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TNF-α gene upregulation and cytokine changes in mice under chronic unpredictable stress

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Abstract

Background: Psychological stress is a major contributing factor to psychiatric and immune system disorders. While tumour necrosis factor-alpha (TNF-α) is recognised as a key inflammatory mediator, its precise gene expression alterations under chronic stress remain incompletely understood. This study aimed to investigate the impact of chronic unpredictable stress (CUS) on anxiety- and depression-like behaviours, hypothalamic-pituitary-adrenal (HPA) axis activity, and systemic inflammation, with a specific focus on TNF-α gene expression in mice.

Methods: Forty male albino mice were randomly divided into a control group and an experimental group subjected to a four-week CUS protocol. Behavioural alterations were assessed using the Elevated Plus Maze (EPM), Forced Swim Test (FST), and Tail Suspension Test (TST). Serum concentrations of HPA axis hormones (ACTH and CRF) and inflammatory cytokines (TNF-α, IL-6, and IL-17) were quantified. Additionally, TNF-α mRNA expression was analysed using RT-qPCR.

Results: CUS induced significant behavioural deficits, evidenced by reduced open-arm exploration in the EPM and increased immobility in the FST and TST ($p<0.05$), indicating exacerbated anxiety- and depression-like states. These behavioural changes were accompanied by HPA axis hyperactivation, marked by a significant increase in serum ACTH and CRF concentrations. Furthermore, CUS triggered a robust systemic inflammatory response, showing significantly elevated levels of TNF-α, IL-6, and IL-17. Crucially, RT-qPCR analysis revealed a significant upregulation of TNF-α gene expression in the CUS group, with an average fold change of 3.22-fold compared to 1.44-fold in the control group.

Conclusion: Chronic unpredictable stress effectively drives anxiety- and depression-like behaviours in mice by hyperactivating the HPA axis and upregulating pro-inflammatory networks. The significant overexpression of the TNF-α gene underscores its pivotal role in stress-induced systemic inflammation, offering potential molecular insights into the neuroendocrine-immune axis.

Keywords: Chronic unpredictable stress; HPA axis; Neuroinflammation; Pro-inflammatory cytokines; Behavioral despair; Gene expression.

1. Introduction

The term “stress” has become very popular in contemporary society, and is commonly invoked to explain a wide variety of psychological and medical problems [1]. Along with this fashionable trend, it is commonly assumed that psychological stressors represent a concern of particularly present-day origins, or at least that they have become much more prominent and pervasive with advances in modern technologies and the apparent quickening pace of life. As a consequence of these perceived pressures, it is also commonly believed that with this accelerating progress of civilisation, more people are succumbing to mental and physical disorders than ever before [1,2].

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Due to the influence of various stressors, psychological stress disrupts the organism's homeostasis. Consequently, the organism reacts, and the sympathetic-adrenal-medullary (SAM) system and the hypothalamic-pituitary-adrenal (HPA) axis are activated, producing and releasing specific hormones [3,4]. Therefore, the psychological response to emotional stress also modulates the functions of the immune system, the autonomic nervous system (ANS), levels of hypothalamic and pituitary hormones, neuropeptides, cytokines, and other factors involved in this network [5,6]. Thus, the short- and long-term effects of stress are associated with changes in HPA axis functioning, which alter glucocorticoid levels and may influence diverse health outcomes [7,8].

The stress response involves bidirectional connections between the central nervous system (CNS) and the immune system. In addition, adaptation to stress is a key mechanism in the body's response. Thus, the effectiveness of the stress response depends on the type, intensity, and duration of stress, as well as the characteristics of the person [9,10]. A growing number of studies have further explored the impact of psychological stress on inflammation, using experimental protocols to examine changes in mediators of inflammation in response to short-term laboratory stressors designed to characterise transient effects. stresses of daily life. Early results suggest that acute stress induces reliable changes in both enumerative and functional aspects of immunity, including increases in both circulating and stimulated markers of inflammation [11,12].

