# Assessment of Pain Perception in Female Wistar Rats Subjected to Acute Restraint Stress

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## Abstract

Pain perception is the interpretation of nociceptive input by the cerebral cortex. Pain perception is influenced by various factors, including stress. Acute exposure to stress produces marked alterations in the behavioral patterns, neurochemistry, physiological functions of animals and modulate nociceptive responses, producing either analgesia or hyperalgesia. However, its effects in female rats remain under-investigated. This study aimed to assess the effect of acute restraint stress on pain perception in Female Wistar Rats. Eighteen (18) female Wistar rats weighing between 180-220g were randomly divided into three groups with six (6) rats in each group: Group I – Control (CTL); Group II – Pain alone (PAL) and Group III - Restrain stress + Pain (RSP). Restraint stress was induced in Group III by keeping the rats in a mesh-wire restrainer, thirty minutes daily for twenty-eight days. Twenty-four (24) hours after the last restraint stress procedure, local pain was induced in Group II and III rats using tail flick immersion test and formalin induced paw licking model. Thereafter, animals in all groups were sacrificed, the control and induced hind paw were removed, weighed, homogenized and analyzed for Nitric oxide, Serotonin, Prostaglandin $E_2$ and C-reactive protein levels. Histological examination of these control and induced paws were carried out using H&E staining.The result showed that the relative paw weight of the PAL group was significantly ($p<0.05$) increased when compared to that of the CTL. There were no significant differences on tail flick latency RSP when compared with PAL group. The pain score in PAL group shows a significant ($p<0.05$) increase when compared to RSP group at both phases. Paw homogenate Prostaglandin $E_2$ and C-reactive protein levels increased significantly ($p<0.05$) in the RSP and PAL group when compared to CTL group individually. However, there was a further increase in Prostaglandin $E_2$ and C-reactive protein levels in RSP group when compared to PAL groups. Nitric oxide level was significantly ($p<0.05$) increased in both RSP and PAL group when compared to CTL group individually. However, there was no significant ($p<0.05$) difference in Nitric oxide level in RSP group when compared to PAL group. Serotonin level was significantly ($p<0.05$) reduced in the RSP and PAL group when compared to the CTL group individually. However, there was no significant ($p<0.05$) difference in Serotonin level in RSP group when compared to PAL group. In conclusion, this study has shown that exposure to restraint stress may significantly affect pain perception by inducing biochemical and histological changes affecting both pain threshold and behavior. This finding also demonstrates the importance of considering stress as a modulating factor in pain research.

**Keywords**: Restraint, Pain, Formalin, Serotonin and C-Reactive.

## 1.0 Introduction

The International Association for the Study of Pain (IASP) adopted the new definition of pain as 'an unpleasant sensory and emotional experience associated with, or resembling that associated with, actual or potential tissue damage (IASP, 2020). A plethora of stimuli, such as heat, inflammation, necrosis, spasm, to name a few, are responsible for the initiation of pain (Wang *et al*., 2023). Numerous chemical mediators that express painful conditions have been identified (Cosman *et al.,* 2014). Fatigue, anxiety and pain are interrelated to each other, increased level of fatigue and anxiety is also responsible for increased perception of pain (Cirillo, 2024). Pain perception involves current stimulation in peripheral nociceptive nerves and the subsequent stimulation of postsynaptic excitatory neurons in the spinal cord (Smith, 2023). Repeated or persistent noxious stimulation results in an enhanced responsiveness, lowered threshold and the development of background activity, known as peripheral sensitization (Raja *et al*., 2020). Clinically it manifests as pain hypersensitivity. This peripheral sensitization is linked to the release of inflammatory mediators such as chemokines/cytokines as well as neuropeptides (such as CGRP and substance P) (Spekker *et al.,* 2021). Stress refers to a series of physiological and behavioral changes that occur in an individual during the process of experiencing negative emotions (Xu *et al*., 2020). Individuals strive to adapt by manipulating the environment to alter the impact caused by the source of stress (Erdem *et al*., 2025). Stress modulates pain perception, resulting in either

