Journal of Research in Pharmacy

Research Article

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Effects of metformin in an experimental chronic unpredictable mild stress model

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Received: 3 May 2025/ Revised: 27 May 2025 / Accepted: 30 May 2025

ABSTRACT: Prolonged interaction with challenging life situations is a well-recognised risk factor for depression, anxiety, and cognitive decline, particularly among menopausal women. This research aimed to assess the effects of metformin (MF) on anxiety-like behavioural changes, cognitive impairment, brain neuroplasticity, and apoptosis induced by chronic unpredictable mild stress (CUMS) in menopausal rats. To achieve this, we conducted the open field test (OFT) to measure anxiety-like behaviours and locomotor activity, the elevated plus-maze (EPM) to assess anxiety-like behaviour, and the novel object recognition test (NORT) to evaluate recognition memory. Additionally, we measured the levels of brain-derived neurotrophic factor (BDNF), sirtuin1 (SIRT1) , synaptophysin, and caspase-9 by ELISA in the hippocampus, prefrontal cortex (PFC), and amygdala. Our experimental groups were categorised into five groups, with six rats in each group, as follows: Control, ovariectomised (OVX), OVX+CUMS, OVX+MF, and OVX+CUMS+MF. Four weeks after the OVX, the CUMS protocol was implemented, and MF treatment commenced following CUMS and continued for 14 days. Chronic administration of MF improved recognition memory and anxiety levels in rats subjected to CUMS, as assessed by the NORT, OFT, and EPM. MF increased BDNF, synaptophysin, and SIRT1, key markers of neuroplasticity in specific brain regions. We also found that MF treatment reduced pro-apoptotic caspase-9 levels. Histopathological analysis of the hippocampal dentate gyrus and prefrontal cortex indicated neurodegenerative changes such as pyknotic nuclei, cytoplasmic sparseness, widespread vacuolisation, and perivascular and pericellular oedema in the OVX and OVX+CUMS groups. However, these pathological features were significantly diminished in the MF groups, resulting in better preservation of neuronal integrity, fewer pyknotic cells, and reduced vacuolisation. In conclusion, metformin appears to be a beneficial therapeutic agent that enhances neural plasticity, decreases apoptosis, and improves recognition memory and anxiety-like behaviours induced by OVX+CUMS

KEYWORDS: CUMS; BDNF; SIRT1; synaptophysin; caspase-9; metformin; brain; rat

1. INTRODUCTION

By 2030, the global population of women who paused menstrual cycles is projected to reach approximately 1.2 billion [1]. Post-menopause predisposes women to neuropsychiatric disorders [2]. Because menopause itself often increases stress and anxiety levels [1]. Therefore, menopause makes women more sensitive and vulnerable to stress. Prolonged interaction with challenging life situations is a well-recognized possibility factor for the development of depression, anxiety, and cognitive impairments in humans and animals [3- 6].

Although antidepressants, anxiolytics, neuroprotective agents, and antioxidants currently exhibit certain clinical efficacy for treating anxiety and neurodegeneration, challenges remain regarding long-term use, relapse, and side effects. Therefore, novel molecular targets and therapeutic approaches are necessary for the effective and safe treatment of stress-associated anxiety and neurodegeneration [3, 7].

Chronic unpredictable mild stress (CUMS) is a widely utilised animal model for studying depression and anxiety [8]. CUMS effectively mimics repeated exposure to stressful life events in humans. The cognitive deficits related to CUMS, associated with frontal lobe dysfunction, are considered core components of depression and anxiety disorders [6]. Sustained glucocorticoid release due to chronic stress has deleterious effects on the prefrontal cortex (PFC), hippocampus, and amygdala [9, 10]. CUMS promotes neuronal apoptosis in the cerebral cortex [11] and hippocampus [12]. Previous studies have demonstrated that exposure to CUMS in rats leads to impaired synaptic plasticity in the hippocampus, as indicated by decreased levels of brain-derived neurotrophic factor (BDNF) [13]. Synaptophysin, a synaptic protein of nerve cell presynaptic vesicles, is typically used as a marker for synaptic-related neuronal plasticity [14]. It is considered an indicator

How to cite this article: Ozsagir MH, Polat EB, Cilingir Kaya OT, Aykac A, Ozbeyli D. Effects of metformin in an experimental chronic unpredictable mild stress model. J Res Pharm. 2026; 30(1): 171-185.

© 2026 Marmara University Press
ISSN: 2630-6344

https://doi.org/10.12991/jrespharm.1690442

of synaptic density and integrity. Chronic stress has been shown to reduce synaptophysin expression in stress-related brain regions, particularly the hippocampus and prefrontal cortex, thereby impairing synaptic plasticity [15]. SIRT-1 (a sirtuin family member) modulates stress response, metabolism, and neuroprotection [16].

Metformin (MF) has recently attracted attention with its positive effects on cognitive impairment and neurodegeneration in different experimental models [17, 18]. It has been shown that metformin can improve memory by repairing oxidative stress and inhibiting neuroinflammation and apoptosis, which may expand its clinical indications in the field of neurodegenerative disorders [19]. The beneficial effects of metformin on depressive behaviours and abnormal morphology in the hippocampus in the rat CUMS model have also been reported [20]. In a metabolic syndrome model, MF increased hippocampal immunoreactivity of synaptophysin, sirtuin-1, and BDNF important plasticity markers [21]. In addition, MF treatment decreased depressive behaviour and oxidative stress in PFC [22]. However, the effects of metformin on anxiety-like behaviours, neuroplasticity, and apoptosis in postmenopausal CUMS-administrated rats are unknown.