Cytokines and other humoral mediators of inflammation are potent activators of the central stress response, and the glucocorticoids released via this mechanism might regulate the recruitment of immune cells into inflamed tissues to cope with psychological stress and depression [13,14]. Although numerous studies have examined the impact of psychological stress on the immune response and elevated levels of inflammatory cytokines, few have directly examined gene expression of cytokines such as TNF under chronic psychological stress in mouse models. This indicates a research gap in understanding the precise molecular mechanisms linking chronic psychological stress to alterations in the gene expression of inflammatory cytokines, which is crucial for developing future therapeutic strategies for psychiatric and immunological disorders [15]. Therefore, the current study aimed to identify biochemical and gene expression changes in TNF in mice exposed to chronic unpredictable stress.

2. Materials and Methods Animal model

40 male albino mice, aged 8–12 weeks, weighing 20–25g, were obtained from the Animal House at the College of Science, University of Kirkuk, for this experiment. The animals were divided into two groups of 20 mice each:

- A control group that was not subjected to chronic unpredictable stress.
- An experimental group that was subjected to a chronic unpredictable stress (CUS) program.

2.1. Chronic unpredictable stress (CUS)

To apply chronic unpredictable stress (CUS), adult mice (8–12 weeks old, weighing 20–35 g) were subjected to psychological stressors twice daily (morning and evening) for four consecutive weeks. The stressors used included temporary food or water deprivation, confinement in designated tubes for 1–2 hours, continuous light exposure, cage tilted at a 30–45° angle overnight, soiled mice litter covering half of the cage (for 12 hours. /day), social isolation (12 hours. /day), and several cage changes over defined hours. The stressors were randomly and unpredictably selected daily to avoid adaptation, taking care not to cause any physical harm [16,17]

2.2. Behavioral tests

2.2.1. Elevated Plus Maze (EPM)

The Elevated Plus Maze (EPM) test is used to assess anxiety levels in mice by balancing curiosity and fear of open spaces. The apparatus consists of two open arms and two closed arms raised off the ground. Both the duration of stay and the number of entry points to the open arms are recorded; increases in these two variables indicate a decrease in anxiety levels [18].

2.2.2. Forced Swim Test (FST)

The mice are placed in a water-filled cylinder (25–30 cm high, 23–25°C) so that they cannot touch the bottom for 6 minutes. Immobility time is recorded during the last 4 minutes of the test. Increased immobility is considered an indicator of depression-like behaviour, while continuous activity and movement reflect frustration resistance.

2.2.3. Tail suspension test (TST)

The tail suspension test (TST) is used to assess depression-like behaviour. Mice is suspended by its tail approximately 50 cm above the ground for 6 minutes. The time of immobility (in seconds) is recorded during the last 4 minutes of the test, with a longer time reflecting greater levels of experimental "depression."

2.3. Sample collection

Mice were anaesthetised with ketamine/xylazine (Alsaad Pharmaceuticals, Syria), and blood was drawn via cardiac access after dissection. One ml of blood was withdrawn from each mice; a portion was separated for serum and frozen pending biochemical analysis, while the remainder was mixed with TRIzol at a 1:3 ratio for RNA extraction for molecular analysis.

2.4. Biochemical assay

Blood cytokine levels were measured using ELISA (Biobase, China). Serum concentrations of ACTH, CRF, TNF-α, IL-6, and IL-17 were determined using pre-prepared ELISA kits from Sunlong Biotech (China) according to the manufacturer's instructions. Samples and titrants were added to the wells with the specified reagents. Measurements were taken at the recommended wavelength using a plate reader (Biobase, China), and concentrations were calculated using the standard calibration curve.

2.5. Molecular detection

RNA was extracted from serum samples using the Total RNA Mini Kit (Transgen, China). cDNA was synthesised with the EasyScript First-Strand cDNA Synthesis SuperMix per the manufacturer's instructions. Total RNA yield was measured at 260 nm, and purity was checked by the 260/280 nm ratio on a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). All steps were performed under sterile, RNase-free conditions at room temperature (25°C). For qPCR, the TNF-α gene was amplified using β-actin as a reference gene and predefined primers (Table 1). Each 20 µL reaction contained Master Mix, primers, cDNA, and nuclease-free water. The SaCycler-96 Real Time PCR System (Sacace, Italy) was used with this program: 94°C for 30 seconds (activation); 45 cycles of 94°C for 5 seconds (denaturation), 60°C for 35 seconds (annealing); then a melt curve at 90°C for 15 seconds. Relative gene expression was calculated by the ΔΔCt method, comparing the target and reference genes against the control group.

