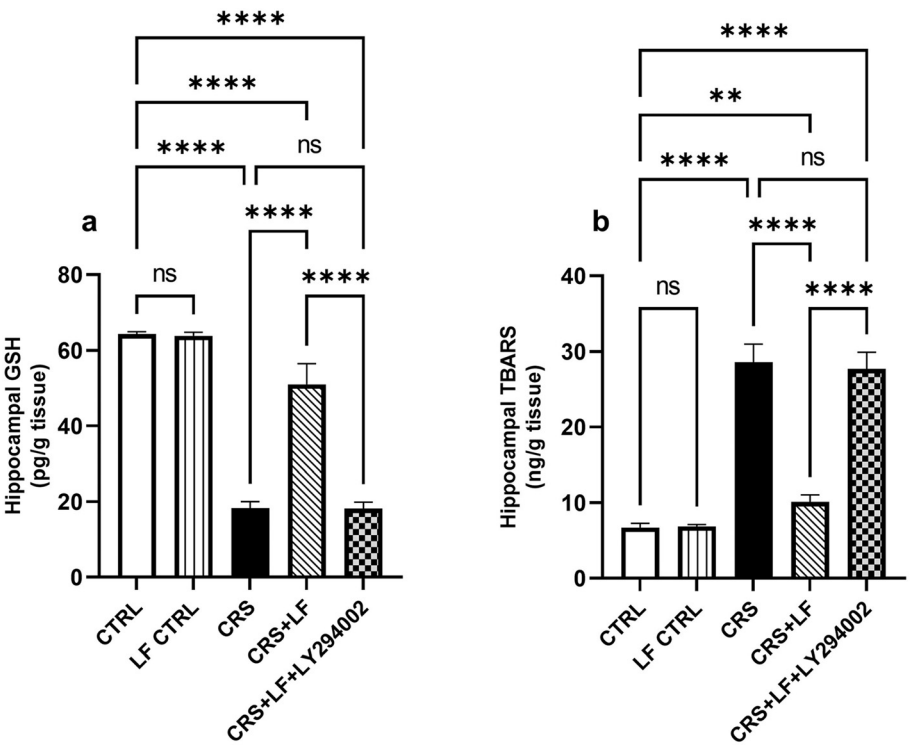


**Fig. 4** Effect of lactoferrin (LF) on hippocampal (a) reduced glutathione (GSH) and (b) thiobarbituric acid reactive substances (TBARS) levels in chronic restraint stressed (CRS) rats. Values are expressed as mean ± SD ($n$ = 6). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant, **$P$ < 0.01, and ****$P$ < 0.0001.

rats showed 282% reduction in the content of TBARS and raised the GSH content by about 179%. However, this improvement in the oxidative milieu was lost after the administration of LY294002 (1.2 mg per kg per day).

### 3.4. Effect of LF on the BDNF content in CRS rats

Rats subjected to restraint stress showed a significant decrease in BDNF content by about 77% compared to the CTRL group. Treatment with LF (300 mg per kg per day) boosted the content of BDNF by 195% compared with the CRS group. However, LY294002 significantly erased the protective effect of LF (Fig. 5b).

### 3.5. Effect of LF on PI3K, Akt, mTOR, and GSK-3β levels in CRS rats

As depicted in Fig. 6(a–d), CRS inhibited the signaling of the PI3K/Akt/mTOR axis. This was demonstrated by a significant decrease in the PI3K, Akt, and mTOR levels to nearly 29%, 64%, and 15%, respectively, *versus* controls. Additionally, chronic stress tripled the level of GSK-3β to that of the CTRL group. On the other hand, LF administration significantly upregulated the levels of PI3K, Akt, and mTOR by about 152%, 41%, and 220%, respectively, compared with the CRS group. Moreover, LF significantly abridged GSK-3β levels by 64% compared to the diseased animals. The favorable effect of LF was cancelled by the administration of LY294002.

