Introduction

The formalin test is an important animal model in the study of acute long-lasting pain, yet the procedures for performing this test have not been standardised, greatly impeding the comparison of data. The response to the test can also be substantially influenced by the conditions and manipulation to which the animal is subjected. The response to the test can also be substantially influenced by the conditions and manipulation to which the animal is subjected before the actual test is performed, and the type and extent of this influence differ among individuals, even when the stimulus is the same. One fundamental factor is the stress to which animals are subjected prior to performing the test. Stress can be induced not only by placing the animal in a restrainer but also by: a) forced awakening; b) separation from the group; c) transportation to another room; d) exposure to a new cage, new odours, and light. Whatever the source, this stress must be reduced. In the present work, we provide information on the procedures used to perform the formalin test and the effects of both these procedures and the conditions and manipulation prior to the test on the animal and its behaviour, with the ultimate goal of improving these procedures and conditions and decreasing the variability among laboratories.

Animals

Different animal species, and thus different means of observing behaviour, are used in the formalin test [1-4]. The most commonly used animals are rats and mice, although few studies explain the reason for choosing the specific rat or mouse strain. With few exceptions males are studied, and those few laboratories that have used females have only in rare cases mentioned the oestrous cycle phase. In most cases, rats weighing 220-280 g or mice weighing 25-30 g are used; these weights correspond to adults just out of puberty. However, younger
animals, such as 90-150 g rats, are also often studied, although the explanation for using younger animals is rarely given, despite the fact that younger animals greatly differ from adults in terms of hormonal status and the amount of pain behaviour [2, 5].

**Surgery**

Exposure to surgery, which entails performing general anaesthesia, implanting devices, and/or creating lesions in areas of the central nervous system (CNS), is an important variable in the state of the animal. Recovery from surgery is also important. In most laboratories, the animal is allowed to recover for 5-10 days. However, under certain conditions, such as when implanting an intravenous catheter or a microdialysis cannula, the animal is allowed to recover for only a few hours (4-6 h) or days (1-2 days). These particular conditions probably greatly affect the state of the animal. In fact, each surgical procedure is per se stressful and probably painful. Thus the neuroendocrine modifications induced by these manipulations must be taken into account when interpreting results: to this end, a control group not exposed to surgery could be useful.

One of the problems related to surgery is the postsurgical isolation of the animal (that can greatly affect the animal’s behaviour) for preventing damage to the cannula through the mutual grooming and scratching that occurs in social groups. A recent study addressed this problem by housing the animal [6] in a cage divided into two parts by a perforated perspex partition: the experimental animal was placed in one section and the companion(s) in the other, allowing the animals to hear, smell, see, and touch (with the tip of the nose) each other. To prevent or at least reduce the autonomic behaviour that can follow rhizotomy, one interesting system is that of placing a female in the cage of the lesioned male [7].

**Handling and habituation procedures**

When an animal encounters a threatening stimulus, such as a new environment, complex physiological changes occur to metabolically prepare the animal to effectively cope with the stimulus [8, 9]. These changes include activation of the hypothalamic-pituitary-adrenal axis and the release of catecholamine from the adrenal medulla and peripheral sympathetic nervous system, both of which modulate pain transmission. Several behavioural aspects are also influenced, in addition to pain and analgesia [4], whereas re-exposure to the same situation increases the animal’s familiarity with the environment. To avoid aspecific novelty-related reactions, methods such as handling animals before the formalin test and/or placing the animal in the observation box some time before the beginning of the test (minutes or hours) have been used. However, in some laboratories, animals are handled for several days prior to the formalin test (e.g., twice a day for a week), and this handling, which constitutes a form of treatment in itself, has been found to have several effects [10-11]. Moreover, if habituation procedures are only carried out in the cage in which the animal is normally housed, the habituation will be limited to the researcher and thus be practically useless in terms of habituating the animal to the experimental procedure.

**Housing conditions**

In order to provide that the animals used in a given experiment are as homogeneous as possible, it is important to avoid exposure to aspecific influences. To this end, the basics of suitable housing must be followed. For example, different species (e.g., rats and mice), different genders, or animals who have been subjected to the experiment and those that have not, should not be housed in the same room, given that animals can communicate via a variety of olfactory and ultrasound systems, which can be a source of anxiety and fear [12, 13]. Once they are delivered by the supplier, animals should be allowed to acclimate to the cage in which they are housed for 1-2 weeks. Regarding the specific type of housing, animals are commonly placed in cages that allow sawdust to be placed at the bottom, although wire-bottom cages are also used. Although the impact of the type of cage has not been evaluated in studies that have performed the formalin test using the paw as the main injection site, both types of cages can interfere. Wire-bottom cages are easier to clean, yet they do not allow the animals to use bedding material, as rodents usually do. On the other hand, the sawdust has to be changed every 2-3 days, which can greatly disturb some animals. For this reason, whether or not to clean the cage just before the animal is subjected to the experiment must be carefully weighed. Moreover, re-housing rodents from established groups in new associations is intensely stressful [12].