stress-induced analgesia or stress-induced hyperalgesia, as reported in both animal and human studies (Zhang *et al*., 2024). The responses to stress include neural, endocrine, and behavioural changes, and built-in coping strategies are in place to address stressors (Bovis, 2024). Peculiar to humans are additional factors that modulate pain that are experienced in times of stress, notably psychological factors that potentially influence the directionality of pain perception (Abudoush, 2023). Restraint stress is a widely used stressor in animals including rodents (Olave *et al.,* 2022). This stress does not involve physical pain but represents a psychologically aversive inescapable environment, also restricting movement (McCarty, 2016). Another aspect of restraint stress is the exposure to a novel environment, such as small individual cages (Molina *et al.,* 2023). Restraint stress also results in anorexia and weight loss in rodents (Scanes *et al.,* 2024). Restraint stress (RS) induces anxiety-like behavior in both mice and rats, with a dysfunction of the amygdala (Mahony *et al.,* 2022). In rodents, the effects of a single session of restraint, lasting from one to three hours, elicited variable effects in different laboratories, ranging from analgesia. no effect, and hyperalgesia (Oliver *et al.,* 2023). Commonly, mice or rats are placed in the restrainer once a day for a period of 1 to 6 h, for 7 to 40 days (Pola *et al.,* 2024). The chronic restraint stress leads to mechanical and thermal allodynia and to thermal hyperalgesia (Eren-Koçak *et al*., 2021). That can be measured using the von Frey filaments, the hot plate and tail flick/withdrawal assays, and the paw withdrawal to cold (Avonts, 2024).

# 2.0 Materials and Methods

### 2.1 Materials used

Plastic Cages, Wood Shavings, Weighing Scale, Needle and Syringe (2ml and 5ml), Insulin Syringe, Plain Bottles, Cotton Wool, Marker, Drinker and Feeders, Wire Mesh Restrainer, Distilled Water, Disposable Gloves, Methylated Spirit, Laboratory Coat, Dissecting Sets, Face Mask, Detergent, Sterile, Razor Blade, Centrifuge Machine, Thermometer, Water Bath, Stop Watch, Ceramic Mortar and Pestle.

### 2.2 Chemicals

Chloroform (Sigma, Germany), 2.5% Formalin (Sigma, Germany), Phosphate buffer, Normal saline.

### 2.4 Study Design

For the assessment of pain perception in rats subjected to restraint stress, eighteen (18) female Wistar rats weighing between 180-220 g were acquired from a commercial sales breeder, in Ogbomoso, Oyo state, Nigeria. All the animals were housed in well-aerated plastic cages and wire gauze while wood shavings were used as bedding to keep each compartment dry. They were allowed free access to standard pelletized feed and water *ad libitum* in a room under natural light. The animals were allowed to acclimatize for two weeks so as to adapt to their new environment.

### 2.5 Animal Grouping

Eighteen (18) female Wistar rats were randomly divided into three groups with six (6) rats in each group. The animals were designated thus: Group I control (CTL), Group II pain alone (PAL) and Group III restrain stress + pain (RSP).

**Table 2.1: Animal grouping and their procedures**

| Groups | Procedures |
|---|---|
| Control (CTL) | Rats were fed with animal feed and water *ad libitum* |
| Pain alone (PAL) | Rats were fed with animal feed and water only for Twenty-Eight (28) days. Twenty-four hours (24) after the experimental period, local pain stimulation was conducted on PAL group rats using tail flick immersion test and formalin induced paw licking model. |
| Restraint stress plus pain (RSP) | Rats were subjected to restraint stress using wire mesh for 30 minutes for Twenty-Eight (28) days. Twenty-four (24) hours after the restraint stress, local pain stimulation was conducted on RSP group rats using tail flick immersion test and formalin induced paw licking model. |

### 2.6 Restraint Stress Procedure

The rats were subjected to daily restraint stress session for 30 minutes daily for 28 days according to the method described by Owolabi *et al*. (2024). The restraint stress was applied by placing the rats individually inside a 25cm by 7cm wire mesh and the end intertwined; this restricts their movement without causing physical harm.