Table 1 The sequence of primers that used this study·

Primer	Primer sequence 5'- - 3'	Tm (°C)	GC%	product length	Ref.
TNF-F	TCTCGAACCCCGAGTGACCAA	63.16	57.14	123 bp	[19]
TNF-R	ACCGCACCTCGACTCCTAT	60.08	57.89		
B-ACTIN-F	AACCCCAAGGCCAACCGCGAG AAGATGACC	73.33	60.00	417 bp	
B-ACTIN-R	GGTGATGACCTGGCCGTCAGG CAGCTCGTA	74.13	63.33		

2.6. Ethical clearance

This study was approved by the Institutional Review Board of [Faculty of Science, University of Kirkuk], in accordance with ethical guidelines outlined for the care and use of laboratory animals. The study strictly followed the ethical principles of the 3R framework—replacement, reduction, and refinement—along with ensuring the welfare of the animals by adhering to the 5F principle (freedom from hunger and thirst; freedom from discomfort; freedom from pain, injury, or disease; freedom from fear and distress; as well as freedom to express normal behaviour. All animal care and procedures were conducted in compliance with international ethical standards, such as those outlined in the Guide for the Care and Use of Laboratory Animals [19].

2.7. Statistical analysis

Parametric data were analyzed by one ways Analysis of Variance (ANOVA) continued with Least Significant Difference (L.S.D.), and $p<0.05$ was considered to be significant. Statistical Package for Social Sciences (SPSS) was used.

3. Results and Discussion Behavioral tests

3.1. Elevated Plus Maze (EPM)

The elevated plus maze test showed a significant decrease in time spent on the open arms in mice exposed to chronic unpredictable stress compared to the control group. The number of times mice entered open arms was also significantly reduced ($p<0.05$), indicating a higher level of anxious behaviour in the stressed mice. These findings align with the work of Walf and Frye [20], who highlighted the Elevated Plus Maze (EPM) as a standardised tool for assessing anxiety-related behaviours in rodents. Their study demonstrated that EPM outcomes are shaped by a conflict between the mouse's curiosity to explore and its fear of open, elevated spaces. Mice that enter open arms more frequently and remain there for longer periods exhibit lower levels of anxiety, while those that tend to stay in closed arms show higher levels of anxiety.

3.2. Forced Swim Test (FST)

The forced swim test revealed that mice exposed to chronic unpredictable stress showed a significant increase in immobility time during the last 4 minutes compared to controls ($p<0.01$). This decrease in activity and movement duration reflects depression-like behaviour in stressed mice. The Forced Swim Test (FST), introduced by Porsolt et al. [21], is a well-established animal model for studying depression in rodents and is sensitive to antidepressant treatments. In this test, a mouse or rat is placed in a water-filled cylinder, unable to touch the bottom, and the duration of immobility is observed over a set period. Prolonged immobility indicates depression-like behaviour, while reduced immobility following antidepressant administration signifies a treatment response.

3.3. Tail Suspension Test (TST)

The tail suspension test showed a significant ($p<0.05$) increase in immobility time in mice exposed to chronic unpredictable stress compared to controls. These results match those observed in the forced swimming test. They indicate the emergence of submissive and depression-like behaviour due to continuous psychological stress. Steru et al. [22] indicated that the Tail Suspension Test (TST) is a good method for assessing depressive-related behaviours in rats and for detecting the effects of antidepressants. The test involves suspending the rat by its tail and observing the duration of its immobility over a set period. Rats with longer immobility show depressive-like behaviour. When antidepressants are administered, this duration decreases, making the test effective for studying emotional responses and antidepressant efficacy.

