Assessment of Plasma Inflammatory Markers' Level after Peripheral Pain Stimulation in Female Wistar Rats Subjected to Restraint Stress

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Abstract

Inflammation is a vital immune response to injury, infection or harmful stimuli, aiming to eliminate threat and initiate healing while pain functions as a protective mechanism signaling potential tissue damage. Peripheral pain stimulation arises when nociceptors detect noxious stimuli from the surface of the body and transmit signals to the brain. Inflammatory responses usually follow pain stimulation. Restraint stress, a model of chronic psychological stress, involves immobilization that simulates uncontrollable stress. This study investigated plasma inflammatory markers' level following peripheral pain stimulation in female Wistar rats exposed to restraint stress. Eighteen female Wistar rats weighing between 180–220 grams were randomly divided into three groups (n=6): Control (CTL), Peripheral Pain (PPN), and Restraint Stress + Peripheral Pain (RPN). After two weeks of acclimatization, rats in the RPN group were restrained using wire mesh for 30 minutes daily. The experiments lasted 28 days. Twenty-four hours after the last restraint stress session, peripheral pain was induced using the hot-water tail-flick and formalin-induced paw licking methods (using the left hind paw). Thereafter all animal were sacrificed, blood (plasma) samples were collected for biochemical assays of Nitric Oxide (NO), Myeloperoxidase (MPO), Tumor Necrosis Factor-alpha (TNF-α), and Interleukin-6 (IL-6). The control and induced paw tissues were removed, weighed and processed for histological examination using hematoxylin and eosin (H&E) staining techniques.

Results showed a significant ($p<0.05$) increase in relative paw weight in PPN group when compared to RPN and CTL independently. There were no significant differences on tail flick latency of RPN when compared to PPN group. Also, there were significant ($p<0.05$) increase in plasma NO, MPO, TNF-α, and IL-6 in both PPN and RPN groups when compared to CTL individually. However, there was no significant difference in these inflammatory markers in the PPN when compared to RPN group. Histological analysis revealed greater inflammatory infiltration, edema, and hyperkeratinization in RPN when compared to PPN group. In conclusion, peripheral Pain stimulation led to elevated plasma inflammatory markers, however, in the presence of restraint stress, peripheral pain does not significantly amplify these markers. Also, restraint stress gave rise to more deleterious histological changes in the pain stimulated tissue.

Keywords: Inflammation, Restraint stress, Peripheral pain, Myeloperoxidase, Interleukin-6.

1.0 Introduction

Inflammation is a fundamental immune defense mechanism that responds to injury, infection, or harmful stimuli to eliminate threats and initiate healing (Kesselman *et al.*, 2020; Ridker, 2022). While inflammatory response is protective in the short term, chronic or dysregulated inflammation is strongly implicated in the development of chronic diseases, including arthritis and cardiovascular disorders (Libby *et al.*, 2023). The vascular changes associated with inflammation such as vasodilation and increased permeability facilitate the migration of immune cells like neutrophils and macrophages to the affected site, where they act to neutralize pathogens (Ricklin *et al.*, 2022). The inflammatory response progresses through distinct phases. During initiation, pattern recognition receptors such as Toll-like receptors (TLRs) detect harmful signals and activate intracellular pathways like nuclear factor kappa B (NF-κB), leading to cytokine release. The amplification phase is characterized by leukocyte recruitment and the generation of reactive oxygen species (ROS) and enzymes that support pathogen clearance (Liu *et al.*, 2023). Finally, the resolution phase involves specialized pro-resolving mediators, including resolvins and lipoxins, which actively suppress inflammation and promote tissue repair. A failure to resolve inflammation disrupts this balance, resulting in chronic inflammation and progressive tissue damage (Serhan and Levy, 2021).