This study sought to evaluate the potential effects of metformin on anxiety-like behavioural alterations, cognitive dysregulation, brain neuroplasticity, and apoptosis induced by CUMS application in menopausal rats. For this purpose, the open field test (OFT) to measure exploratory, anxiety-like behaviours and locomotor action, the elevated plus-maze (EPM) to assess anxiety-like behaviour, and the novel object recognition test (NORT) to determine recognition memory was performed. In addition, the levels of BDNF, SIRT1, synaptophysin, and caspase-9 were measured by ELISA in the hippocampus, PFC, and amygdala.

2. RESULTS

2.1. Behavioural and cognition tests

2..1.1. Novel object recognition test (NORT)

The difference score obtained from the NORT results showed that recognition memory was significantly reduced in the OVX and OVX+CUMS groups compared to the control group ($p<0.05$ and $p<0.001$, respectively). MF treatment significantly increased the difference score in the OVX+CUMS+MF groups compared with the OVX+CUMS groups ($p<0.001$). In addition, the OVX+CUMS group's different score was higher than the OVX group ($p<0.001$) (Figure 1a).

2.1.3. Open field test (OFT)

The time spent in the central region was lower in the OVX+CUMS group than in the control and OVX groups ($p<0.01$), while there was a significant increase in the metformin-treated OVX and OVX+CUMS groups compared to the untreated ones ($p<0.01$, Figure 1b). The number of rearing decreased in the OVX+CUMS group compared to the control ($p<0.01$) and was significantly higher in the OVX+CUMS+MF group than in the OVX+CUMS group ($p<0.01$, Figure 1c). While the number of grooming in the OVX+CUMS group decreased compared to the control group ($p<0.05$), the number of grooming in the OVX+CUMS+MF group increased compared to the OVX+CUMS group ($p<0.01$, Figure 1d).

2.1.3. Elevated plus maze (EPM) test

The anxiety index ($N_{anxiety}$) in the experimental groups was determined as follows for control, OVX, OVX+CUMS, OVX+MF, OVX+CUMS+MF, respectively: 0.503±0.00, 0.623±0.01, 0.674±0.03, 0.51±0.0, 0.607±0.01. When anxiety-like behaviours were evaluated by EPM, $N_{anxiety}$ of the OVX, and OVX+CUMS groups were found to be significantly higher than the control group ($p<0.01$ and $p<0.001$, respectively; Figure 2a). MF treatment significantly attenuated the $N_{anxiety}$ in the OVX+MF and OVX+CUMS+MF groups when compared with OVX and OVX+CUMS groups ($p<0.01$ and $p<0.05$, respectively) (Figure 2a). Cumulative freezing time (CFT) in the groups was determined as follows for control, OVX, OVX+CUMS, OVX+MF, OVX+CUMS+MF, respectively: 58.33±2.09, 90.30±2.09, 113.0±3.50, 74.67±3.39, 70.5±5.19. CFT of the OVX and OVX+CUMS groups were found to be significantly higher than the control group ($p<0.001$ for both groups, Figure 2b). MF treatment significantly attenuated the CFT in the OVX+CUMS+MF group when compared with OVX+CUMS groups ($p<0.001$, Figure 2b).

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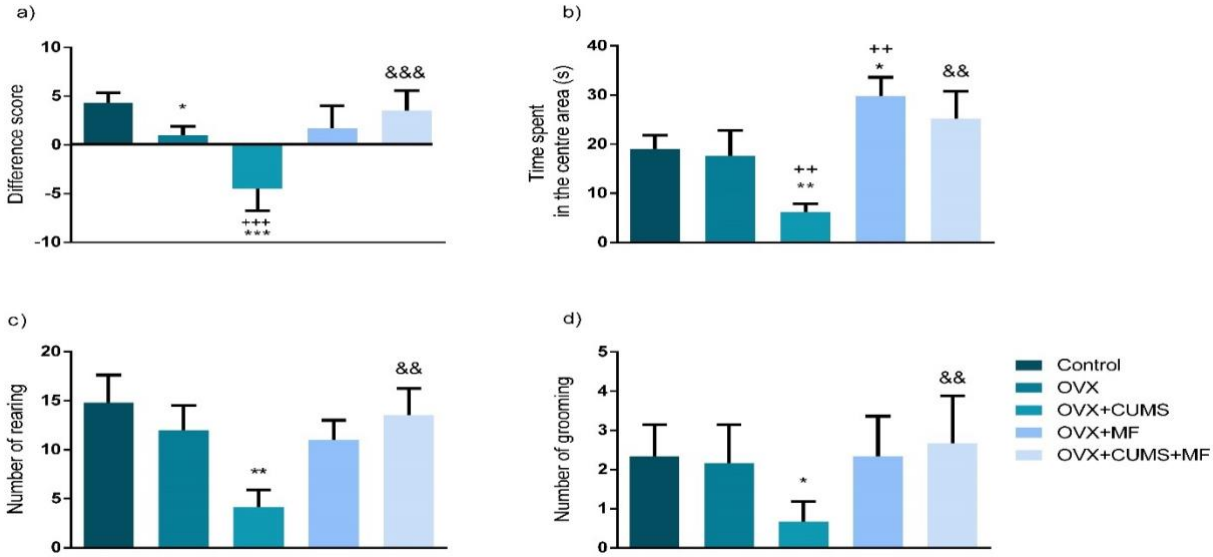


Figure 1. Results of the NORT and OFT. a) Difference score, b) Time spent in the centre area, c) Number of rearing, d) Number of grooming. OFT: Open field test, NORT: Novel object recognition test, OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin, s: Second. *p<0.05, **p<0.01, ***p<0.001 compared to control group; ++p<0.01, +++p<0.001 compared to OVX group; &&p<0.01, &&&p<0.001 compared to OVX+CUMS group. Each group consists of 6 animals.

