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Assessing Inflammatory Pain in Animal Studies: An Overview of the Formalin Test

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ABSTRACT

Pain, a complex and debilitating experience, significantly impacts the quality of life. It is considered as a primary reason for seeking medical care. Inflammatory pain, resulting from tissue injury, is characterized by the release of inflammatory mediators that activate nociceptive pathways, leading to heightened sensitivity. Assessment of inflammatory pain is necessary for understanding its mechanism and developing effective treatment strategies. This study provides an overview of the formalin test, a widely used animal model for assessing inflammatory pain. The test comprises distinct phases (phase I, interphase, and phase II), which reflect acute and tonic pain responses. As a tool for evaluating analgesic efficacy and pain mechanisms, the formalin test has been instrumental in advancing our understanding of pain biology and the development of novel therapeutic approaches.

Keywords:

pain, inflammation, formalin, tonic pain.

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1. Introduction

Pain is a fundamental physiological function that serves as a vital warning system to alert individuals to actual or potential harms (1). This complex and multifaceted experience affects millions of people worldwide, resulting in a significant economic burden (2). Pain is not just a physical sensation; it also profoundly impacts an individual's psychological, social, and emotional well-being. It impairs daily activities and overall quality of life, leading to increased distress, anxiety, and disorientation (1,3,4).

Pain can result from various causes, including injury, disease, or inflammation. One specific type of pain is inflammatory pain, which occurs when tissues are subjected to chemical, mechanical, or thermal stimuli, leading to tissue damage. This damage triggers a cascade of pain and inflammation, prompting individuals to seek immediate relief and treatment for their symptoms (5). Acute inflammation plays an important role in protecting the body from infection and promoting tissue repair. However, when inflammation persists for a while, it becomes chronic, losing its beneficial effects and instead causing tissue damage and pain. Chronic inflammatory pain is often associated with various conditions, such as arthritis, tissue injury, or infection (6,7).

Investigating pain control and developing effective treatment strategies necessitate rigorous research in animal models. However, accurately measuring and assessing pain, particularly in animals, poses a significant challenge due to its subjective nature, encompassing both physical and emotional aspects (8). Despite these difficulties, multiple methodologies are available for assessing inflammatory pain in animal models. The formalin test is a widely used and notable method for evaluating inflammatory pain. It is characterized by its ability to distinguish between both acute and tonic phases of pain, as well as its sustained effects (9). The formalin test has been instrumental in advancing our understanding of the mechanisms of inflammatory pain and has provided a valuable means of evaluating the efficacy of analgesic interventions. This review aims to provide an in-depth assessment of the formalin test as a practical tool for assessing and evaluating inflammatory pain.

2. Pain: Definition and classification

According to the International Association for

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the Study of Pain (IASP), pain is defined as "an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage" (10). This complex process begins when tissue damage, chemical irritation, or abnormal immune responses activate nociceptors (11). These specialized primary sensory neurons have free nerve terminals that detect and respond to noxious or potentially damaging stimuli, converting these into electrical signals (12). The signals then converge at the spinal cord's dorsal horn, a critical gateway to the ascending pain pathway. From there, the signals are relayed through multiple parallel pathways to higher brain centers, including the brainstem, diencephalon, and potentially the cortex (13). Descending signals from these higher brain centers also modulate the pain response based on internal states, medical and drug history, and external stimuli. These processes ultimately shape the complex experience of pain (14–16).

Pain is traditionally categorized into two primary types, acute and chronic, based on its onset and duration (17). Acute pain is a self-limiting, adaptive response to tissue damage, which serves as a warning sign of an underlying medical condition or injury. It is typically linked to a specific cause, such as an injury or surgical trauma, and has a relatively brief duration. In contrast, chronic pain is marked by persistent and prolonged discomfort that lasts or recurs for more than three months, often coexisting with underlying medical conditions. Notably, the primary origin of chronic pain remains poorly understood, and its complexity warrants further investigation (3,11,18).

Additionally, pain can be further divided into three distinct categories based on its underlying mechanisms, including nociceptive, inflammatory, and pathological pain (19). Nociceptive pain is a high-threshold pain responding to intense noxious stimuli, such as extreme temperatures or sharp objects. It is designed to signal impending or actual tissue damage, serving a protective role that demands immediate attention and action. Inflammatory pain is an adaptive response of the immune system to tissue injury or infection, heightening sensory sensitivity and creating tenderness to promote recovery and prevent further damage. Pathological pain is a maladaptive type of pain resulting from abnormal functioning of the nervous system, often occurring in conditions such as fibromyalgia and irritable bowel syndrome, characterized by amplified sensory signals and low-threshold pain without any actual noxious stimuli or inflammation (19,20). Despite the common outcome of pain, the underlying processes for nociceptive, inflammatory, and pathological pain are unique, necessitating targeted treatment approaches that address the specific mechanisms to play for each type of pain.