The International Association for the Study of Pain (IASP) defines pain as "an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage" (IASP, 2020). Pain may be classified into nociceptive, neuropathic, and nociplastic types. Nociceptive pain arises from tissue injury or inflammation, neuropathic pain stems from nervous system dysfunction, while nociplastic pain reflects altered central pain processing without obvious tissue or nerve damage, as in fibromyalgia (Fitzcharles *et al.*, 2021). The physiology of nociceptive pain involves four stages. In transduction, noxious thermal, chemical, or mechanical stimuli activate nociceptors via ion channels such as Transient Receptor Potential Vanilloid (TRPV1) (Basbaum *et al.*, 2019). During

transmission, Aδ fibers carry sharp pain signals and C fibers convey dull, burning sensations to the spinal cord and brain. Modulation occurs through descending neural pathways, which release neurotransmitters like serotonin, norepinephrine, and endorphins that can either inhibit or amplify pain signals (Martikainen *et al.*, 2022). Finally, perception arises as the brain integrates these inputs through regions including the somatosensory cortex, limbic system, and prefrontal cortex, producing the conscious experience of pain (Bushnell *et al.*, 2020).

Stress represents the body's physiological and psychological response to challenges that disrupt homeostasis. It primarily activates the hypothalamic–pituitary–adrenal (HPA) axis and the sympathetic–adrenal–medullary (SAM) system. The HPA axis promotes the release of glucocorticoids such as cortisol, while the SAM system produces catecholamines such as adrenaline, both of which regulate immunity and inflammation (Babb *et al.,* 2019). Acute stress can temporarily enhance immune surveillance, but chronic stress is often detrimental, promoting the secretion of pro-inflammatory cytokines including IL-6, TNF-α, and IL-1β (Liu *et al.*, 2025). Restraint stress is a widely used experimental model of chronic psychological stress, achieved by physically restricting animal movement. This model consistently activates the HPA axis, elevating glucocorticoids that suppress lymphocyte proliferation and alter cytokine patterns (Zefferino *et al.*, 2023). Interestingly, while restraint stress generally dampens immune activity, it can sometimes enhance resistance to infections via glucocorticoid-mediated anti-inflammatory pathways (Kiecolt-Glaser *et al.*, 2018). In other contexts, such as IL-10-deficient models, restraint stress worsens intestinal inflammation, implicating autoimmune and bowel diseases. Additionally, it has been shown to induce oxidative stress and inflammatory cell infiltration in gastric tissues, underscoring its broad systemic effects (Gue *et al.*, 2018). The study aims to assess the plasma inflammatory markers level after peripheral pain stimulation in female Wistar rats subjected to restraint stress.

2.0 Materials and Methods

2.1 Study Area

The study was carried out at the animal laboratory house around Ladoke Akintola University of Technology, located at Ogbomoso North Local Government, Ogbomoso, Oyo State, Nigeria.

2.2 Study design

For the assessment of plasma inflammatory markers' level in female Wistar rats subjected to peripheral pain stimulation and restraint stress, a total of eighteen (18) pathogen-free female Wistar rats with an average weight of 180 – 220 grams were used for the study after being purchase from a commercial rat breeder in Ogbomoso Oyo State, Nigeria. They were grouped into 3 different groups, with each group containing six (6) rats, and were kept at the Animal Laboratory House in Ogbomoso, Oyo State, Nigeria. The acclimatization of the rats was for two weeks at the study site before the commencement of the experimental procedure, and they were kept under standardized animal house conditions.

2.3 Animal Dieting and Housing

The study rats were fed with standardized and substantial feeds (Breed Well-Grower pellet) and were allowed free access to feeds and water *ad libitum* throughout the acclimatization and experimental period. They were kept in a well-aerated plastic cage and wire gauze while wood shavings were used for their bedding, and daily routines were observed, with changing of beddings, feeding the rats, changing of drinkers, and cleaning of their environments, with proper handling of the animals ensured.

2.4 Grouping

The group designations are: I. = Control (CTL), II. = Peripheral Pain Alone (PPN), III= Restraint Stress + Pain (RPN). The body weights of all the rats in all the groups were monitored daily using a digital weighing balance and were recorded in grams.

The table below shows how the study rats are grouped into different cages;

Table 2.1 Animal groupings and their specific subjections:

GROUPS (n= 6)	SUBJECTIONS
Control (CTL)	Animals were fed animal feed and water *ad libitum*
Peripheral Pain Alone (PPN)	This group were subjected to peripheral pain stimulation, which was induced firstly, by the hot water tail-flick model followed by the formalin-induced paw licking pain model.
Restraint stress + pain (RPN)	Rats were subjected to restraint stress using a wire mesh for 30 minutes daily for twenty-eight (28) days. Twenty-four hours after the last restraint stress induction, peripheral pain stimulation was induced using the hot water tail-flick model firstly and then the formalin-induced paw licking pain model.