3.4. HPA Hormones

The findings showed the concentrations of HPA hormones in studied groups, where ACTH concentration in CUS group (231.7± 37.8) demonstrated significant ($P<0.05$) increase compared with control (46.27± 8.395), as shown in figure (1). The levels of CRF demonstrated significant ($P<0.05$) elevated in CUS group (268.4 ± 14.8) compared with control (71.19 ± 12.11), as shown in figure (2).

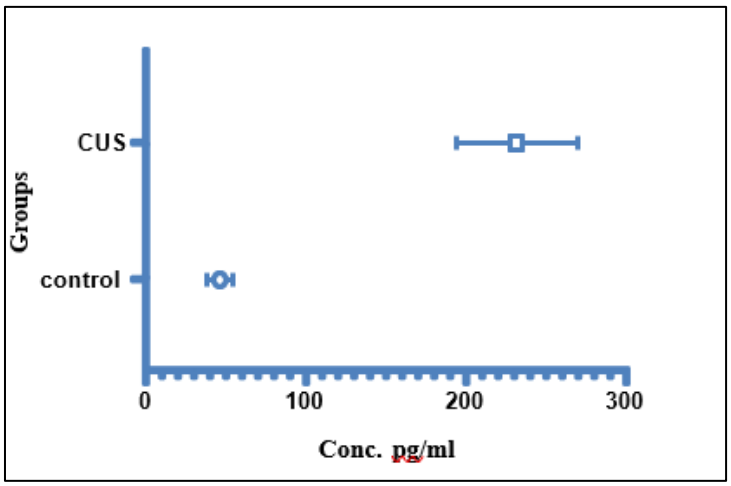

Figure 1 The concentrations of ACTH in studied groups

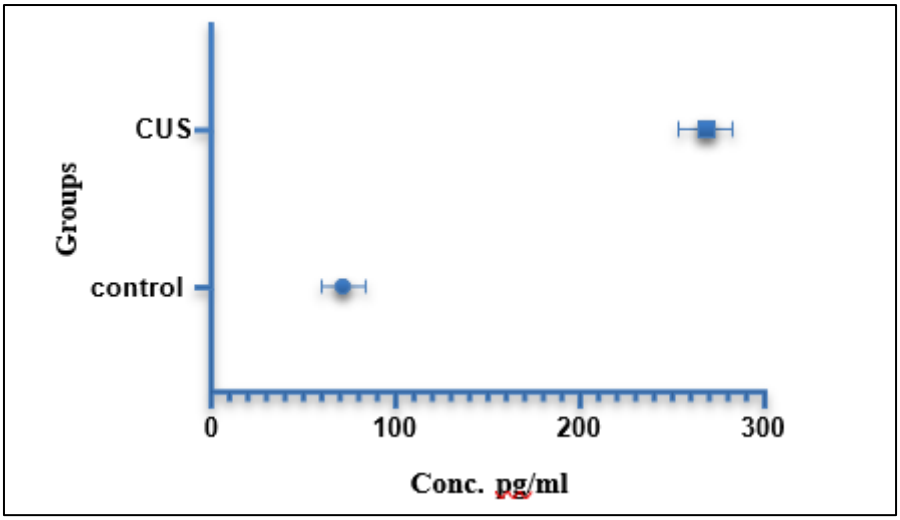

Figure 2 The concentrations of CRF in studied groups

In a study by Bakry and Hamada [23], chronic stress was shown to increase ACTH levels in animal models, suggesting that activation of the HPA axis is a key biological mechanism linking stress to physiological and behavioural changes. These findings support the effect of chronic unpredictable stress (CUS) on mice compared to healthy mice, demonstrating the direct relationship between psychological stress and elevated ACTH. Belo-Silva *et al*. [24] demonstrated that chronic stress in mice leads to a marked increase in ACTH levels, suggesting that activation of the HPA axis is a key biological mechanism linking stress to physiological and behavioural changes. A study examining the effects of chronic unpredictable stress on an enriched environment showed that stress led to elevated ACTH levels, while environmental and therapeutic modifications helped reduce the intensity of the hormonal and behavioural response.