### 3.6. Effect of LF on histopathological aberrations observed in CRS rats

Fig. 7 shows the histopathological examination of the hippocampus of the different groups of rats. The CTRL group and the LF CTRL showed a normal hippocampal histology with well-preserved pyramidal neurons with large vesicular nuclei in the Cornu Ammonis (CA1 and CA3) and dentate gyrus (DG) regions (Fig. 7a–f). In contrast, the CRS group had severe hippocampal damage, characterized by shrinkage, pyknosis, and necrosis of pyramidal neurons, as well as vacuolation of molecular cells and neuronophagia which was also evidenced in histological lesion score (Fig. 7g–i and p). Otherwise, marked regression of the neuropathic changes was recorded in the examined sections of the CRS + LF group with mild hippocampal damage and less pyknosis and necrosis of pyramidal neurons as well as amelioration in the total damage score (Fig. 7j–l and p). However, the hippocampal section of the LY294002 group showed moderate hippocampal damage with pyknosis, neuronophagia and necrosis of pyramidal neurons (Fig. 7m–p).

### 3.7. Effect of LF on inflammatory mediators (NF-κB (p65), TNF-α and IL-10) in CRS rats

Neuroinflammation was evidenced in the CRS group by prominent hippocampal immunohistochemical expression of NF-κB (p65) and TNF-α reaching 48- and 35-times that of the controls (Fig. 8 and 9). Moreover, the anti-inflammatory marker, IL-10,

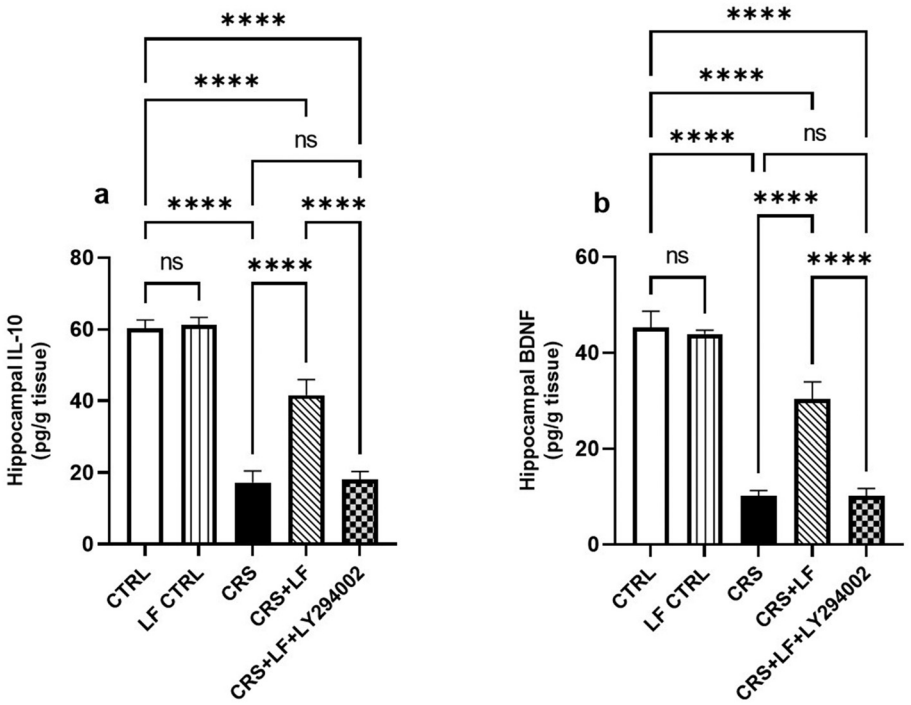


Fig. 5 Effect of lactoferrin (LF) on hippocampal (a) Interleukin-10 (IL-10) and (b) brain-derived neurotrophic factor (BDNF) levels in chronic restraint stressed (CRS) rats. Values are expressed as mean ± SD ($n$ = 6). Statistical analysis was performed using one-way ANOVA followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant and ****$P$ < 0.0001.