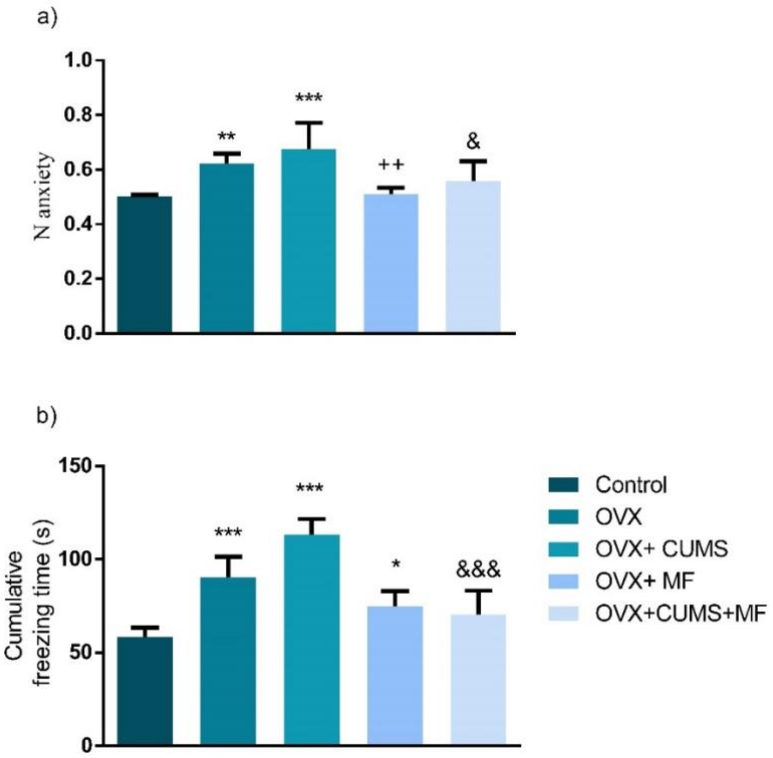


Figure 2. Results of the EPM test. a) Anxiety index, b) Cumulative freezing time (s). EPM: elevated plus-maze, OVX: ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin, s: second. **p<0.01, ***p<0.001 compared to control group; ++p < 0.01 compared to OVX group; &p<0.05, &&&p<0.001 compared to OVX+CUMS group. Each group consists of 6 animals.

2.2. Brain BDNF, sirtuin1, synaptophysin, and caspase-9 levels

2.2.1. Brain BDNF levels

The results of the ELISA test showed that only the OVX+CUMS group had a decline in the hippocampal levels of BDNF ($p<0.01$) in comparison to the control group (Figure 3a). No difference was observed in the other groups compared to the control. Hippocampal BDNF level increased significantly in the OVX+CUMS+MF group compared to the OVX+CUMS group ($p<0.05$) (Figure 3a).

In the PFC, both the OVX and OVX+CUMS groups showed decreased BDNF levels compared to the control ($p<0.01$). However, BDNF levels in both the OVX+MF and OVX+CUMS+MF groups increased ($p<0.05$ and $p<0.01$, respectively) (Figure 3b).

In the amygdala, in all groups, BDNF levels showed a decline in comparison to the control group ($p<0.05$- 0.01) (Figure 3c).

2.2.2. Brain sirtuin levels

While no difference was observed in hippocampal sirtuin levels in the OVX group compared to the control group, there was a significant decrease in the OVX+CUMS group ($p<0.05$). The OVX+MF and OVX+CUMS+MF groups' sirtuin levels increased in comparison to the OVX and OVX+CUMS groups ($p<0.05$) (Figure 4a).

In the PFC, there was no difference in sirtuin levels of the OVX, OVX+CUMS group in comparison to the control ones. Only the OVX+CUMS+MF groups had significantly increased sirtuin levels compared to the OVX+CUMS group ($p<0.01$) (Figure 4b).

In the amygdala, only the OVX+CUMS+MF group showed an increase in sirtuin levels compared to the OVX+CUMS group ($p<0.01$). The other groups were no different from the control group (Figure 4c).

2.2.3. Brain Synaptophysin levels

In the hippocampus, compared with the control group, there was a significant decrease only in the OVX+CUMS group ($p<0.05$). The OVX and OVX+MF and OVX+CUMS groups were no different from the control (Figure 5a).

Compared to the control, synaptophysin levels decreased in the OVX and OVX+CUMS groups. A significant increase was recorded in the metformin-treated groups compared to the OVX and OVX+CUMS groups ($p<0.001$) (Figure 5b).

Compared to the control, synaptophysin levels in the amygdala increased only in the OVX+MF group. No significant difference was found between the other groups ($p<0.05$) (Figure 5c).

2.2.4. Brain caspase-9 levels

Hippocampal caspase-9 levels were elevated in the OVX and OVX+CUMS groups compared to the control ones, meanwhile, OVX+MF and OVX+CUMS groups' caspase-9 levels were reduced compared to the OVX and OVX+CUMS groups ($p<0.05$ and $p<0.01$, respectively) (Figure 6a).

In the PFC, there was a significant increase in the OVX+CUMS group compared with the control group ($p<0.01$). OVX+MF and OVX+CUMS groups' caspase-9 levels were reduced compared to the OVX and OVX+CUMS groups ($p<0.01$) (Figure 6b).

The caspase-9 level in the amygdala was lower in the OVX+CUMS+MF group compared to the control group, while no significant differences were observed among the other groups (Figure 6c).