2.5 Restraint Stress Induction

Restraint stress was induced according to the method described by Owolabi *et al.* (2024). The animals were subjected to daily restraint stress sessions using a wire mesh for 30 minutes daily for 28 days. Each rat was kept in a wire mesh which restricted their movement without causing physical harm, and was gently placed in a prone position such that the rats had free access to air and were not pained.

2.6 Administration

The rats in the peripheral pain group and Restraint Stress + Pain group were subjected to both restraint stress and peripheral pain stimulation.

2.7 Method of pain assessment

Both thermal and chemical methods of pain assessment were employed;

2.7.1 Thermal Method of Pain Assessment

The thermal method of pain assessment, also called the tail immersion latency test, was carried out according to the modified method described by Chopra *et al.* (2010). The tail of the rat was immersed in a hot water bath (55°C ± 5 °C) until tail withdrawal (flicking response) or signs of struggle were observed. Shortening of the tail-withdrawal time indicates hyperalgesia (Umar *et al.*, 2015). The steps involved are;

- Hot water of a constant temperature between 50–60°C was prepared in a water bath.
- The rat was restrained in such a way that the head of the rat was facing superior while the tail was facing downward.
- About ¾ of the rat tail was dipped into the hot water, and the time taken to flick the tail off the hot water was recorded in seconds.

2.7.2 Chemical Method of Pain Assessment

The Chemical method of pain assessment, also called the formalin-induced paw licking pain model. This test permits monitoring pain-related responses such as itching, paw-licking, etc. caused by subcutaneous injection of an inflammatory agent, such as 2.5% formalin solution, into the left hind paw as described by Lopez-Cano *et al.*, (2017). After the injection, two distinct periods or phases of licking behavior occur, which are separated by a quiescent period. The steps involved are;

- The rat was gently restrained with the hind limb well-exposed.
- 0.02 ml of 2.5% formalin was injected subcutaneously into the plantar aspect of the left hind paw.
- The rat was gently placed in a cage in a serene environment to study the response, i.e., paw-licking over a total of one hour, which was divided into two phases; the first phase lasted for 15 minutes, then another 15 minutes rest, and the second phase lasted for 30 minutes.

2.8 Procedure for Sacrifice

Immediately after the pain procedures, the chemical method (chloroform) of sacrificing experimental animals was employed to sacrifice the rats. The procedure followed that of Edem *et al.,* (2022). The following method was used to anesthetize every rat in the experimental groups: each rat was allowed to inhale chloroform from a chloroform-soaked cotton wool in a desiccator until it was completely unconscious within 1--2 minutes.

2.8.1 Sample Collection

Blood samples were collected through a direct cardiac puncture into EDTA bottles. Plasma from the blood was used for biochemical analysis.

3.8.2 Histological Procedure

The control and induced left hind paws were removed, weighed by a digital weighing balance (recorded in grams) and then processed for further histological analysis. The histology sample was preserved with 2.5% formalin. The method used for the histological analysis are hematoxylin and eosin staining techniques

2.8.3 Biochemical Assay

The parameters measured in the investigation of the plasma inflammatory markers' level in rats included: Plasma nitric oxide, plasma interleukin-6, plasma TNF-Alpha, and plasma myeloperoxidase using rat-specific enzyme-linked immunosorbent assay (ELISA) KIT.

2.9 Analysis of Statistical Data

The study's numerical data were represented as mean ± Standard Error of the mean (SEM). SPSS (version 16.0) was used for Statistical Analysis. One-Way Analysis of Variance (ANOVA) was used to compare between groups of three followed by Duncan's posthoc multiple comparison test and paired t-test was used to compare between two groups. $p< 0.05$ was considered significant in both categories.