 

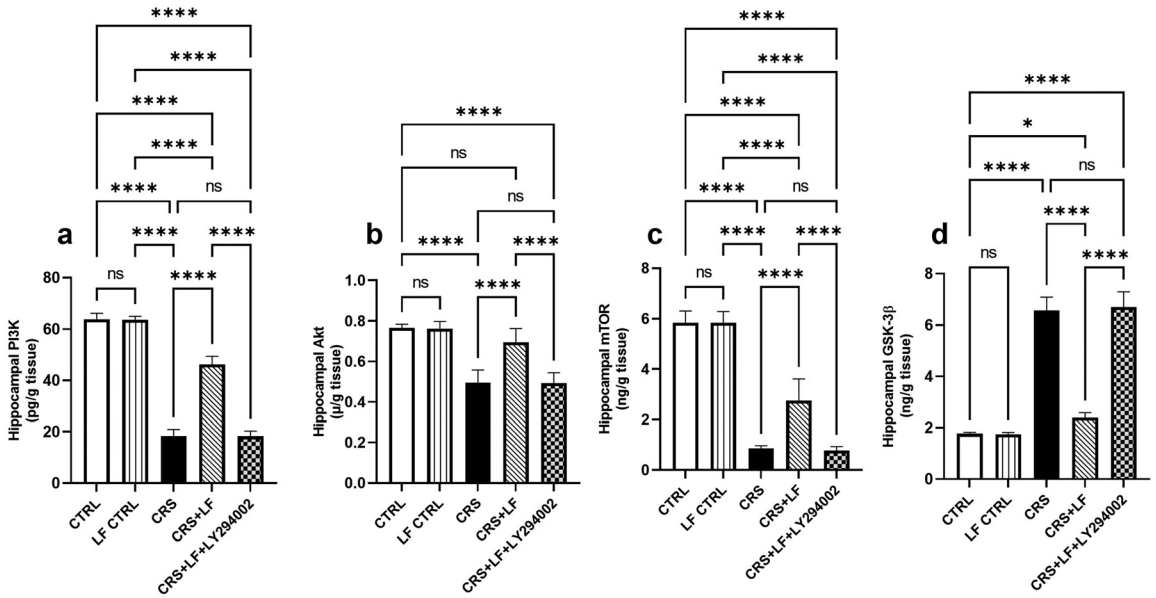


**Fig. 6** Effect of lactoferrin (LF) on hippocampal (a) PI3K, (b) Akt, (c) mTOR, and (d) GSK-3β levels in chronic restraint stressed (CRS) rats. Values are expressed as mean ± SD ($n$ = 6). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant, *$P$ < 0.05, and ****$P$ < 0.0001.