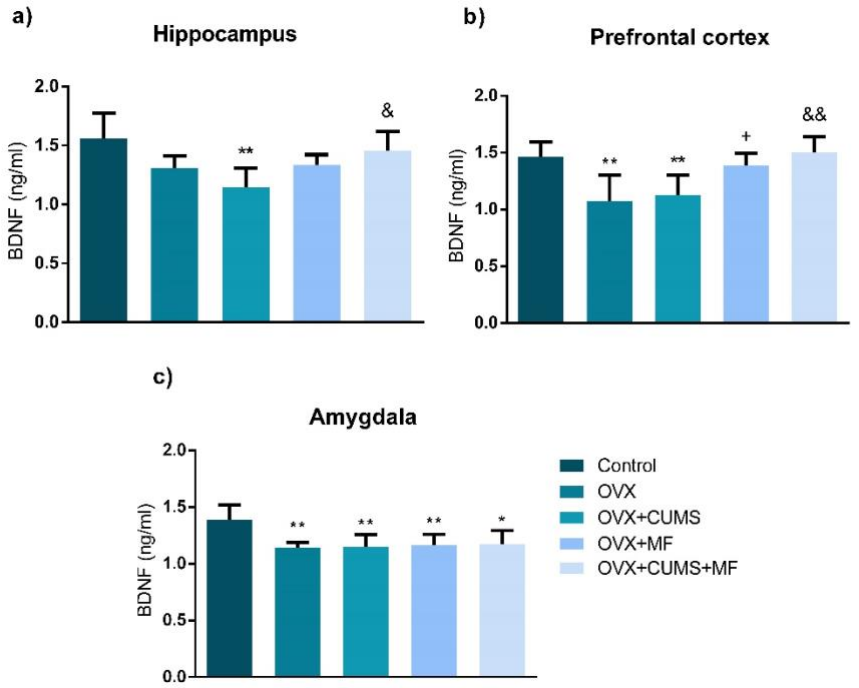


Figure 3. Results of the BDNF ELISA test. a) Hippocampus, b) Prefrontal cortex, c) Amygdala. BDNF: brain-derived neurotrophic factor, OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin. *p<0.05, **p<0.01 compared to control group; +p<0.05, compared to OVX group; &p<0.05, &&p<0.01 compared to OVX+CUMS group. Each group consists of 6 animals.

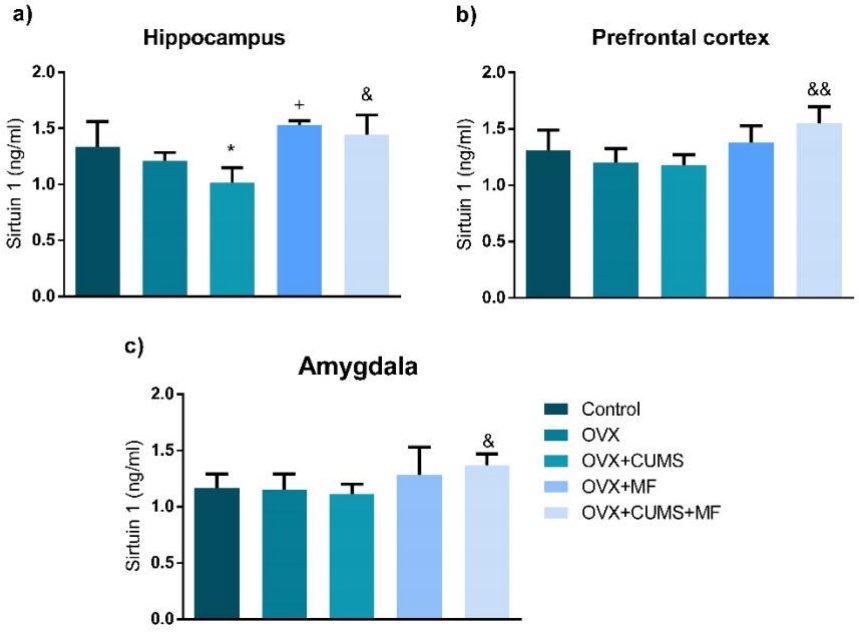


Figure 4. Results of the sirtuin1 ELISA test. a) Hippocampus, b) Prefrontal cortex, c) Amygdala. OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin. *p<0.05 compared to control group; +p<0.05, compared to OVX group; &p<0.05, &&p<0.01 compared to OVX+CUMS group. Each group consists of 6 animals.

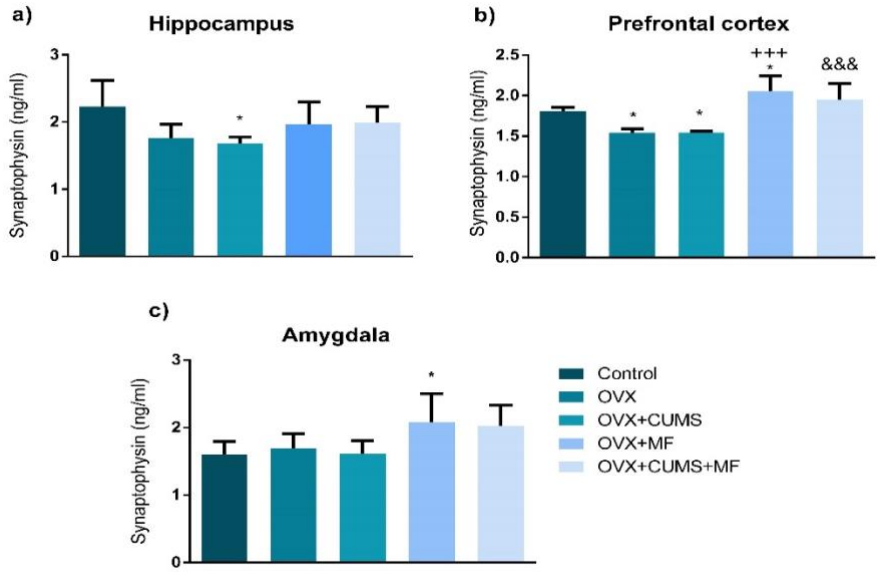


Figure 5. Results of the synaptophysin ELISA test. a) Hippocampus, b) Prefrontal cortex, c) Amygdala. OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin. *p<0.05 compared to control group; +++p<0.001, compared to OVX group; &&&p<0.001 compared to OVX+CUMS group. Each group consists of 6 animals.