Rats and mice are often housed in groups, and it is commonly believed that housing them singly is stressful, although the response to being housed singly seems to vary by species and the stage of development [14-15]. When attempting to provide suitable housing, it should also be kept in mind that rodents tend to form a hierarchy in a group [16]. In other words, the appropriate choice depends on the characteristics of the animals and the experimental procedure adopted.
The temperature of both the cage in which the animal lives and the experimental room can greatly affect the expression of pain-induced behaviour [17]. If the experimental room is not located in proximity to the animal’s cage, the animals can be exposed to different temperatures and must thus be allowed to adjust to the new environment for several hours before beginning the experiment.

Experimental apparatus

The observation box in which the formalin test is carried out generally consists of a perspex box with featureless surfaces surrounded by a wall to prevent escape. A mirror is generally positioned under the floor so that the paw can be observed. The dimensions of the cage are usually around 20x20x30 cm for mice and 30x30x30 cm for rats, although the formalin test has also been carried out in a tube or in disproportionately small cages (a 10x20 cm box for rats weighing 250-300 g). However, the specific dimensions of the observation box have never been considered as important to the aim of the experiment, also because the animal’s behaviour is in most cases video-taped, and better-defined images can be obtained when small cages are used. Immediately after receiving the injection, the animal moves around the cage, rears, and tries to escape from the pain, and small cages and tubes, which prevent the animal from moving, could produce fear and discomfort, ultimately altering the sensitivity to pain and biasing the results.

With regard to the features of the experimental room, these have not been described in most published studies. The animal to be tested has to be transported singly to the experimental room, so as to avoid any influence upon other animals. Light, temperature, and odour must also be kept constant. The use of white noise, such as that produced by running water from a nearby tap [1], could help to avoid interference from sudden noises in other rooms. Moreover, the detergent used to wash the observation box must be strong enough to eliminate odours that may disturb the animal yet not so strong as to leave its own disturbing odour.

Formalin test

In performing the formalin test, the timing of the light/dark cycle is very important. Both sensitivity to pain and behaviour are known to change with the time of day [18], and the decision to use the dark phase to study pain is based on the consideration that the circadian phases induce a series of modifications that cannot be uninfluential in pain perception and therapy. As mentioned, few studies have allowed the animals to become adequately habituated to the experimental apparatus; although animals are commonly placed in the experimental cage a few minutes before the formalin injection (10-15 min are required for a decrease in exploration and locomotion), this may not be sufficient for the CNS to adjust the levels of arousal and hormones. Although what truly matters is that the experiment be consistently performed at the same time of day, some effort should be made to change the habit of testing animals during their resting period.

Injection procedures

Depending on the specific goal of the experiment, formalin can be injected into different body regions and either subcutaneously or intramuscularly. The paw is the most common site, although plantar or ventral injection is also performed. At times, both paws are injected simultaneously. In choosing the injection site, it must be considered that the level of pain response can change according to the specific site. Although the most suitable injection site for the formalin test has yet to be defined, for analyses of pain-evoked responses the treatment should be limited to a single injection in the back of the hindpaw, for a number of reasons, specifically: the forelimbs are often used in grooming behaviour; it is easier to inject formalin into the soft cutaneous tissue of the back of the hindpaw, as opposed to the ventral paw or the ankle; and the animal’s walking is not modified by the presence of the fluid in this site.

Particular attention must also be placed on the actual performance of the injection, in that this is rather subject to user variability and several studies have indicated that pain-evoked behaviours change when the same amount of formalin is injected into different parts of the same paw [19]. An important part of performing the injection is how the animal is restrained. In injecting formalin, the animal is most commonly restrained by hand for a short time, which requires two people. Some studies have used a restrainer or, for mice, a murine holder. Based on ethical considerations, it has recently been suggested that brief anaesthesia be induced immediately before injection, which is probably the best option when the site of formalin injection is crucial.

Unless the effects of repeated painful stimuli are being studied, the formalin test is rarely performed more than once on the same animal. However, to reduce the number of animals used, some laboratories perform 2 or 3 formalin-test procedures on the same animal at 7-day intervals. Although several reports have described the long-lasting effects of formalin on the CNS [20, 21], studies carried out in mice with very low concentrations of formalin (0.02-0.2%) have shown that the repetition of the test does not induce any tissue change that could affect results [22].
Apart from the concentration, the specific dose of formalin varies among laboratories and according to the objectives of the experiment. The average dose is 10-20 µl for mice and 50 µl for rats, although in rats, doses of 80-150 µl have often been used, and in some cases it has been as high as 250 or 400 µl. The decision to use high doses must be carefully evaluated. Although there are no specific studies concerning the effects of different doses, it is reasonable to believe that low doses allow the injection to be performed more rapidly and more accurately. Moreover, high doses cause greater necrosis of the tissue, making it more difficult to interpret the results. Furthermore, formalin often tends to leak from the injection site and higher doses produce more leakage, which is important when considering that the animals lick the injection site and can taste and smell the formalin, consequently involving different neural systems.