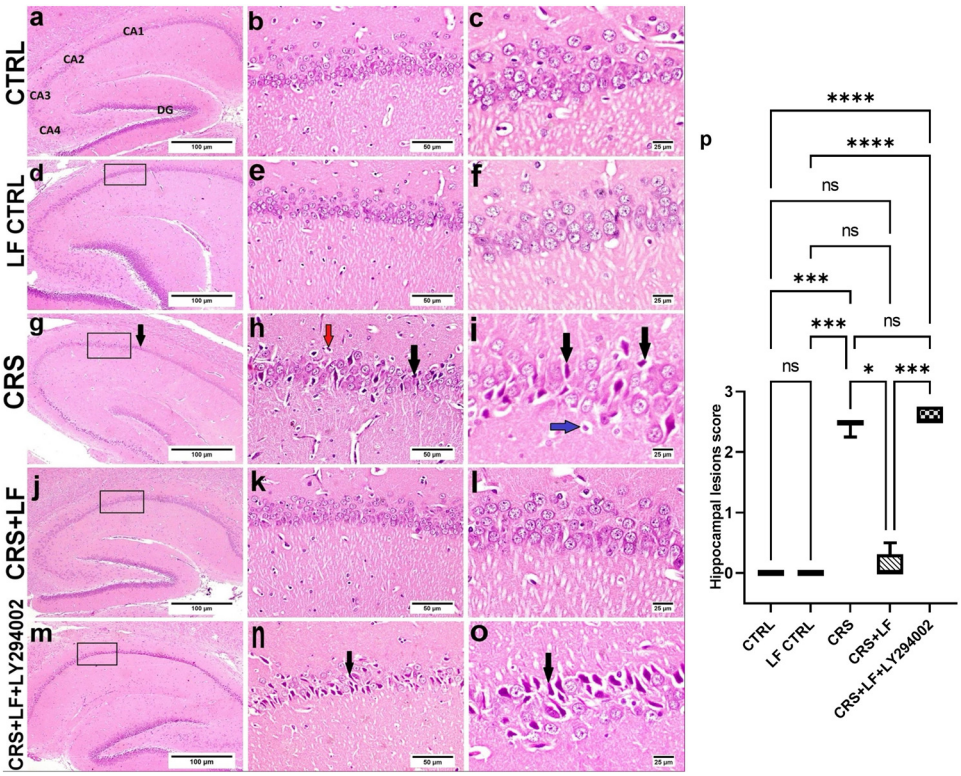


**Fig. 7** Effect of LF on histopathological aberrations observed in the hippocampi of rats stained with H&E. (a, b & c) CTRL rats showing the normal structure and morphology of pyramidal neurons with large vesicular nuclei in the hippocampus. (d, e & f) The LF CTRL group showing no histopathological alterations. (g, h & i) CRS rats display degenerative changes in pyramidal neurons such as shrinkage, pyknosis, necrosis (black arrow), necrosis of pleomorphic cells (red arrow), and vacuolation of molecular cells (blue arrow). (j, k & l) The CRS + LF group revealing preservation of the normal histological structure with no histopathological alterations. (m, n & o) CRS + LF + LY294002 group showing severe degenerative changes in pyramidal neurons such as pyknosis and necrosis (black arrow) (scale bar 100 μm, 50 μm & 25 μm). (p) Total histological scoring of hippocampal lesions analyzed using the Kruskal–Wallis ANOVA method followed by Dunn's *post hoc* test, with a significant value set at ns: non-significant, *$P$ < 0.05, ***$P$ < 0.001, and ****$P$ < 0.0001.