### 2.7 Pain Stimulation Method

#### 2.7.1 Thermal Method of Pain stimulation (Hot water tail immersion test)

The tail immersion test was carried out according to the method described by Xu *et al*. (2013). Each animal in group II and III were gently hand-held with the lower 2/3 of the tail immersed in hot water maintained at a constant temperature of 55±0.5°C. The time taken for the animal to flick its tail out of water (tail withdrawal latency) was measured in and recorded in seconds. Changes in nociception were determined by the changes in the latency between the tail immersion and withdrawal from the hot water bath.

### 2.7.2 Chemical Method of Pain stimulation

20µL of 2.5% Formalin was used to induce neuropathic pain as described by Eto et al. (2024). The rats in group II and III were each injected with 0.02 ml of 2.5% v/v formalin solution subcutaneously under the surface of the left hind paw. The amount of time the animals spent licking the injected paw was recorded for two distinct periods, results are shown as the total time spent on licking in each phase. Phase 1 was defined as the period of time beginning immediately after the formalin injection and lasting for 15 min. Phase 2 was defined as beginning 20 min post-formalin injection and lasting until 30 min post-injection. Licking behaviors during each phase are presented as the sum of the total seconds spent on licking during that phase.

### 2.8 Animal Sacrifice and Tissue Processing

### 2.8.1 Animal Sacrifice

Immediately after the last pain assessment animals were anesthetized and sacrificed, the chemical method (chloroform) of sacrificing experimental animals was used in sacrificing the rats according to the method of Di lanni *et al*. (2024). The rats were placed in a desiccator with a cotton wool soaked in chloroform on the average of 2ml for about 1-2 minutes.

### 2.8.2 Sample Collection

The left hind paw of the rats of all groups were collected and weighed (recorded in grams).

### 2.8.3 Homogenization

The paw was homogenized in phosphate buffer (10% w/v, 0.1MPBS, PH= 7.4) using ceramic mortar and pestle which was placed on ice block to maintain the integrity of the pasw tissue. The homogenates were centrifuged at 4,000 rpm for 3 minutes at 4$^{o}$C. The supernatant was then processed for biochemical analysis.

### 2.8.4 Histological Analysis

The left hind paw of the rats of all groups were removed and weighed (recorded in grams) and then processed for further histological examination. The histology sample was preserved with 2.5% formalin paw tissues. The method use for the histological analysis are hematoxylin and Eosin (H&E) staining

### 2.8.5 Biochemical Assay

The serotonin, prostaglandin, C-Reactive protein, and nitric oxide activity in the paw homogenate was determined with rat-specific Enzyme Linked Immunosorbent Assay (ELISA) kit.

### 2.9 Statistical Analysis

The results were represented as Mean ± Standard Error of the Mean (SEM). Data was analyzed using SPSS (version 16.0) for statistical analysis. One way analysis of variance (ANOVA) to assess the differences between groups, followed by paired T-test. $p< 0.05$ was considered significant.

# 3.0 Results

### 3.1 Effects of Pain stimulation and Acute Restraint stress on Relative Paw weight of female Wistar Rats

Table 3.1 show that there was a statistically significant ($p< 0.05$) increase in relative paw weight in the PAL group when compared to the CTL group. There was no significant difference in RSP group when compared to both CTL and PAL groups.

| **Groups** | CTL | PAL | RSP |
|---|---|---|---|
| **Relative Paw Weight (%)** | $0.52\pm0.02^{a}$ | $0.65\pm0.03^{b}$ | $0.56\pm0.03^{ab}$ |

### 3.2 Effects of Acute Restraint Stress on Hot Water Immersion Tail Flick Model in Female Wistar Rats

Table 3.2 show that there was no statistically significant ($p< 0.05$) increase in reaction time of RSP group when compared to the PAL group.

| **Groups** | PAL | RSP |
|---|---|---|
| **Reaction time (secs)** | $3.02\pm0.24^{a}$ | $3.91\pm0.035^{a}$ |

### 3.3 Effects of acute restraint stress on pain score in formalin induced pain test at first phase and second phase in Female Wistar Rats