3. Inflammatory pain

Inflammatory pain, also considered a subset of nociceptive pain, is a complex phenomenon characterized by heightened sensory sensitivity and emotional reactivity to painful stimuli, resulting from the body's inflammatory response to tissue injury or damage (21). This biological response, triggered by various causes such as trauma, infection, or disease, aims to defend the affected tissue, clear away debris, and facilitate the healing process. During inflammation, a diverse array of immune cells and blood vessels release a complex mixture of pro-inflammatory mediators, which includes prostaglandins, cytokines, chemokines, nerve growth factors, lipids and lipoxygenase products, ATP, proteases, and neuropeptides. These mediators can either directly stimulate nociceptors (causing spontaneous pain) or increase the sensitivity of primary sensory nerves, rendering them more prone to activation and pain transmission. This cascade of events contributes to the development of hypersensitivity, manifesting as allodynia, where normally non-painful stimuli become painful, and hyperalgesia, where painful stimuli become even more intense (6,22,23). The heightened sensitivity of nociceptors, known as peripheral sensitization, is a critical component of this process. Furthermore, sustained activation of primary afferents leads to profound changes in the central nervous system, resulting in central sensitization, where the transmission of pain impulses is amplified along the spinal cord and in the brain, ultimately exacerbating the pain experience. This intricate interplay between peripheral and central mechanisms underlies the complex phenomenon of inflammatory pain (6,24).

Since examining the mechanisms of pain in

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humans is not entirely feasible, animal models are used to study pain. Several preclinical animal models are utilized to assess inflammatory pain, focusing on three particularly susceptible organ systems: the skin, joints, and gut. For cutaneous inflammatory pain, models include capsaicin-induced pain, mustard oil-induced pain, formalin-induced pain, and acid-induced pain. Joint inflammatory pain is evaluated using models such as Freund's complete adjuvant (FCA)-induced hyperalgesia, kaolin-carrageenan-induced pain, and collagen-induced arthritis pain. Visceral inflammatory pain is assessed using models including capsaicin-induced visceral pain, mustard oil-induced visceral pain, and acetic acid-induced writhing, providing a comprehensive toolkit for understanding and developing treatments for inflammatory pain in various organ systems (22). Here, we will focus specifically on the formalin test, a widely used model for assessing cutaneous inflammatory pain in animal models.

4. Formalin test

The formalin test, developed by Dubuisson and Dennis in 1977, is a well-established model for evaluating acute, long-lasting pain and hyperalgesia in animal subjects. This test has been widely employed in research settings to investigate the mechanisms of pain and develop effective treatments (25).

Formalin, an aqueous solution containing 37% (w/w) formaldehyde, is used as the nociceptive stimulus in this model. When diluted to a 10% formalin solution, it contains approximately 3.7% formaldehyde. Commercial stock formalin, typically containing 37% formaldehyde, is used as the starting material and is often diluted in normal saline (0.9% sodium chloride solution) to achieve the desired concentration, usually ranging from 0.5% to 5% formalin for experimental purposes (26). A 1% or lower formalin concentration is often used to detect the effects of mild analgesic agents and avoid ceiling effects (27).

The formalin test has been used in various species, including rodents (primarily rats and mice), cats, primates, rabbits, and guinea pigs (26,28). However, this review will focus on the use of rats as the experimental subjects.

4.1. Procedure

The procedure involves placing animals in plexiglass boxes. A mirror positioned below the floor at a 45-degree angle provides an unobstructed view of the animal's paws. The outline of the experimental setup is shown in Figure 1. Prior to the test, each animal is acclimated to the chamber for 15 minutes. Next, a formalin solution (usually 5%) is administered subcutaneously to the animal's paw. Following the injection, the animal is returned to the chamber, and its behavioral responses to pain are recorded for a specified period (30-90 min). The observed responses can include lifting, licking, or biting of the affected paw, which are indicative of pain and discomfort (25–27). It is worth noting that the procedure of the formalin test is refined and may be varied based on the purpose of the experiment (27).