Füchsl et al. [25] showed that chronic social stress (CSC) leads to elevated ACTH levels, which are associated with changes in pituitary structure and adrenal hormone receptors. This confirms that the HPA axis and ACTH are sensitive indicators of the effects of chronic stress and can be modulated by both environmental and therapeutic factors, in line with the current study's findings. Similarly, Bravo and Dinan [26] found that both acute and chronic stress increase hypothalamic corticotropin-releasing factor (CRF) secretion, which in turn stimulates pituitary ACTH release and activates the HPA axis. During chronic stress, elevated CRF levels were also detected in additional brain regions, including the BNST. Furthermore, a review by Backström and Weinberg [27] clarifies that CRF_1 and CRF_2 receptors serve distinct roles: CRF_1 is mainly associated with anxiety-related responses, while CRF_2 is involved in coping mechanisms. Together, these observations indicate that the CRF system is a central biological marker for psychological stress and a promising target for pharmacological and behavioural interventions.

3.5. Cytokines

The findings showed cytokine concentrations in the studied groups, with TNF-α in the CUS group (483.8±48.7) demonstrating a significant ($P < 0.05$) elevation compared with the control (156.4±20.21), as shown in Figure 3. The concentration of IL-6 was significantly elevated in the CUS group (229.0 ± 27.61) compared with the control (83.85 ± 3.39), as shown in Figure 4 ($P < 0.05$). The concentration of IL-17 was significantly elevated in the CUS group (198.1 ± 21.2) compared with the control (67.01 ± 7.55), as shown in Figure 5 ($P < 0.05$).