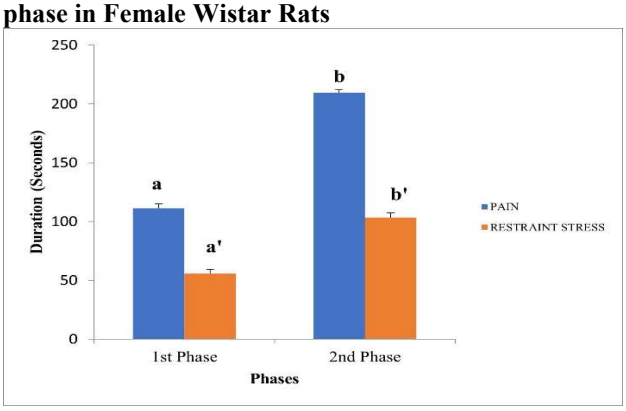



**Fig 3.1 Effects of acute restraint stress on pain score in formalin induced pain test in female Wistar rat**

The pain score in formalin induced pain test in Figure 3.1 showed a significant ($p< 0.05$) increase in pain score of PAL group when compared to RSP group at both first phase and second phase. Also, there was significant ($p<0.05$) increase in pain score of the second phase when compared to the first phase.

### 3.4 Effects of acute restraint stress on Pain Mediators in paw tissue of Female Wistar rats subjected to Formalin induced pain model