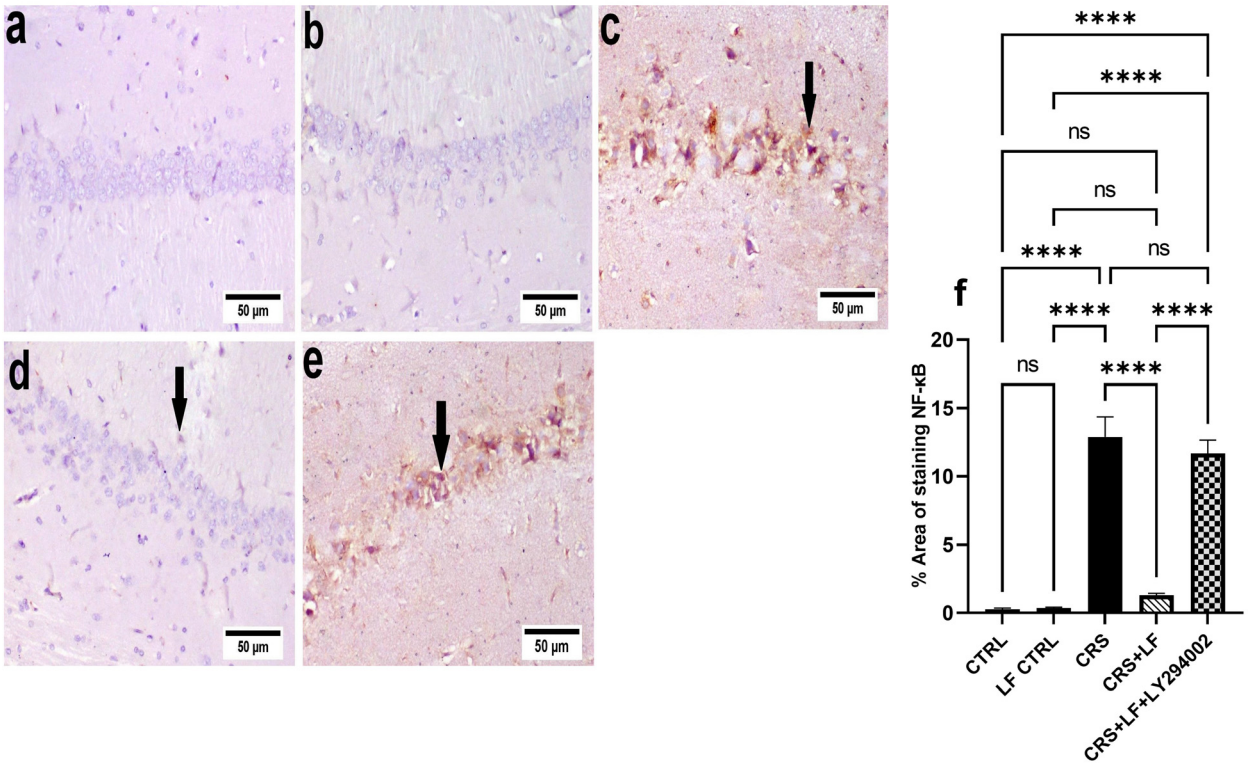


**Fig. 8** Representative photomicrographs of the immunohistochemical staining of NF-κB (p65) in the hippocampi of rats from different groups (scale bar 100μm (ESI Fig. 1†) & 50 μm). (a and b) CTRL and LF CTRL groups showed no expression of NF-κB (p65). (c) The CRS group indicating high expression of NF-κB (p65). (d) The CRS + LF group showing low expression of NF-κB (p65). (e) The CRS + LF + LY294002 group showing moderate expression of NF-κB (p65). (f) Represents the positive area percent of the NF-κB (p65) expression. Values are calculated for each group as mean ± SD ($n$ = 5). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant and ****$P$ < 0.0001.

was significantly hampered by 70% in the diseased rats (Fig. 5a). In contrast to CRS rats, LF treatment halted the inflammatory reaction as revealed by the weak hippocampal stain of both NF-κB (p65) and TNF-α by 90% and 95%, respectively (Fig. 8 and 9). This anti-inflammatory effect of LF was further confirmed by a 240% rise in the IL-10 level when compared to the diseased rats (Fig. 5a). Administration of LY294002 abolished the anti-inflammatory and neuroprotective effects of LF and imprinted a strong NF-κB (p65) and TNF-α stain comparable to that of the CRS group (Fig. 8 and 9).

## 4. Discussion

This study aimed to evaluate the efficacy of LF in halting depressive-like behavior in rats exposed to CRS. Moreover, the study provides strong evidence that the possible antidepressant potential of LF may be mediated, in part, to the activation of the PI3K/Akt/mTOR pathway which was confirmed *via* the administration of LY294002, a specific inhibitor of the PI3K pathway, that antagonized and reversed all the beneficial effects of LF.

The first step in conducting depression is to establish a successful depressive-like model. The CRS has been used in the current research as a model of generating depressive-like symptoms because it induces behavioral, neurological, and hormonal changes that are similar in some extent to how humans can experience depression.[33–35]

The current experiment showed that CRS increased depressive-like behavior as evidenced by reduced sucrose grooming time in the splash test and swimming time in the FST as well as increased immobility time in the TST. Consistent with our results, in previous studies, CRS led to decreased sucrose consumption and grooming frequency in splash tests, and extended immobility time in the FST[36] and TST.[37] In contrast, three weeks-administration of LF significantly improved the rats' free movement in the TST, swimming duration, and sucrose intake in the splash test.[38]

Recent studies have shown a strong correlation between abnormal hypothalamus-pituitary-adrenal axis (HPA) activity and depression.[39,40] Stress can harm rodents' prefrontal cortex and hippocampus, stimulate the HPA axis, impair negative feedback, and boost the production of serum corticotropin-releasing hormones like ACTH and corticosterone.[41] Our data revealed that rats subjected to CRS had increased levels of corticosterone in their blood and reduced levels of serotonin, which may occur due to hastened breakdown of monoamines or delayed synthesis because of the loss of monoamine