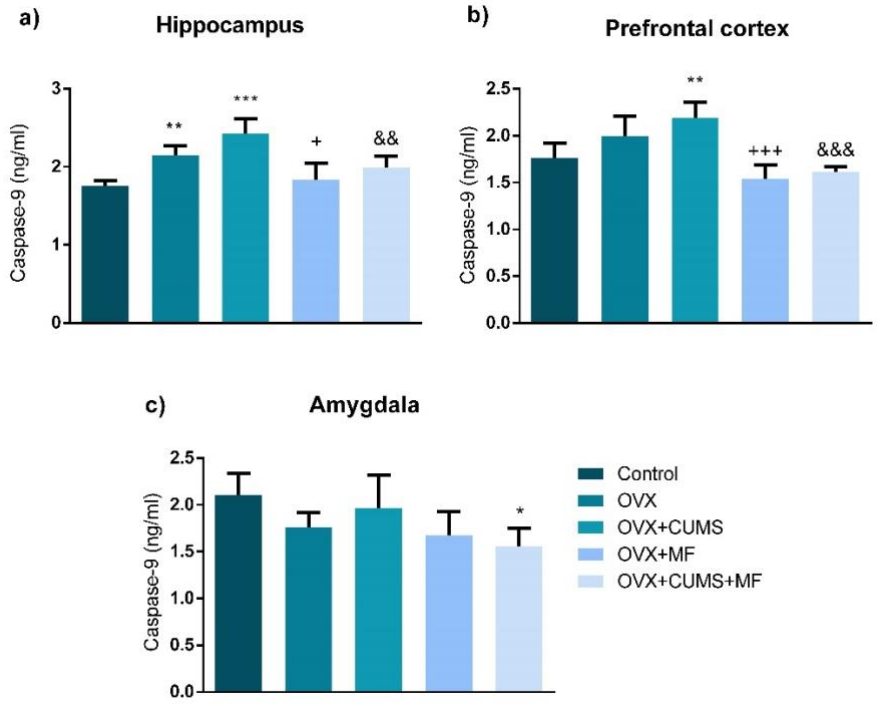


Figure 6. Results of the caspase-9 ELISA test. a) Hippocampus, b) Prefrontal cortex, c) Amygdala. OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin. *p<0.05, **p<0.01, ***p<0.001 compared to control group; +p<0.05, +++p<0.001 compared to OVX group; &&p<0.01, &&&p<0.001, compared to OVX+CUMS group. Each group consists of 6 animals.

2.3. Histopathologic evaluation

Histopathological analysis of the hippocampal dentate gyrus and prefrontal cortical regions in the OVX, and OVX+CUMS groups revealed prominent neurodegenerative changes, including pyknotic nuclei, cytoplasmic rarefaction, and widespread vacuolization. Perivascular and pericellular oedema were also observed. In contrast the OVX+MF and OVX+CUMS+MF groups exhibited marked reductions in these pathological features. Neuronal integrity was better preserved, with fewer pyknotic cells and reduced vacuolization.