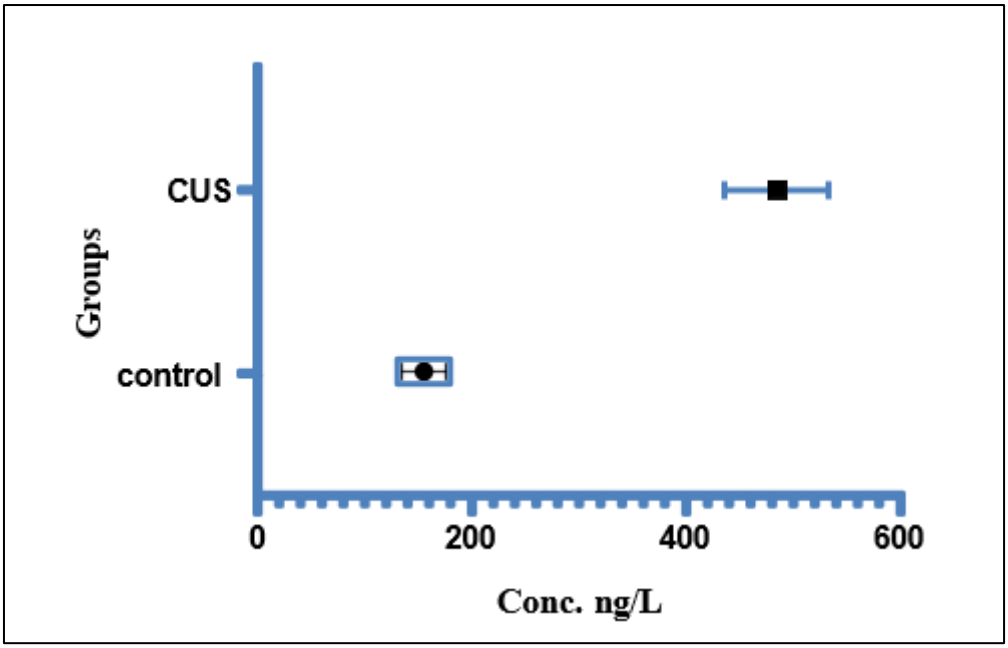

Figure 3 The concentrations of TNF-α in studied groups

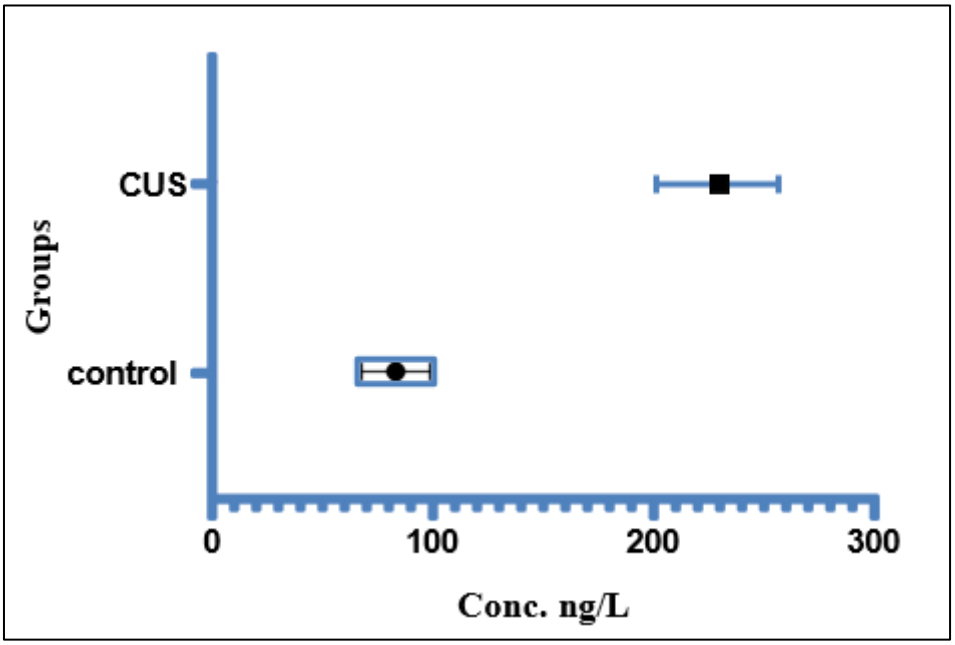

Figure 4 The concentrations of IL-6 in studied groups

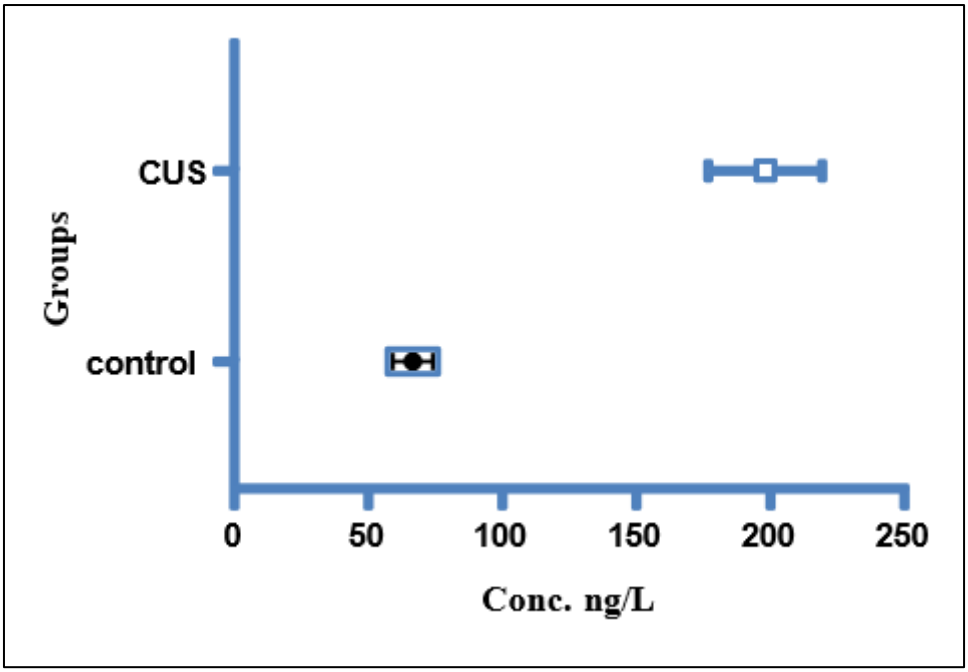

Figure 5 The concentrations of IL-17 in studied groups

The current results showed a significant increase ($P < 0.05$) in the concentrations of the cytokines TNF-α, IL-6, and IL-17 in the CUS-exposed group compared to the control group. These results are consistent with those of Wohleb et al. [28]. That study demonstrated that acute psychological stress exposure in mice, according to the "learned deficit" model, led to a marked increase in plasma levels of the inflammatory cytokines TNF-α, IL-6, and IL-17A. These cytokines peaked within 6–12 hours of stress exposure. On the other hand, this increase was accelerated by pre-conditioning stress. In these cases, the rise occurred within 1 hour of subsequent stress exposure. This acceleration is explained by an immunoconditioning process. This process makes the immune system more responsive to neurohormonal stimuli. It results from activation of the HPA axis and the sympathetic nervous system. These changes lead to the release of corticosteroids and catecholamines. These substances, in turn, stimulate cytokine release from macrophages and lymphocytes. These findings are supported by other studies. Gouin and Kiecolt-Glaser [29], showed that chronic stress increases the sensitivity of immune cells to produce IL-6 and TNF-α. Oh et al. [30], demonstrated that repeated accumulation of mild stress elevates IL-17A in the brain, leading to depressive behaviours. These data suggest that inflammatory responses to stress represent a crucial link between physiological stress and neurological and immunological disorders.

3.6. TNF-α gene expression