 

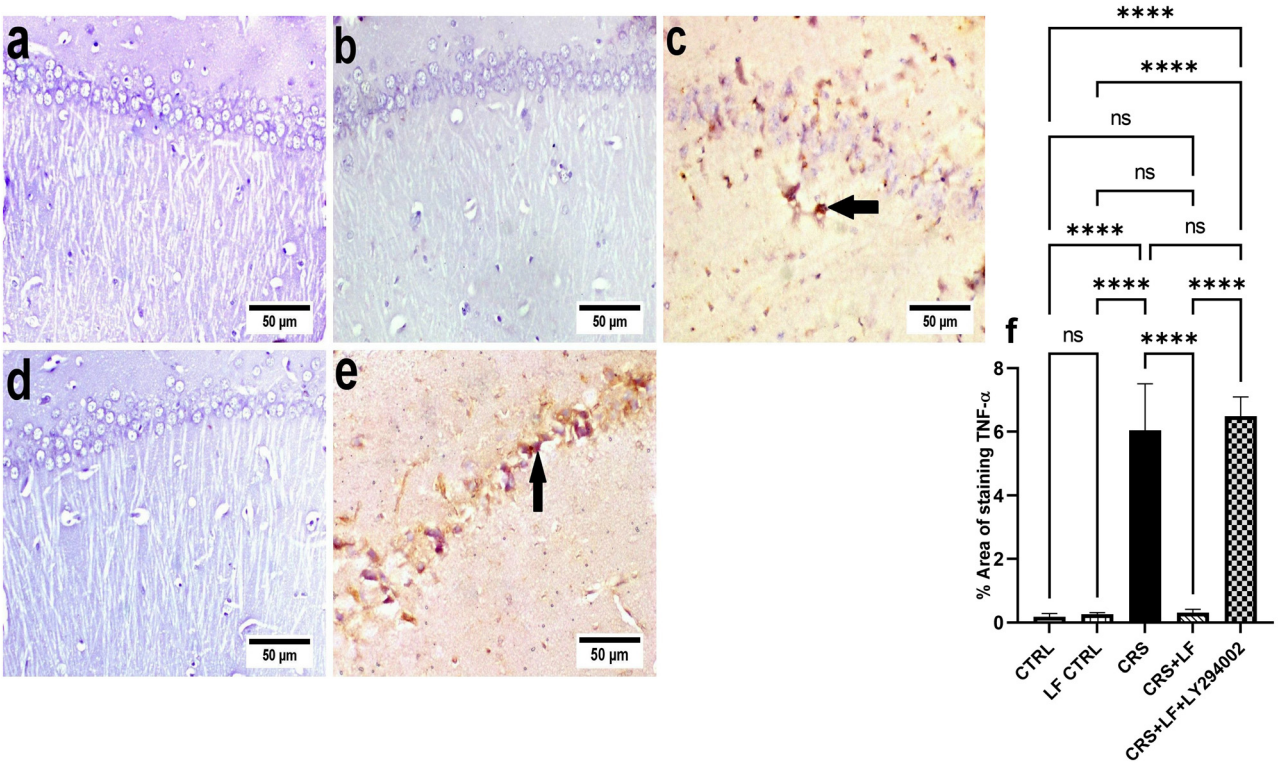


**Fig. 9** Representative photomicrographs of the immunohistochemical staining of TNF-α in the hippocampi of rats from different groups (scale bar 100 μm (ESI Fig. 2†) & 50 μm). (a and b) CTRL and LF CTRL groups showed no expression of TNF-α. (c) The CRS group indicating high expression of TNF-α. (d) The CRS + LF group showing low expression of TNF-α. (e) The CRS + LF + LY294002 group showing moderate expression of TNF-α. (f) Represents the positive area percent of the TNF-α expression. Values are calculated for each group as mean ± SD ($n$ = 5). Statistical analysis was performed using the one-way ANOVA technique followed by Tukey's *post-hoc* test, with a significant value set at ns: non-significant, and ****$P$ < 0.0001.