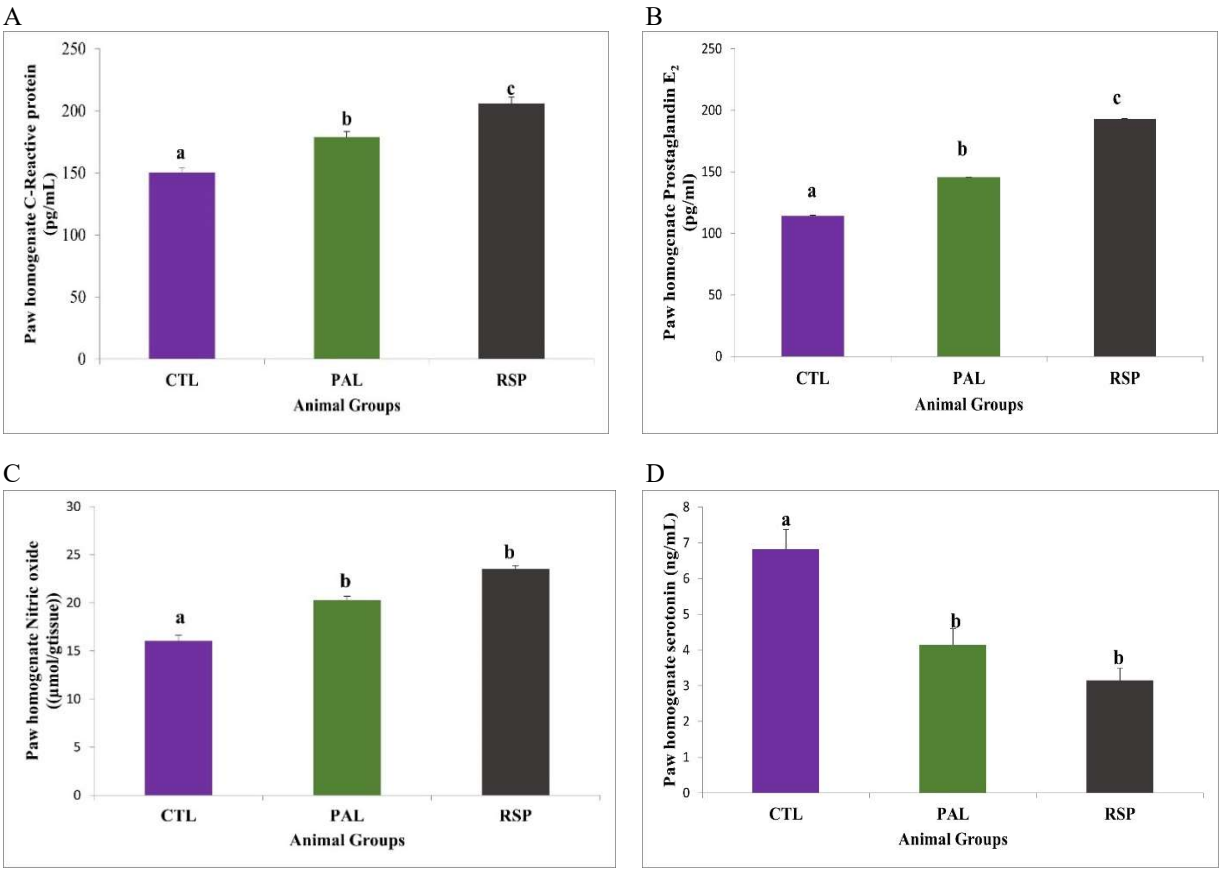



**Fig 3.1 Effects of acute restraint stress on (A) C-Reactive Protein (B) Prostaglandin (C) Nitric oxide (D) Serotonin activity in paw tissue of Female Wistar rats subjected to Formalin induced pain model.**

Examining the effects of acute restraint stress on Pain Mediators found in paw tissue of Female Wistar rats subjected to Formalin induced pain model, the result shows that there was a statistically significant ($p < 0.05$) increase in Prostaglandin $E_2$ level in RSP group and PAL group when compared to the CTL individually. However, there is further

increase in RSP group when compared to PAL group. Similarly, there was a statistically significant ($p < 0.05$) increase in CRP level in the RSP group and PAL group when compared to the CTL group individually. However, there was a further increase in RSP group when compared to PAL group. Furthermore, there was a statistically significant ($p < 0.05$) increase in Nitric oxide level in RSP group and PAL group when compared to the CTL group independently. While, there was no significant difference in RSP group when compared to PAL groups. Contrastly, there was a statistically significant ($p < 0.05$) decrease in Serotonin level in RSP group and PAL group when compared to the CTL group independently. However, is no significant difference in RSP group when compared to PAL groups.

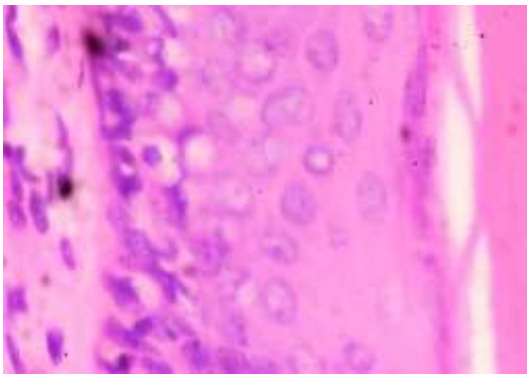
### 3.5 Effect of Acute Restraint Stress and local Pain stimulation on the Paw Histology of Female Wistar Rats

**Plate A:** A section of the paw of rat (Control)showing the epidermal layer appears intact and healthy, the dermal layer shows a normal structure with collagen fibre and intact articular cartilage with no evidence of inflammation (H&E 400).

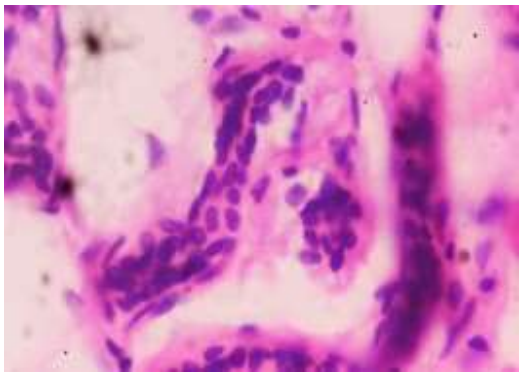
**Plate B:** A section of the paw of rat (Pain Alone) showing the epidermal layer with subepidermal vacuolation seen and hyper keratinization. The dermal layer shows normal collagen with mildly infiltrating inflammatory cells admixed fat layer (H&E 400).

**Plate C:** A section of the paw of rat (Restraint Stress plus Pain) showing the epidermal layer with sub epidermal infiltration of inflammatory cells and hyper keratinization. The dermal layer shows mildly infiltrating inflammatory cells admixed fat lobules (H&E 400).