3.0 Results

Table 3.1 (a) Effect of Peripheral pain stimulation and Acute Restraint Stress on the relative paw weight of female Wistar rats. (b) Effect of acute restraint stress on the hot water immersion tail flick model in female Wistar rats. (c) Effect of peripheral pain stimulation and Acute Restraint stress on plasma Nitric Oxide (NO) and Myeloperoxidase (MPO) in female Wistar rats.

GROUPS	CTL	PPN	RPN
Relative Paw weights (%)	0.52±0.02[a]	0.65±0.03[b]	0.56±0.03[ab]

a

GROUPS	PPN	RPN
Hot water immersion tail flick response (seconds)	3.02±0.24[a]	3.91±0.35[a]

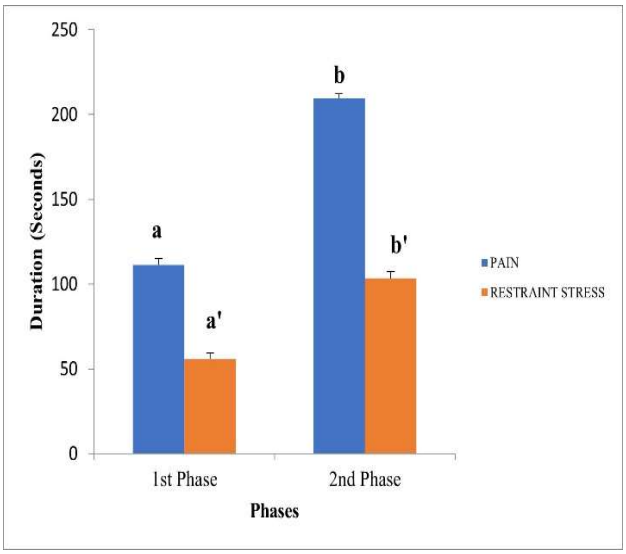
b

GROUPS	CTL	PPN	RPN
Plasma Nitric Oxide (NO) (µmol/ml)	12.86 ± 1.05[a]	18.49 ± 1.26[b]	20.16 ± 1.18[b]
Plasma Myeloperoxidase (MPO) (µmol/ml)	13.67 ± 1.11[a]	24.32 ± 1.65[b]	26.32 ± 1.48[b]

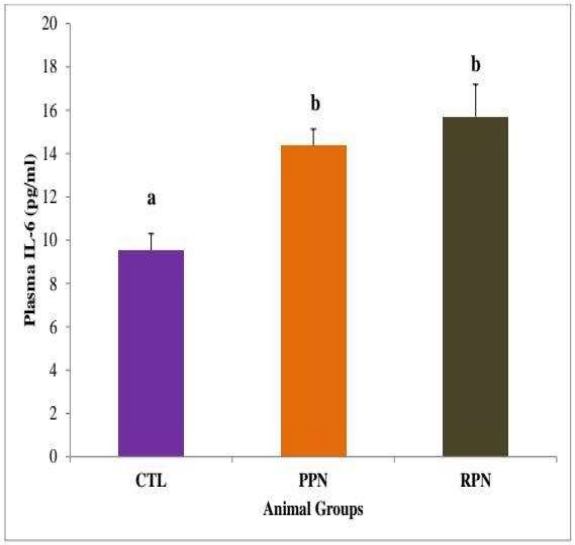
c

(a) The relative paw weight showed there was a significant ($p<0.05$) increase in the PPN group when compared to the CTL group. However, there was no significant difference in RPN when compared to both CTL and PPN groups independently. **(b)** The hot water tail immersion showed there was no statistically significant difference in PPN when compared to RPN group. **(c)** The plasma nitric oxide and myeloperoxidase both showed that there was a significant ($p<0.05$) increase in PPN and RPN groups when compared to CTL group individually. However, PPN group showed no statistical significance difference when compared to RPN group.