neurons.[42] There is also evidence that high corticosterone levels cause reduced norepinephrine production. The current research revealed that LF considerably reversed and restored corticosterone and 5-HT to normal levels, which suggests that LF may have a protective impact on depressed behaviors in rats by modulating corticosterone and monoamine secretion. These results are in alignment with those of previous studies.[43–45] The interplay between the HPA axis and the monoamine system in the hippocampus has a substantial role in the pathophysiology of sadness by depleting monoamine neurotransmitters.[46–48] Similarly, it has been noted that reduced norepinephrine production is caused by elevated corticosterone levels.[49,50]

Previous studies have shown that CRS triggers an increased oxidative stress response in various rat brain regions.[51–53] In accordance with this approach, the current data showed that rats exposed to CRS developed higher levels of reactive oxygen species (ROS) (TBARS) and lower levels of antioxidants in the brain (GSH).[34] The increased oxidative stress is likely related to the elevated glucocorticoid levels and increased glucose metabolism and oxygen consumption in response to stress, as well as the decreased antioxidant defense mechanisms in the brain that cause different generation of ROS.[54] Moreover, neuroinflammation, a significant factor in the development of neural injury, may be induced by oxidative stress in the central nervous system.[55]

Our results in the histopathological examination showed variable neuropathological alterations. According to the "inflammatory theory of depression", stress-related stimuli can initiate inflammatory processes, which can then cause abnormalities in the serotonin and HPA physiological axis and ultimately, depressive-like behavior.[56] In the same setting, the current findings showed that rats' hippocampi had an enhanced pro-inflammatory response, as indicated by the enhanced immunoexpression of NF-κB and TNF-α, thus causing neuroinflammation which results in depression.[57] The activation of the NF-κB signaling pathway contributes to the development of depressive-like symptoms.[58] High ROS concentrations have been shown to function as upstream messengers that activate the NF-κB inflammatory signaling pathways.[59] It has been reported that LF improved depression-like symptoms in rats *via* reducing the serum levels of IL-6 and elevating IL-10 levels.[60] Overall, these results suggest that LF may be a promising candidate for reducing inflammation in the hippocampus induced by chronic restraint stress.[61]

The observed findings support earlier research that showed psychological stresses, including CRS, increase the production of pro-inflammatory cytokines in the brain's hippocampus.

Increased free radical generation and the loss of neurotransmitters like dopamine and norepinephrine are linked to improved inflammatory indicators in the central nervous system.[62,63] Several studies indicate that neuroinflammation plays a vital role in the pathogenesis of depressive disorders.[64] In light of this, studies suggested that the sharp increase in pro-oxidants and pro-inflammatory cytokines in the hippocampus of the brain may trigger a negative loop perpetuating the disruption of the HPA axis, neuronal cell death, and depressive-like behavioral outcomes.[59] However, in the current study, when rats were given LF, their pro-inflammatory cytokines and oxidative stress indicators were reduced, thereby breaking the loop and improving behavioral outcomes.[65] Other studies have also found that LF improves the immune system performance and ROS modulating capacity, making it beneficial for CNS illnesses. It is worth noting that LF considerably decreased hippocampal TBARS levels and increased reduced GSH, which is consistent with its ROS-lowering abilities and effects on mitochondrial maintenance in various diseases.[66,67]

Brain-derived neurotrophic factor (BDNF) governs several brain functions and is integral to treating mental disorders like depression. The primary signal transduction receptor for BDNF is tropomyosin-related kinase B (TrkB), a neurotrophin receptor tyrosine kinase expressed in high amounts in the brain. Multiple lines of evidence point to the importance of BDNF-TrkB signaling in the etiology of depression, and antidepressants influence BDNF and TrkB levels.[68,69] The hippocampi of rats subjected to CRS expressed low BDNF protein which upon therapy with antidepressants was normalized according to previous research.[70] In the present study, the BDNF content was decreased in the hippocampus of CRS rats, which may have been caused by oxidative damage and suggested that the growth and nourishment of neurons were hampered which is congruent with previous studies.[71,72] Patients with depression have lower levels of BDNF in their peripheral blood and central nervous system.[73] However, LF can inhibit NF-κB with subsequent anti-apoptotic effects and increased BDNF levels.[74] In line with our results, LF regulates neurogenesis, such as neuronal cell proliferation, differentiation, migration, and synaptic connections.[75,76]