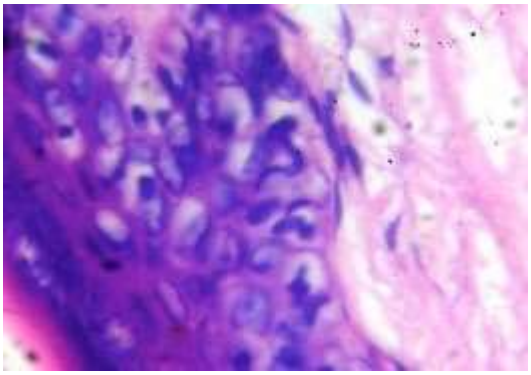
**$A^{I}$**



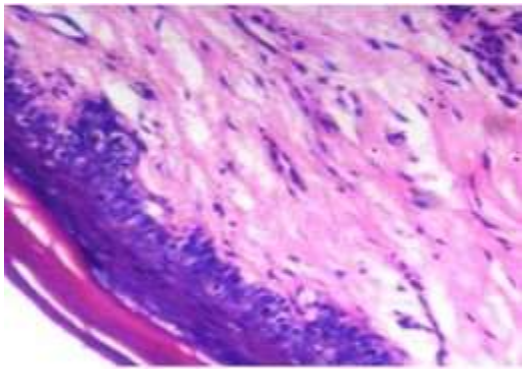
**$A^{II}$**



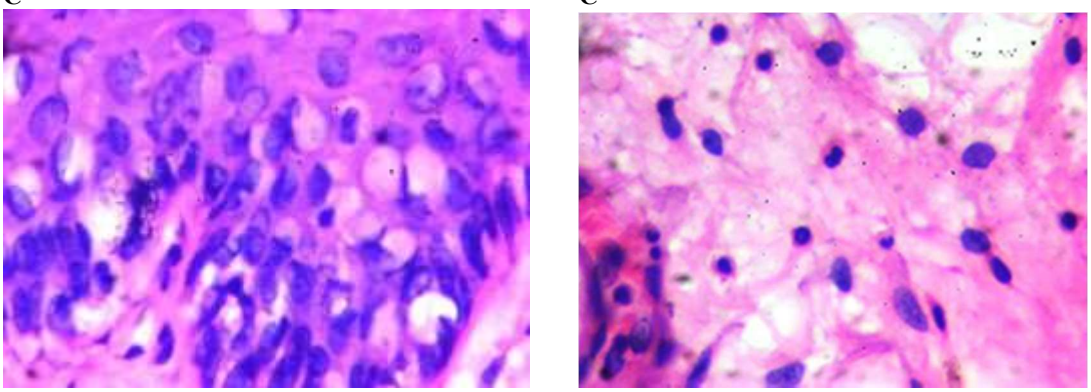
**$B^{I}$**



**$B^{II}$**



$C^{I}$ $C^{II}$



**4.0 Discussion**

Stress and pain are two distinct yet overlapping processes that share several conceptual and physiological similarities (Abudoush, 2023). Exacerbation of pain by stress, and co-morbidity of pain with stress-related psychiatric disorders including anxiety and depression represent very significant clinical challenges (Aboushaar ·*et al.,* 2024). So far, stress effects on pain perception were evaluated mainly with pain threshold/tolerance, static measurements that are considered highly variable between as well as within subjects (Makovac *et al.,* 2020). Because pain perception is dynamic, and influenced by inhibitory and excitatory mechanisms, it is valuable to study stress effects also using "dynamic measurements" that evaluate traits of central pain modulation (Merces *et al*. 2024). This study aimed to assess pain perception in female Wistar rats subjected to acute restraint stress.

The significant increase in relative paw weight shown in the pain group when compared to control group suggest that pain has a more profound inflammatory effect particularly when induced by tissue damage or inflammation which often leads to increased blood flow and fluid accumulation in the affected area, resulting to swelling and an increase in paw weight. This aligns with a recent study by Elgendy *et al*. (2025) that examined the effects of formalin injection in rats and found that it rapidly induces peripheral inflammation, evidenced by paw edema (swelling) within 5 minutes and confirmed statistically significant increases in Paw size measurements compared to controls rats. However, the moderate increase in paw weight seen in combined restraint stress and pain group when compared to pain alone and control groups independently may be attributed to activation of the hypothalamic-pituitary-adrenal (HPA) axis which suppresses the inflammatory cascade thereby leading to less fluid accumulation and swelling which is responsible for the moderate increase. This supports the findings of Herman *et al*. (2020), that identified restraint stress as a well-established activator of the hypothalamic-pituitary-adrenal (HPA) axis, leading to increased release of glucocorticoids like corticosterone which suppresses the peripheral inflammatory cascade.