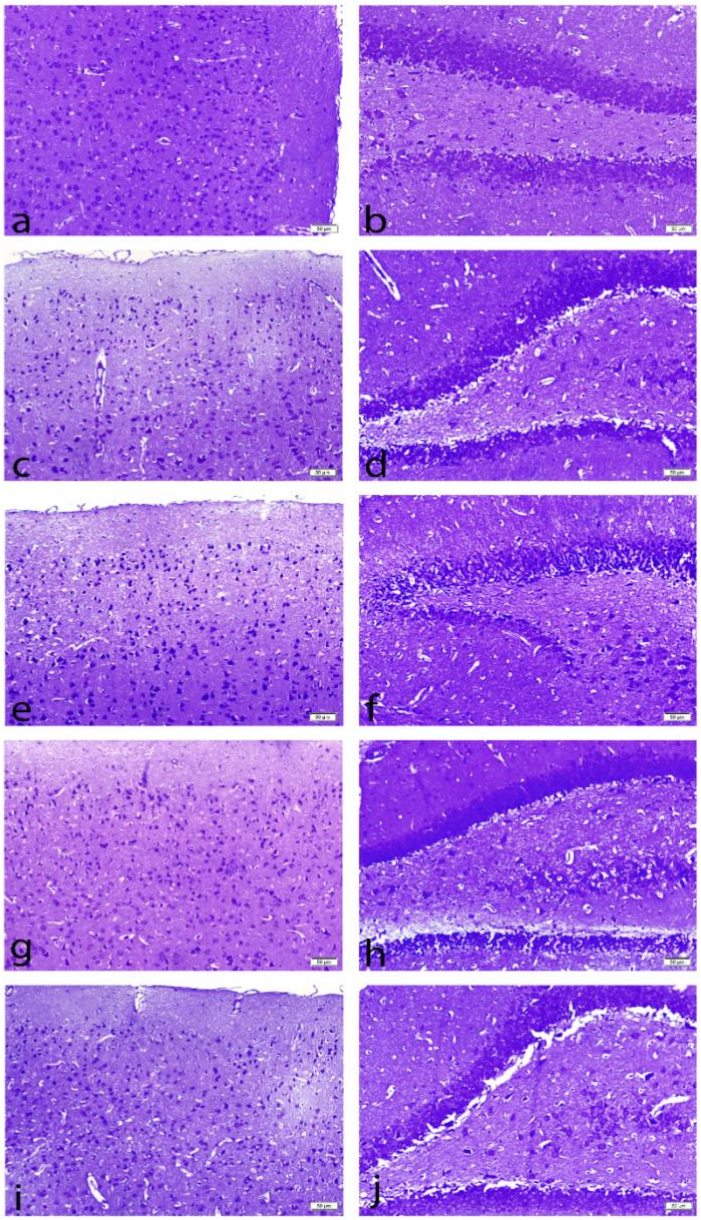


Figure 7. Results of the histopathological experiment of the groups. a) Prefrontal cortex of the control, b) Hippocampus of the control, c) Prefrontal cortex of the OVX, d) Hippocampus of the OVX, e) Prefrontal cortex of the OVX+CUMS, f) Hippocampus of the OVX+MF, g) Prefrontal cortex of the OVX+MF, h) Hippocampus of the OVX+CUMS+MF, i) Prefrontal cortex of the OVX+CUMS+MF. Cresyl violet stain. OVX: Ovariectomised, CUMS: Chronic unpredictable stress, MF: Metformin. Bars: 50 µm.

3. DISCUSSION

CUMS causes cognitive impairment in rats [6, 23]. Consistent with previous studies, our data showed that rats exposed to CUMS demonstrated decreased recognition memory compared to the control groups [23-25]. In prior research, metformin improved recognition memory impaired by experimental metabolic syndrome [21], methotrexate chemotherapy [26], doxorubicin-induced memory dysfunction [27], diabetes [28], and ageing [29] models. In the current study, both OVX and CUMS applications depleted recognition memory. Furthermore, there was a significant decrease in the OVX group that received CUMS compared to the OVX group. However, MF treatment improved memory impaired by OVX+CUMS.

We found increased anxiety levels in the OVX + CUMS group, as determined by the decreased time spent in the centre area, rearing, and grooming numbers in the OFT. In addition, the anxiety index and cumulative freezing time increased with OVX and OVX + CUMS applications in the EPM. All of these improved with MF treatment. Anxiety disorders are less common in diabetic patients using metformin [30]. Zhang et al. have emphasised that AMPK activation in medial PFC inhibitory neurons mediates the anxiolytic effects of metformin and that metformin has potential as a therapeutic option for anxiety treatment [30]. Furthermore, other studies show that MF produces antidepressant-like activities when administered alone [31] or in combination with fluoxetine [32] in chronically stressed animals. Research suggests that the neuroprotective properties of metformin may also mediate anxiety reduction. For example, a previous study has shown that metformin prevents methamphetamine-induced neurodegeneration, including symptoms of anxiety and depression, and reduces oxidative stress and inflammation in the brain. That study suggested that the neuroprotective effects are mediated by CREB/BDNF and Akt/GSK 3 signalling pathways [18]. Similarly, in the current study, MF increased BDNF in the hippocampus and PFC. Another mechanism could be that metformin facilitates the transport of GABAA receptors to the cell membrane. This action is crucial because GABA activity is essential for inhibition in the brain regarding anxiety disorders [33]. In a previous study, MF treatment significantly reduced anxiety-like behaviours in ovariectomized rats, and this effect was attributed to the drug's ability to reduce oxidative stress in the PFC and hippocampus [2]. In our study, elevated rearing and grooming numbers with MF treatment compared to OVX+CUMS also indicate increased locomotor activity. The locomotor function-enhancing effect of metformin in stressed animals has been reported previously. Chronic MF treatment increased locomotor activity in mice subjected to restraint stress, but this effect was determined in the forced swim test, not in the OFT. The researchers linked these effects to AMPK activation in the hippocampus [34]. Another study has shown that MF treatment increased locomotor activity in male rats with inflammation induced by lipopolysaccharide in the OFT. These effects were attributed to MF's reduction of neuroinflammation and oxidative stress [35].

BDNF plays a critical role in synaptic transmission and memory enhancement. It is found at high levels in both rats and humans' hippocampus, amygdala, cerebellum, and cerebral cortex. BDNF binds to tyrosine kinase receptors, promotes cell survival, and increases dendritic spine complexity [36]. Furthermore, BDNF enhances neurogenesis by boosting cell survival and increasing neurogenesis [37]. At the molecular level, the effects of stress and depression disrupt the synaptic structure and function responsible for neural plasticity, with decreased BDNF levels having a significant impact. In experimental stress models, BDNF levels are reduced in both the cortex and hippocampus [38]. Conversely, some drugs can increase BDNF levels. Metformin treatment led to a marked increase in hippocampal BDNF expression in rats with methamphetamine-induced anxiety and depression [18]. In another model of social defeat stress, metformin administered to mice (200 mg/kg) elevated hippocampal BDNF levels and counteracted stress-induced synaptic maladaptation by increasing BDNF levels [39]. The PFC and amygdala integrate sensory stimuli with emotional value. In the current study, only OVX and stress application reduced hippocampal BDNF levels, while either OVX alone or OVX combined with CUMS decreased BDNF levels in the PFC. BDNF levels are higher in the hippocampus than in the PFC [36]. Therefore, OVX applied alone may not have decreased hippocampal BDNF levels. A more substantial effect was observed when stress was combined with OVX, decreasing BDNF levels. MF treatment reversed the decreased values and restored them to control levels. Wu et al. reported that metformin administration increased hippocampal BDNF by activating the PI 3 K/AKT signalling pathway in a model of postoperative cognitive dysfunction in mice [40]. Additionally, MF has been shown to enhance the expression of BDNF genes by increasing CREB phosphorylation in various experimental models of neurodegeneration [18]. Conversely, our results in the amygdala indicated that OVX and OVX + CUMS treatments reduced BDNF levels, but these treatments did not restore levels to control. The amygdala and PFC are interconnected through reciprocal glutamate projections, and the PFC stabilises amygdala activity

[41]. Furthermore, BDNF signalling in the amygdala is complex. While some stressors increase BDNF levels [42], this expression is decreased in the amygdala of rats undergoing chronic stress [43]. Conversely, neuroprotective treatments such as ginsenoside elevate BDNF levels in the amygdala of chronically stressed rats [44].