Figure 3.1 (a) Effect of acute restraint stress on pain score in Formalin-Induced model at first phase and second phase in female Wistar rats (b) Effect of Peripheral pain stimulation and Acute Restraint Stress on the plasma interleukin-6 of female Wistar rats (c) Effect of Peripheral pain stimulation and Acute Restraint Stress on the plasma Tumor Necrosis Factor-Alpha (TNF-α) of female Wistar rats (d) Effect of Peripheral Pain Stimulation and Restraint Stress on the induced Paw Histology of Female Wistar Rats.



a



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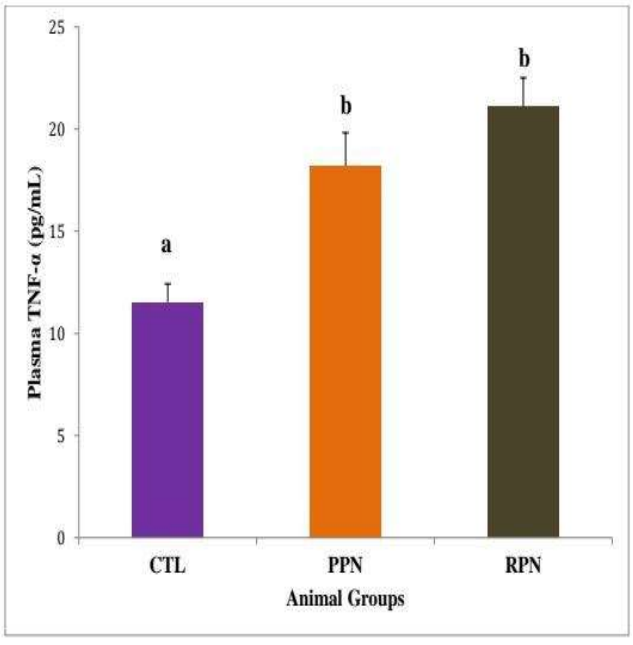

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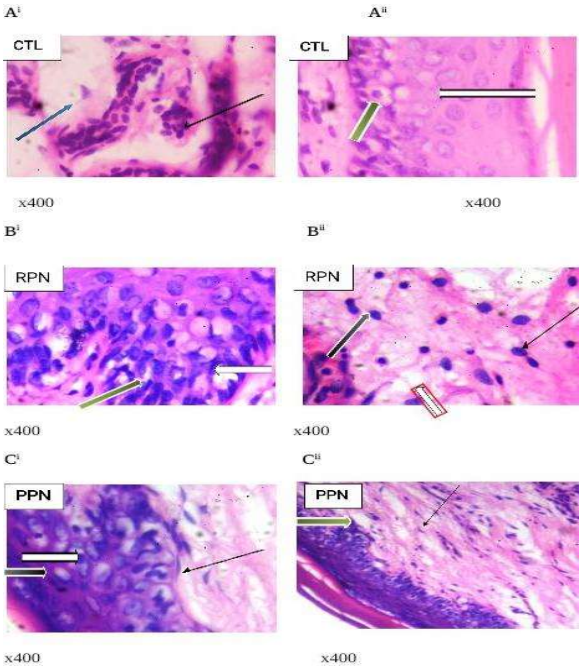

d

(a) The pain score in formalin-induced model shows there was a significant ($p<0.05$) increase in pain threshold of PPN when compare to RPN group during the first phase (15minutes). Also, there is significant ($p<0.05$) increase in pain threshold of PPN when compared to RPN during the second phase (30minutes). Furthermore, there is significant ($p<0.05$) increase in pain threshold in second phase when compared to first phase. **(b)** The plasma interleukin-6 result showed there was a significant ($p<0.05$) increase in the plasma interleukin-6 of PPN and RPN groups when compared to CTL individually. However, PPN level showed no statistical significance when compared to RPN group. **(c)** The plasma TNF-α result showed there was a significant ($p<0.05$) increase in PPN and RPN groups when compared to CTL individually. However, PPN level showed no statistical significance when compared to RPN**. (d)** Plate A showed normal histological structures, Plate B showed the epidermal layer (white arrow) with sub-epidermal infiltration of inflammatory cells (green arrow) and hyperkeratinization (black arrow)., The dermal layer (white arrow with red outline) shows mildly infiltrating inflammatory cells admixed with fat lobules (slender arrow). Plate C showed the epidermal layer (white arrow) with vacuolation (green arrow) seen and hyperkeratinization (black arrow) with mildly infiltrating inflammatory cells admixed fat layer (slender arrow).