Gene expression analysis using RT-qPCR showed that chronic exposure to CUS led to a significant increase in TNF-α gene expression compared to the control group. The mean fold change in the control group was approximately 1.44, while it increased to 3.22-fold in the CUS group, indicating a clear activation of the inflammatory pathway in response to chronic stress (Table 2). A decrease in ΔCT values was also observed in the CUS group, indicating greater TNF-α gene transcription relative to the reference gene β-ACTIN. These results support the hypothesis that chronic stress promotes peripheral and central inflammatory activity by increasing the expression of pro-inflammatory cytokines, such as TNF-α, consistent with previous studies demonstrating the role of the HPA axis in activating immune pathways under prolonged stress conditions. In Figure 6, the X-axis represents the number of cycles, while the Y-axis represents fluorescence intensity measured during the RT-qPCR analysis. Positive samples showed a curve of amplification at different cycle numbers on the Roy channel, while negative specimens displayed a known curve of amplification at any cycle number and remained flat below the threshold level of amplification.

Table 2 Analysis of TNF-α gene expression in mice with CUS compared to the control group

Groups	TNF-α	B-ACTIN	ΔCT	ΔΔCT	2-ΔΔCt	Folding	Mean
Control	32.5	25.1	7.4	0.48	0.716977624	0.716977624	1.442534
	31.3	25.3	6	-0.92	1.892115293	1.892115293	
	31.5	25.4	6.1	-0.82	1.765405993	1.765405993	
	32.3	25.3	7	0.08	0.946057647	0.946057647	
	31.5	25.5	6	-0.92	1.892115293	1.892115293	
CUS	31.3	24.2	7.1	0.18	0.882702996	0.882702996	3.222602
	31.1	25.1	6	-0.92	1.892115293	1.892115293	
	30.8	24.9	5.9	-1.02	2.02791896	2.02791896	
	30.3	25.6	4.7	-2.22	4.658934346	4.658934346	
	31.1	25.7	5.4	-1.52	2.867910496	2.867910496	
	30.2	25.6	4.6	-2.32	4.993322196	4.993322196	
	31.4	25.9	5.5	-1.42	2.67585511	2.67585511	
	31.1	25.2	5.9	-1.02	2.02791896	2.02791896	
	31.3	25.1	6.2	-0.72	1.647182035	1.647182035	
	30.1	25.2	4.9	-2.02	4.055837919	4.055837919	
	31.4	25.3	6.1	-0.82	1.765405993	1.765405993	
	29.4	25.5	3.9	-3.02	8.111675838	8.111675838	
	30.1	25.2	4.9	-2.02	4.055837919	4.055837919	
	29.1	24.3	4.8	-2.12	4.34693945	4.34693945	
	30.2	24.5	5.7	-1.22	2.329467173	2.329467173	