Phosphatidylinositol kinase (PI3K) is an enzyme that plays a vital role in signal transmission mechanisms within cells. The downstream Akt-dependent signaling pathway is critical for PI3K because Akt is a target protein for this pathway. Akt regulates intracellular metabolism and is involved in the regulation of cell development and apoptosis.[77] Interestingly, the signaling system PI3K/Akt is involved in many physiological processes in the brain, including neuronal learning, cell survival and growth, and apoptosis suppression.[77] BDNF/TrkB-induced PI3K activity triggers Akt activation, which phosphorylates mTOR at serine 2448, thereby enhancing the activity of downstream molecules.[78] According to Wu *et al.* (2018) work, the prefrontal cortex and hippocampus exhibit constant and quick antidepressant-like effects when PI3K/Akt/mTOR signaling increases. In line with earlier research, CRS brought on depressive signs and a corresponding downregulation of PI3K/Akt/mTOR signaling. The results of this study demonstrate that chronic stress inhibits the CREB signaling of the PI3K/Akt/mTOR axis, as evidenced by a significant decrease in the levels of PI3K, Akt, and mTOR. This inhibition was reversed by the administration of LF (300 mg $kg^{-1}$), which significantly increased the levels of these proteins.

It has been demonstrated that chronic restraint stress enhances GSK-3β activity and elevates the expression of pro-inflammatory cytokines such as TNF-α and IL-6, resulting in the onset of depressive- and anxiety-like disorders, which is consistent with several reports.[79,80] Moreover, LF effectively decreased the level of GSK-3β in stressed rats. Mood stabilizers and antidepressants may augment the PI3K/Akt/mTOR signaling.[81] Furthermore, studies have indicated that inhibition of GSK-3β or activation of the PI3K/Akt/mTOR pathway can attenuate the deleterious effect of chronic restraint stress on behavior and physiology[82,83] which was shown in the current research on the administration of LF, an effect that was abolished by pretreatment with the PI3K inhibitor, LY294002 (1.2 mg per kg per day). However, it should be stated that the effect of LY294002 alone in CRS rats was not investigated, which is a limitation in the present work. This issue will be carefully considered in future research. Consequently, these results suggest that LF may be a potential therapeutic candidate for treating diseases associated with chronic stress-induced dysregulation of the PI3K/Akt/mTOR pathway.

In conclusion, the current study demonstrated that CRS instigated depressive-like behavior, increased oxidative stress and neuroinflammation, and dysregulated rats' HPA axis and monoamine neurotransmitters. Moreover, the PI3K/Akt/mTOR signaling as well as BDNF expression were impeded by CRS. However, LF ameliorated these effects by mitigating ROS and pro-inflammatory cytokines, restoring corticosterone, 5-HT and BDNF levels, and modulating the hippocampal PI3K/Akt/mTOR/GSK-3β pathway. Therefore, these findings suggest that LF may possess considerable antidepressant and neuroprotective properties that could aid in the management of depression and related disorders. Nevertheless, to fully comprehend the precise mechanism of LF, additional studies are required to assess its effectiveness and safety in clinical settings.

## Author contributions

The authors confirm their contribution to the manuscript as follows: M. F. E, A. E. E, and R. M. E were responsible for study conceptualization, supervision and reviewing and editing the manuscript. A. E. E and R. M. E designed the methodology and the mechanistic pathway. H. H. A performed the experiment, collected, analyzed and interpreted the data as well as wrote the original draft of the manuscript. In addition, K. A. A was responsible for performing the histological and immunohistochemical examination, visualizing and interpreting the data. All authors approved the final version of the manuscript.

 

## Conflicts of interest

The authors declare no conflict of interest.

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