The tail-flick test is a widely used method to evaluate nociception in rodents, particularly in thermal pain models. In this test, noxious heat is applied to the tail, and the latency period before the animal withdraws its tail is recorded as a measure of pain sensitivity (Barrot, 2012). The test shows that the combined restraint stress and pain group showed no significant ($P<0.05$) difference in reaction time compared to the pain group, indicating a ceiling effect which explains that the combined restraint stress plus pain group may already be under acute *stress induced analgesia*, so adding pain doesn't elevate latency further. This concur with the study of Sadler *et al*. (2022) that *acute* restraint stress alone is sufficient to induce analgesia supporting the idea that if pain is introduced afterward, there may be no further increase in latency because the *stress induced analgesia* is already activated.

The formalin-induced pain model is widely used to assess pain behaviors triggered by moderately intense and persistent noxious stimuli (Jalali *et al*., 2024). The result in figure 4.1 showed the pain response curve induced by 2.5% formalin was biphasic, having 2 peaks, from 0 to 5 min (first phase) and 30 min (second phase). While the first phase involves pain and is not primarily driven by an inflammatory response, the inflammatory reaction develops later in the second phase (Hoffmann *et al*. 2022). The pain group displayed a significant ($p<0.05$) increase in latency compared to the combined restraint stress and pain group in the first phase, this suggests that the reduced latency in the combined restraint stress and pain group is likely due to the stress-induced analgesia (SIA) phenomenon. The restraint stress, experienced before or during the formalin injection, activates endogenous pain-inhibitory mechanisms, raising the pain threshold and delaying the initial behavioral response to the formalin injection in the stressed group.

Stress, like that induced by restraint, can activate descending pain-modulatory pathways, releasing neurotransmitters like endorphins and enkephalins, which inhibit pain signaling. (Karcz *et al*., 2024). Similarly, the pain group displayed a higher latency compared to the combined restraint stress and pain group in the second phase. The delayed or reduced response in combined restraint stress plus pain group in the second phase suggest stress-induced analgesic effect which might be more pronounced during this inflammatory phase. This is likely mediated by the activation of the endogenous opioid system and spinal cord pathways involved in pain modulation during stress. (Tobaldini, *et al*., 2020).

Prostaglandin E2 (PGE2) is a major inflammatory mediator that lowers the activation threshold of pain receptors, contributing to both peripheral and central sensitization during formalin-induced pain (Buisseret *et al*., 2019). C-Reactive Protein (CRP) serves as a reliable marker of inflammation, helping to indicate the inflammatory response triggered by formalin injection, although it does not directly reflect pain intensity (Stanimirovic *et al*., 2022). Nitric oxide (NO) is a reactive signaling molecule involved in nociceptive transmission**,** vascular changes**,** and inflammation **(**Andrabi *et al*., 2023). In pain processing and modulation, Serotonin (5-HT) has both pain-enhancing (hyperalgesic) and pain-reducing (analgesic) effects depending on the receptor type and site of action, making it crucial in both peripheral and central pain modulation (Lee *et al*., 2021). The study further shows pain group showed a significant ($p<0.05$) increase in PGE2, CRP, NO level and decrease in Serotonin level when compared to control group confirming that formalin-induced nociception pain triggers prostaglandin production, release of inflammatory cytokines which signal the liver to produce CRP, activation of nitric oxide–mediated pathways and that inflammation associated with pain can lower serotonin levels through increased serotonin reuptake and reduced serotonin synthesis respectively. This increase is consistent with DeMarco *et al*. (2019) studies that formalin injection activates COX enzymes, leading to elevated PGE2 production in inflamed tissue. Friščić et al. (2025) demonstrated that formalin injection into rodent paws leads to a significant rise in local inflammatory markers, including CRP, through sustained cytokine activation. These cytokines stimulate hepatic and possibly local CRP synthesis at the site of injury (Poddar, 2023). These findings are also consistent with study of singh et al. (2019) that reported that formalin triggers both immediate and sustained activation of NO production in the spinal cord, contributing to nociceptive signaling which facilitate inflammatory signaling. This also align with recent study by Tokunaga *et al*., (2024) that reported that local depletion of serotonin may occur after sustained noxious stimulation, especially when serotonin turnover is not matched by synthesis. The combined restraint stress and pain group demonstrated a significant ($P<0.05$) increase in PGE2, CRP level, but significant($P<0.05$) difference NO and serotonin level when compared to Pain group. This result indicates that the combined effect of psychological stress on pain leads to a greater release of PGE2.and CRP. Strekalova *et al*. (2022) also demostrated that stress hormones released during restraint can directly influence the activity of COX enzymes, further contributing to increased PGE2 synthesis. Balakin et al. (2025) further confirmed that restraint stress augments peripheral inflammation by enhancing IL-6 and TNF-α production, which are key inducers of CRP synthesis. However, no significance difference in serotonin and NO level between the two groups suggest that acute restraint stress may not provide enough time to induce major alterations in peripheral nitric oxide synthesis. The study by Coelho *et al*. (2022*) also* demonstrated how acute restraint stress may preferentially alter central NOS activity without peripheral increases.