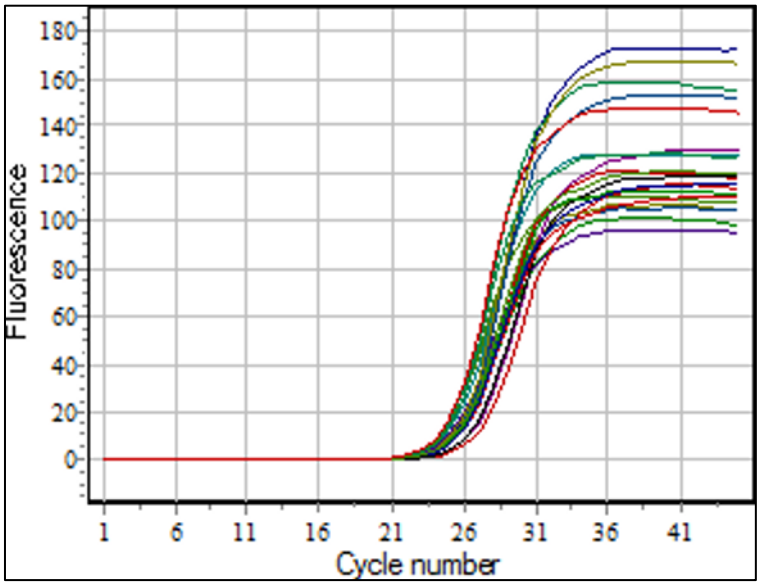

Figure 6 Amplification of TNF-α gene on Cy5 channel indicating positive sample with curve shape at different cycle number.

The results of the current study showed that unpredictable stress (CUS) increased TNF-α gene expression, consistent with previous studies demonstrating similar effects of chronic stress on the expression of inflammatory cytokines. For example, a study in Sprague-Dawley rats subjected to compression stress for 7 or 14 days showed increased gene expression of TNF-α and IL-6 in the liver, as well as increased plasma levels of these cytokines. This supports the relationship between continuous stress exposure and alterations in the peripheral inflammatory immune response [31]. Similarly, another study by Xiong et al. [32] in Wistar rats subjected to moderate chronic stress for four weeks showed an increase in the gene expression of inflammatory cytokines such as TNF-α and IL-6 in brain structures such as the hippocampus, cerebral cortex, and splenic appendage, while a decrease in anti-inflammatory cytokines was observed. These results suggest that chronic stress can shift immune homeostasis toward an inflammatory state, consistent with our findings of increased TNF-α gene expression following exposure to unpredictable stress. Furthermore, Reiche et al. [33] demonstrated that chronic stress stimulates the release of stress hormones, such as catecholamines, which activate transcription factors like NF-κB and STAT-3, leading to increased secretion of TNF-α and IL-6 in the blood, thereby maintaining a low-grade chronic inflammatory state. This mechanism highlights how chronic stress links neurohormonal activity to immune changes, supporting our findings of increased TNF-α levels following unpredictable stress. These studies demonstrate the pivotal role of the HPA axis in triggering the inflammatory response under conditions of sustained stress. Therefore, these studies contribute to advancing scientific understanding of how chronic stress affects immune activity, suggesting that elevated levels of inflammatory cytokines such as TNF-α and IL-6 may be part of the mechanism that triggers chronic stress-related inflammation.

4. Conclusions

The results of this study indicate that exposure to chronic unpredictable stress (CUS) leads to clear behavioural disturbances. These are manifested by increased anxiety and depressive-like behaviour. CUS is also accompanied by physiological and molecular changes, including elevated levels of ACTH and CRF. There is a marked increase in concentrations of inflammatory cytokines such as TNF-α, IL-6, and IL-17. Molecular analysis showed increased TNF-α gene expression. This indicates that chronic unpredictable stress promotes activation of the hypothalamic-pituitary-adrenal (HPA) axis and stimulates systemic and central inflammatory responses. These effects may link prolonged psychological stress exposure to the development of neuroimmune disorders.

Compliance with ethical standards

Disclosure of conflict of interest

No conflict of interest to be disclosed.

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