The site of injection in the original study by Dubuisson and Dennis was the dorsal surface of the forepaw (25). However, most subsequent studies used the hind paw due to forepaw licking during grooming. Both dorsal and plantar surfaces of the paw are used, with plantar injections being more common and stronger reactions (26,27). The formalin test has also been used to study orofacial pain by injecting formalin into the lip or temporomandibular joint (29).

Several factors can affect the severity of an animal's response to pain, including environmental factors (temperature, noise, light, smells, air pressure, and human presence and activity), handling, the animal’s age and breed, the injection site (location and surface), and the testing environment (27).

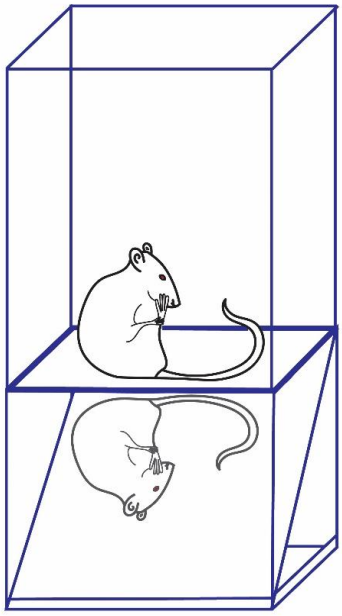

Figure 1: The outline of the experimental setup

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4.2. Scores and parameters

During the assessment period after the injection of formalin into the animal's paw, various pain-related behaviors may be observed, including lifting, licking, biting, flinching, shaking, rubbing, and protecting. The definition of each behavior is provided in Table 1.

Table 1. Definition of pain-related behaviors observed during formalin test

Behavior	Definition
Lifting	The animal may repeatedly lift the affected paw, indicating pain or discomfort.
Licking	The animal may lick the affected paw, which is a common behavior indicating pain or discomfort.
Biting	The animal may bite the affected paw or surrounding areas, which can be a sign of intense pain or discomfort.
Flinching	The animal may flinch or withdraw the affected paw in response to touch or movement, indicating pain-related reflexes.
Shaking	The animal may shake the affected paw, which can be a sign of pain or discomfort.
Rubbing	The animal may rub the affected paw on the ground or against other objects, which can be a sign of pain or discomfort.
Protecting	The animal may protect the affected paw by holding it in a flexed position or avoiding weight-bearing on it, indicating pain or discomfort.

There are several approaches to assessing behavioral parameters in the formalin test. These include:

1. **Frequency-based scoring**: Calculating the number of specific behaviors (e.g., licking and flinching) during a predetermined period after formalin injection (30).
2. **Timing-based scoring**: Recording the duration of specific behaviors, allowing for the assessment of the temporal aspects of pain-related behaviors (31).
3. **Categorical or weighted scoring**: Assigning scores based on the presence or absence of specific behaviors, using a scoring system (25,27) such as:
   - Score 0 (Normal behavior): The animal walks on its paw which received the formalin injection without any apparent discomfort.
   - Score 1 (Mild discomfort): The injected paw is gently placed on the ground or limps during locomotion, indicating some pain or discomfort.
   - Score 2 (Moderate pain): The injected paw is elevated above the surface, touching the floor with the nail, indicating increased pain or discomfort.
   - Score 3 (Severe pain): The injected paw is licked, bitten, or cleaned by the mouth, indicating intense pain or discomfort.

4.3. Phases

The formalin test is typically conducted in 60 minutes following formalin injection. Researchers have divided the phases into different timeframes (26,27). Two approaches to phase division in the formalin test include the two-phase and four-phase models. The two-phase model considers the acute phase (0-6 minutes) and the chronic phase (10-60 minutes), providing a simplified framework for analyzing the formalin test results (32). In contrast, the four-phase model segments the phases into two main phases: phase I (0-9 minutes) and phase II (10-60 minutes), with phase II further subdivided into phase IIA (10-39 minutes) and phase IIB (40-60 minutes), offering a more detailed analysis of the formalin test responses (33). However, most studies agree on the presence of three main phases (26,27,34), which is the most common approach for analysis:

1. **Acute phase** (also known as phase 1): This phase typically lasts from 1-7 minutes and is characterized by an intense, acute painful response to the formalin injection.
2. **Interphase**: This phase usually occurs between 8-14 minutes after the initial injection and is a transitional period between the first and second phases.
3. **Tonic phase** (also known as phase 2): This phase typically lasts from 15-60 minutes and is characterized by a sustained, chronic painful response to the formalin injection.