4.0 Discussion

Inflammation is a critical immune response to injury, infection, or harmful stimuli, aiming to eliminate threats and initiate healing. Pain is a multifaceted physiological and protective mechanism that alerts the body to actual or potential harm through the activation of nociceptors by mechanical, thermal, or chemical stimuli. Stress is a psychological and physiological response to perceived challenges or threats, known as stressors. It can be acute (short-term) or restraint (long-term), and its effect can influence mental, emotional, and physical well-being. The study aimed to assess the plasma inflammatory markers level after peripheral pain stimulation in female Wistar rats subjected to restraint stress.

The results in Table 3.1 **(a)** showed a significant increase in relative paw weight in the Peripheral pain group when compared to the control, possibly reflecting localized edema from nociceptive activation. This aligns with Silva and Campinho (2023), who found that peripheral pain stimulation in female Wistar rats increased paw edema and inflammatory cell infiltration. Also, Zhang *et al.* (2022), reported that carrageenan or formalin-induced inflammatory pain elevated paw weight and volume. Furthermore, the combined restraint stress and peripheral pain group showed an intermediate change in paw weight, not significantly different from either the peripheral pain group or control group. This might be a reflection of a dual phenomenon, stress-enhanced central nociception alongside glucocorticoid-mediated suppression of peripheral edema. A study by Butler and Finn (2018) documented that restraint stress dampens inflammatory paw responses. Another study by Liu *et al.* (2020) also reported that restraint stress partially attenuates the peripheral inflammatory response to pain, probably due to the immunosuppressive effect of stress-induced glucocorticoid (via HPA axis activation). Thus, this suggests that while pain alone elicits an increase in physiological response, the presence of concurrent stress may blunt or modify this effect, highlighting the complex interaction between stress and pain pathways, emphasizing that stress does not uniformly exacerbate pain but can shift its expression from peripheral inflammation to central sensitization.

In Table 3.1 **(b)** there was no significant difference in tail flick latency of combined restraint stress and pain group when compared to peripheral pain only group. This may be due to stimulus intensity applied, tail immersion length, or sex-related coping differences. This correlates with a recent study by Sadler *et al.* (2022), which shows that acute restraint stress elicits physiological stress responses without altering immediate nociceptive threshold in male rats while longer-term or repeated stress shows sex-dependent effects with females often showing no significant modulation of thermal pain thresholds. With respect to Table 3.1 **(c)**; NO, a potent signaling molecule, regulates vasodilation and nociception and is typically elevated in response to painful and inflammatory stimuli (Förstermann and Sessa, 2022). The present result showed significantly increased plasma NO levels in both the peripheral pain and combined restraint stress with pain groups when compared to control group. This rise likely reflects formalin-induced activation of peripheral nociceptors and inducible nitric oxide synthase (iNOS) expression. Al-Rikabi *et al.* (2021) similarly demonstrated heightened plasma and spinal cord NO following formalin administration, highlighting its role in sustained inflammatory pain and hyperalgesia while Espejo *et al.* (2022) reported increased NOS expression with repeated nociceptive stimulation, implicating central sensitization. MPO, a neutrophil-derived heme enzyme, contributes to oxidative stress via reactive oxygen species generation (Khan *et al.*, 2022). The elevated plasma MPO in both the peripheral pain as well as combined restraint stress and pain groups may result from immune cell activation due to tissue injury caused by formalin, aligning with findings by Ahmed *et al.* (2022), who observed increased MPO following acute restraint stress. This reflects a compounded response involving both nociceptive and psychological

stress components. Together, the elevations in NO and MPO highlight integrated neuroimmune responses to nociception and stress. However, no significant differences were observed between the peripheral pain group and combined restraint stress and pain group, suggesting a plateau effect. Liu *et al.* (2020) and Zang *et al.* (2024) also noted that combining stress with pain did not further increase NO or MPO, possibly due to feedback regulation or ceiling effects. Similarly, Sotiropoulos *et al.* (2022), also proposed that acute stress modulates immune responses without amplifying neutrophil enzymes in early nociceptive phases.