Previous studies have shown that chronic stress reduces SIRT1 expression in the hippocampus [14, 45]. Conversely, stimulating SIRT1 inhibited the formation of abnormal dendritic structures caused by prolonged stress exposure in mice [45]. SIRT1 activation in the hippocampus can reverse anxiety-like behaviours associated with chronic stress [14, 45]. In addition, SIRT1 suppression in the hippocampus promotes anxiety [46]. The SIRT1-dependent signalling pathway (PGC-1α/NRF1/TFAM) regulates biogenesis and reduces anxiety [47]. Consistent with prior research, the present study found a marked decrease in hippocampal SIRT1 levels solely in the OVX + CUMS group when compared to the control group [14, 45]. While there was a significant increase in SIRT1 levels in the hippocampus across all metformin-treated groups, in the PFC and amygdala, SIRT1 levels increased only in the OVX+CUMS+MF group. In our study, the parallel between increased hippocampal SIRT1 levels and reduced anxiety levels in the EPM across all metformin-treated groups is noteworthy and aligns with previous findings [14, 45]. Furthermore, a previous study has shown that increasing SIRT1 and SIRT7 improves hyper-anxiety related to prediabetes [48].

Synaptophysin serves as a specific marker for presynaptic terminals, with its expression level reflecting synaptic density. In rats, CUMS leads to a reduction in hippocampal BDNF and synaptophysin levels, which play key roles in synaptic function, learning, and memory [49]. Previous studies found no difference in hippocampal synaptophysin levels in the CUMS model compared to controls in mice and rats [50, 51]. In our study, there was a significant decrease in the CUMS model combined with OVX compared to the control in the hippocampus. However, there was no difference in the group that received OVX alone. On the other hand, while synaptophysin levels in the PFC decrease with OVX and OVX+CUMS application, all MF treatments significantly elevated these levels. In a D-galactose ageing rat model, metformin increased hippocampal synaptophysin expression. This increase was associated with improved synaptic plasticity and enhanced cognitive functions [52]. Metformin reduced synaptic loss in a chronic restriction stress model by increasing synaptophysin expression [15]. MF increases BDNF levels by enhancing AMPK activation and activating the PI3K pathway, which promotes synaptic plasticity and synaptophysin expression [52]. Furthermore, MF reduces neuroinflammation and inhibits neuronal apoptosis, which contributes to preserving synaptic proteins such as synaptophysin [53].

CUMS impairs hippocampal neurogenesis and induces anxiety by increasing apoptosis and inflammation in rats [54]. In the CUMS rat model, an increase in Caspase-9 levels in the hippocampus and cortex has been demonstrated [39, 40]. An increase in apoptosis in the amygdala has been reported in the single prolonged stress model [55]. Social isolation stress has been reported to increase dendritic arborisation in the rats' basolateral amygdala, accompanied by social avoidance behaviour [56]. Restraint stress may also promote damage to astrocytes in the amygdala [57]. In our study, we investigated how CUMS affects apoptosis in different brain regions. Caspase-9 is one of the main initiator caspases involved in the intrinsic pathway of intracellular apoptosis. Activated caspase-9 triggers other executioner caspases, such as caspases 3, 6, and 7, initiating apoptosis [58, 59]. Hippocampal caspase-9 levels increased with OVX and OVX + CUMS, while they decreased significantly in the metformin groups. In the PFC, caspase-9 increased only in the OVX + CUMS group. There was a significant decrease in caspase-9 levels in all MF groups. There is no study in the literature showing the effects of metformin on apoptosis in the CUMS model or stress. Nonetheless, metformin has been shown to inhibit morphine-induced apoptosis in diabetic neuropathy models in rats [60] and limits apoptosis in rat cortex astrocytes under oxygen and glucose deprivation [61]. Our data indicate that MF may reduce apoptosis in the hippocampus and PFC by decreasing the increased caspase-9 levels in the hippocampus and PFC with CUMS. CUMS-treated animals exhibit depressive behaviours similar to human mental disorders, such as decreased pleasure, agitation, and cognitive impairment [62].

4. CONCLUSION

The present study demonstrated that chronic MF treatment improved levels of BDNF, SIRT1, synaptophysin, and caspase-9, as well as histological alterations in the rat brain with OVX+CUMS. It also enhanced recognition memory and reduced anxiety. In conclusion, we assert that metformin is a beneficial therapeutic agent that enhances neural plasticity, reduces apoptosis, and improves recognition memory and anxiety-related behaviours induced by OVX+CUMS.

5. MATERIALS AND METHODS

5.1. The rats and experimental groups

Before the experiments, approval was obtained from the institutional ethics committee (MUHDEK approval no: 31.2024mar). Wistar female rats weighing 200-250 g obtained from Marmara University Animal Health Centre (DEHAMER) were used in the study. Rats were housed at 21±3 °C and 55±5 % humidity, with unlimited access to standard rat food and water. Our experimental groups were randomly divided into five groups, with six rats in each group, as follows:

i) Sham control

ii) OVX-group (Ovariectomy, no treatment)

iii) OVX+CUMS-group (Four weeks after the OVX procedure, the CUMS protocol was applied, and no treatment)

iv) OVX+MF-group (Four weeks after the OVX procedure, MF was administered at a dose of 200 mg/kg/day via oral gavage for 14 days) The MF dose used in the study was determined according to a previous study, which showed that metformin administration for 1-3 weeks had positive effects on neuronal damage, oxidative stress markers, and memory in rat brain [63].

v) OVX+CUMS+MF-group (MF administration was initiated concurrently with the CUMS protocol and continued for a total of 15 days).