A study utilized a unique approach to the division of phases in the formalin test, differing from traditional methods that rely on time post-formalin injection. The method consisted of three well-defined periods: phase 1, which spanned from the start of the test until nociceptive scores decreased below 0.5; the interphase, a period of minimal pain-related behavior, which occurred between the end of phase 1 and the point where scores rose above 0.5; and phase 2, which followed the interphase and extended to the end of the experiment, during which pain-related behaviors increased (35).

Generally, the formalin injection elicits a

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complex biphasic response characterized by two distinct phases (acute and tonic phases) separated by an interphase. Studies have shed new light on the mechanisms underlying each phase. The acute phase is primarily caused by the direct activation of primary nociceptive afferents, specifically the formalin-induced activation of transient receptor potential channel A1 (TRPA1) receptors (36–38). In contrast, the tonic phase is a more prolonged response, resulting from inflammation-induced central sensitization in the dorsal horn of the spinal cord. This process is mediated by the release of inflammatory mediators and alterations in descending regulatory systems, ultimately leading to spinal sensitization and amplified peripheral input. Notably, the tonic phase exhibits a dissociation between primary sensory input and the resulting nocifensive behavior. Despite decreased sensory input, the behavioral response remains elevated and persists for several hours following the initial injection (26,27,38–40).

Contrary to previous assumptions, recent findings suggest that the interphase is not an inactive period, but rather an active process that plays a significant role in modulating pain response (41,42). During this stage, the body's endogenous pain-suppressing mechanisms, including the opioid system, are activated to mitigate pain-related behaviors (34,41,42). As our understanding of the interphase has evolved, it is clear that all three periods of the formalin test, including the interphase, warrant equal attention.

A notable disparity exists between species in their responses to the formalin test, with biphasic pain responses being characteristic of rodents (rats, mice, and guinea pigs), whereas larger mammals (cats, rabbits, dogs, and monkeys) predominantly exhibit a sustained, monophasic response that can persist for sixty minutes or longer (43–46). This species-specific difference in response patterns highlights the limitations of extrapolating findings from small mammals to larger species.

4.4. Applications

The applications of the formalin test are multifaceted and significant in the field of pain research. This model has been instrumental in elucidating the mechanisms underlying inflammatory pain processing and the complex processes of pain relief. The formalin test has been employed to assess the analgesic efficacy of various pharmacological agents, including opioids (such as morphine and fentanyl) (47), nonsteroidal anti-inflammatory drugs (NSAIDs) (such as ibuprofen and naproxen) (48), and other analgesic compounds (such as gabapentin) (49). Additionally, this model has facilitated investigations into drug interactions and synergistic effects (50,51), thereby enhancing our understanding of how different compounds can be combined to achieve optimal pain management. The applications of the formalin test also extend to identifying potential therapeutic targets for pain management, making it a valuable tool in the development of novel analgesic therapies and personalized treatment approaches.

4.5. Limitations

The formalin test, despite its widespread use and utility, has several limitations that must be considered when interpreting results. One of the primary limitations is its reliance on a single nociceptive stimulus, which may not accurately reflect the complexity of pain experiences. In addition, the test is primarily used to assess the efficacy of analgesic treatments in rodents, which may not translate to other species or human pain conditions. The species-specific limitation raises concerns about the test's ability to predict clinical efficacy and highlights the need for further validation in human studies. Moreover, the test's subjectivity and variability, which can be influenced by factors such as animal handling, environmental conditions, and observer bias, can impact the reproducibility of the results. Additionally, the use of a single endpoint measure, such as paw licking or flinching, may not capture the full range of pain-related behaviors and may overlook other important aspects of pain, such as emotional and cognitive components. To optimize the use of the formalin test, it is essential to carefully consider the study purpose and design the experiment accordingly. This may involve modifying the test protocol to better reflect the specific research question, such as using different concentrations of formalin or assessing pain-related behaviors over a longer period.

5. Conclusion

Overall, the formalin test is a widely used and well-established model for evaluating inflammatory pain, allowing researchers to investigate the underlying mechanisms of pain

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and develop effective treatments for managing pain. With multifaceted applications in pain research, including assessing analgesic efficacy, exploring drug interactions, and identifying therapeutic targets, the formalin test has significantly contributed to the field. Although species-specific limitations exist, refining the test protocol to enhance validity and reliability, as well as integrating it with other research approaches like imaging and molecular biology techniques, will be crucial for advancing our understanding of pain mechanisms.

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Conflict of interest

The authors declare no conflict of interest related to this study.

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