This study also assessed the histopathological effects of pain and combined effect of restraint stress and pain on skin tissue architecture to evaluate structural and inflammatory changes. Notably, according to plate 4.1, histoarchitecture of both experimental groups revealed shared features, including the presence of well-defined epidermal and dermal layers, hyperkeratinization, and mild infiltration of inflammatory cells within the dermis. Hyperkeratinization is widely recognized as an epidermal defense mechanism in response to chronic irritation or injury (Deo *et al*., 2018), while the dermal infiltration of inflammatory cells likely reflects the activation of local immune responses, a hallmark of cutaneous inflammation induced by pain stimuli (Lowy *et al*., 2021). These findings are consistent with inflammatory responses to nociceptive stimulation. However, the combined restraint stress and pain group displayed a major subepidermal infiltration of inflammatory cells indicating a stronger inflammatory response. This suggest that the combination of psychological stress and pain may activate both the hypothalamic pituitary adrenal (HPA) axis and sympathetic adrenal medullary (SAM) axis, which influence immune responses and disrupt skin homeostasis. Acute stress has been shown to enhance inflammatory cell migration and cytokine production in the skin, aggravating tissue damage and immune activation (Pondeljak *et al*., 2020). Meanwhile, the pain group exhibited subepidermal vacuolation, a sign of basal keratinocyte injury or intercellular fluid accumulation, commonly associated with cytotoxic effects or mild epidermal degeneration. Minimal vacuolar changes may reflect early-stage epidermal stress or damage without the compounding effects of systemic stress hormones (Affolter, 2023). These changes have been documented in acute pain or irritation models where mechaniical or thermal stimuli affect keratinocyte integrity

(Talagas and Misery, 2019). The absence of heavy inflammatory infiltrates in this group suggests that pain alone may initiate structural compromise at the epidermal level but not the strong immune cell infiltration seen with added stress (Maarouf *et al*., 2020). Furthermore, the dermis of the pain group retained normal collagen architecture, indicating the preservation of extracellular matrix (ECM) integrity. Collagen preservation in the presence of mild inflammation may suggest an acute or self-limiting response, typical of short-term nociceptive stimuli (Lee *et al*., 2002).

# Conclusion

Conclusively, this study provides comprehensive evidence of the combined effect of acute restraint stress and local peripheral pain stimulation on pain perception in female Wistar rats. Elevated paw level of prostaglandin E2 and C-Reactive Protein in the stressed group indicate intensified pain response. However, serotonin and nitric oxide didn't follow this trend suggesting that acute restraint stress showed minimal or no effect on serotonin and nitric oxide level. Histological findings revealed stress contribute to a stronger inflammatory response. Together, these results suggest that psychological stress can trigger reduction in pain sensitivity known as stress induced analgesia or amplify pain perception.

# Conflict of interest

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

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