The formalin-induced pain model is a biphasic test for assessing acute and persistent pain in rodents. The first phase (0–15 minutes) is neurogenic, driven by direct nociceptor activation, while the second phase (15–30 minutes) is inflammatory, involving central sensitization and mediator release (Kim *et al.*, 2023). Figure 3.1 **(a)** showed that restraint stress combined with peripheral pain reduced pain threshold across both phases, suggesting stress-induced hyperalgesia (SIH) which can activate the hypothalamic-pituitary-adrenal axis (HPA) and sympathoadrenal system (SAM), leading to the release of glucocorticoids such as cortisol. Alvarenga *et al.* (2022), similarly reported restraint stress elevates corticosterone and amplifies acute nociception. Also, Zang *et al.* (2022), reported that restraint stress exaggerates inflammatory pain responses during the second phase due to spinal glial activation and increased cytokine production (IL-1β, TNF-α). Furthermore, Park *et al.* (2020), also reported a reduced nociceptive behavior (higher threshold) in the second phase due to internal adaptation, even in the presence of stress. The increase in peripheral pain in both phases may suggest that restraint stress exacerbates both acute and inflammatory pain, while the absence of stress allows for adaptive or protective mechanisms to raise the pain threshold over time. With respect to Figure 3.1 **(b and c);** IL-6 and TNF-α are well-known pro-inflammatory cytokines involved in systemic inflammation and pain sensitization. IL-6 is usually the first to rise in response to tissue damage or stress, while TNF-α mediates both acute and chronic inflammation (Xie *et al.*, 2023; Han *et al.*, 2023). Both markers were elevated in the peripheral pain and combined Restraint stress and pain groups when compared to control group individually. The observed increase suggests activation of peripheral nociceptors and immune cells in response to pain-induced tissue injury. This aligns with Zhou *et al.* (2021), who reported increased IL-6 after formalin injection, and Cui *et al.* (2021), who linked TNF-α to pain-induced neuroinflammation. Furthermore, a study by Wang *et al.* (2022) also confirmed IL-6 upregulation in tail-flick models while Zhang *et al.* (2023) linked TNF-α rise mainly to peripheral pain stimuli. The elevated cytokine levels may result from the chemical nature of the painful stimuli applied (formalin-induced), which is known to provoke local inflammation and systemic cytokine release. However, there was no significant difference is observed in peripheral pain group when compared to combined Restraint stress and pain group, supporting the idea that restraint stress and pain activate overlapping immune pathways. Also, Kim *et al.* (2024) similarly suggested that once cytokine expression is maximally activated by pain, additional stress does not further elevate circulating IL-6 or TNF-α. reflecting a shared ceiling effect in the body's inflammatory response. Overall, these findings demonstrated that IL-6, and TNF-α are consistently elevated by nociceptive stimulation and stress, but their responses plateau when both stimuli coexist, indicating convergent but not additive neuroimmune mechanisms.

The histoarchitecture findings revealed that both the restraint stress plus pain group and the peripheral pain group exhibited marked hyperkeratinization with sub-epidermal inflammatory cell infiltration in the epidermis. The dermal layer also showed mild inflammatory infiltrates among fat lobules, suggesting localized yet systemic inflammation triggered by combined stress and nociceptive input. A recent study by Sotiropoulos *et al.* (2022), reported that restraint stress amplifies immune responses, increasing cutaneous inflammatory cells even without external injury. Similarly, the peripheral pain group displayed epidermal hyperkeratinization with vacuolation and mild inflammatory infiltration in subcutaneous fat, though less pronounced than in the combined group. These findings indicate irritation or barrier dysfunction due to nociceptive stimuli alone, aligning with Yoon *et al.* (2021), who linked pain-induced oxidative stress to structural skin alterations.

Conclusion

Conclusively, this study highlights the impact of combined peripheral pain and restraint stress on systemic inflammation in female Wistar rats. Elevated plasma levels of nitric oxide (NO), myeloperoxidase (MPO), interleukin-6 (IL-6), and tumor necrosis factor-alpha (TNF-α) indicate heightened inflammatory responses. Pain models like tail-flick latency and formalin-induced pain confirmed both neurogenic and inflammatory contributions to pain. Histological findings also revealed stress-induced tissue changes. These results suggest that psychological stress does not intensify pain-related inflammation.

Conflict of interest

The authors declare that there is no conflict of interest regarding the publication of this article.

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