5.2. The ovariectomy procedure

The ovariectomy technique was performed under anaesthesia (Ketamine, 100 mg/kg, Pfizer Co. Türkiye, and xylazine, 10 mg/kg, Bayer Co. Türkiye). After a small abdominal incision, the ovaries were tightly ligated around the oviduct and ovarian artery. The ovaries were excised. The control rats underwent sham operation [64].

5.3. CUMS protocol

Four weeks following the OVX procedure, the CUMS protocol was implemented. This protocol involved a series of stressors, including restraint (confinement within a plastic tube), housing in a cage tilted at a 45° angle, placement in an empty cage without nesting material, overcrowded housing, and exposure to light during the dark phase. To prevent the animals from anticipating the source of stress, the stressors were applied in a randomized order and were not administered for more than two consecutive days [65] (Table 1).

5.4. Behavioural Tests

NOR, OFT and EPM tests were used on the last day of treatment to evaluate anxiety-like behaviours, movement activities, and recognition.

5.4.1. *NORT*

The NORT was used to examine recognition memory functions. The test has familiarizing, sample object, and novel object steps. All procedures are according to Bevins and Besher's protocol. The dimensions of the test box were 50×50×40 cm. In the NOR test, rats were first exposed to two identical objects for 10 min. After a 1-h delay, one familiar object was replaced with a novel one, and rats were allowed to explore for 3 min. Recognition memory was assessed by comparing the time spent with the novel and familiar objects, with a positive difference score indicating successful memory performance [66].

Difference score (s): Time spent with the novel object−time spent with the sample object.

5.4.2. *OFT*

OFT was used to assess both anxiety and locomotor activity. The decreases in time spent in the centre, grooming, and rearing were considered indicators of increased anxiety and depleted locomotor activity (19). A 5-minute observation period was used for the test, and the total time spent in the centre, the number of rearing, and grooming behaviors were recorded [67].

5.4.3. *EPM*

Rats were placed on the central platform facing an open arm on the EPM apparatus and their movements were recorded for 5 min. Cumulative freezing times and anxiety index were calculated for each rat using the formula given below [68]. N = 1 - ½ × [(Time spent in the open arms/300s) + (Number of entries into the open arms/Total number of entries into all arms)]
The entry of all four legs of the rat into the arm was considered a complete entry into that arm.

Table 1. CUMS protocol schedule

Day	Stress Factors	Duration (min)
1	Cage Tilting	120
2	Placement in an Empty Cage Without Nesting Material	480
3	Cage Tilting	120
4	Cage Tilting	120
5	Restraint in a Plastic Tube	480
6	Placement in an Empty Cage Without Nesting Material	480
7	Housing in Overcrowded Cages	180
8	Housing in Overcrowded Cages	180
9	Light Exposure During the Dark Phase	120
10	Light Exposure During the Dark Phase	120
11	Restraint in a Plastic Tube	120
12	Restraint in a Plastic Tube	120
13	Cage Tilting	120
14	Cage Tilting	120

5.5. Neuronal plasticity and apoptosis measurement in different brain tissues

Brain tissues of rats euthanized with pentobarbital (50 mg/kg) were removed. Brain regions, the prefrontal cortex, hippocampus, and amygdala, were dissected according to the coordinates given in the Paxinos rat brain atlas [69]. (Paxinos, 1986). BDNF (Cat. No. E0476Ra), sirtuin1(Cat. No. E1214Ra), synaptophysin (Cat. No. E1496RA), and caspase-9 (Cat. No. E1898Ra) levels were evaluated for PFC, hippocampus, and amygdala (located between 13.20–11.20 mm, 6.70–4.70 mm and 7.20–5.70 mm anterior to the interaural line, respectively) regions using the ELISA method according to the manufacturer's instructions.

5.6. Histopathological evaluations

The brains of euthanized animals were harvested for histological evaluation. The brains were embedded in paraffin after a routine histological procedure. Coronal sections (5 m-thick) of the hippocampus

and prefrontal cortex were stained with Cresyl violet. Blinded observers performed histopathological evaluations for neuronal degeneration, cytoplasmic vacuolization, perivascular oedema, and nuclear pyknosis and photographed on an Olympus BX51 photomicroscope.

5.7. Statistical Analysis

Statistical analysis was performed using Prism 6.0 (Graph Pad Software, San Diego, California, USA). The results were expressed as mean ± SEM, and p values of less than 0.05 were considered signifcant.

Acknowledgements: This study has been supported by Marmara University Scientific Research Projects Coordination Unit (BABKO) under grant number: 11465.

Author contributions: Concept – M.H.O., D.O.; Design – M.H.O., D.O.; Supervision – D.O.; Resources – M.H.O., D.O.; Materials – M.H.O., D.O., E.B.P., O.T.C.K., H.K.E.; Data Collection and/or Processing – M.H.O., E.B.P, O.C.K, O.T.C.K., H.K.E.; Analysis and/or Interpretation – M.H.O., D.O. O.T.C.K., A.A.; Literature Search – M.H.O., D.O.; Writing – M.H.O., O.C.K., A.A. D.O.; Critical Reviews – D.O., A.A.

Conflict of interest statement: The authors declared no conflict of interest.

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