Control groups are not always used when performing the formalin test, probably because the pain-evoked responses, such as the licking or flexing of the limb, are very low in non-formalin-treated animals, including those treated with distilled water or saline. However, the importance of a control group lies more in its use as a baseline for “spontaneous” behaviours. In our laboratory, we treat the control group with a sham injection (i.e., inserting the needle without injecting any substance): in this way, the control animals will experience all of the experimental phases except formalin.

**Pain-evoked behaviour**

Formalin-induced pain evokes three main behavioural responses: licking, tonic flexion, and phasic flexion of the injected limb (“paw jerk”), the frequency, duration, and level of which depend on the specific concentration used and the site of injection. All pain-evoked responses appear immediately after treatment and disappear within 1-2 hours, depending on the concentration, although the swelling induced by the inflammation can last for several days. Interestingly, the formalin-induced inflammation of the injured paw tissue can be assessed by evaluating the degree of plasma extravasation using Evans Blue dye leakage [23].

Grooming and rearing have also been reported to be pain-evoked behaviours. Although grooming is directly related to signs of pain, such as licking, we do not consider it to be a pain-evoked behaviour. It is also not clear whether or not rearing (i.e., investigatory upright posture) can be considered as a pain-evoked behaviour, since it is commonly present in the “spontaneous” behaviours shown by animals when exploring an environment.

The nociceptive responses to formalin are commonly assessed by the weighted-scores method of behavioural rating [24]. However, when using cumulative scores, changes in single behavioural responses can be overlooked. To this regard, we have often observed that only jerking or licking is affected by certain experimental conditions (i.e., exposure to a novel environment, the presence of food) [25, 26] and that, when analysing separately licking, flexing, and jerking, the time course differs for the same formalin concentration. For instance, in animals treated with 50 ml of a 5% or 10% formalin concentration, the duration of licking gradually decreases after 40 min, whereas flexing and paw jerk remain elevated during the last part of the test [27]. Ceiling effects or even reductions in licking or paw jerk have also been described [2, 17, 28]. Since both the separate analysis of responses and the use of cumulative measures can provide important information, the choice of the approach should be based on what best suits the experiment.

Although mice are being increasingly used for the formalin test, only licking has been commonly reported for this species [29]. However, all pain-evoked responses have been recently studied in response to different formalin concentrations [30].

In evaluating pain-evoked responses, an important aspect is the time interval during which behaviours are recorded. The observation period, which generally lasts 60 min, is generally divided into two phases (i.e., the first 0-10 min and the last 20-60 min), and either the total time of each of these two phases is considered or the phases are divided into separate 2- or 5-min periods. The latter approach is particularly useful, in that it allows different periods to be compared and transient pain-evoked changes in behaviour to be investigated. However, the use of samples selected from specific phases or points in time, such as evaluating licking for 2 or 5 min at the beginning of the test, could lead to the loss of important information, or to the inappropriate generalisation of the results collected in those few minutes. Differences in the time intervals in which behaviours are recorded further impede the comparison of results from different laboratories.

Formalin has also been used to induce deep and visceral pain by injecting it into the back muscles [31] or instilling it in the colon [32], in which cases different behavioural responses have been described.

A system of automated analysis [33] is of use in executing the experiments, yet it can result in important information being lost. For example, only personal and direct observation allowed us to realise that formalin-treated animals were licking not only the injected paw but also the contralateral one [34].

Some of the experimental conditions that can affect pain-evoked responses are: exposure to a novel environment (novelty), gender, and the presence of food.
in the experimental apparatus. That novelty can decrease the behavioural reactions to formalin was first suggested by Abbott and co-workers [35]. Although these effects could be attributed to novelty (stress)-induced analgesia, the lack of a reduction in licking indicates that caution should be used before concluding that decreases in nociceptive responses indicate analgesia. In fact, the lack of responses could be due to other factors such as the conflict between the motivation to explore an unknown environment and the need to react to a painful stimulus [25, 36]. It is possible that rats are distracted by the novel apparatus, resulting in an immediate reduction in pain-related responses. For example, the novel cues may command attentional or processing resources necessary for the detection of the formalin-induced pain [37].

Of the variables that can influence the formalin test, gender is the most important [38]. In our laboratory, although formalin-induced pain has been observed to result in the same behavioural responses when comparing male and female rats, with a comparable time-course, the time spent licking and flexing the injected paw has consistently been observed to be longer in females [25]. Although these differences can easily be attributed to the different neural circuits underlying the behavioural response to a nociceptive stimulus, it must also be emphasised that nociception is modulated by common laboratory procedures and that male and female rats are known to behave differently in response to a series of emotional and aversive procedures [39-41].

In contrast with our findings, it has been reported that female mice show lower pain-related responses than males in the formalin test [42]. The discrepancy between these results and ours is an example of how different animal species (rat vs mouse), different sampling times (60 min in our study vs 10 min in the other), and different formalin concentrations (10% vs 5%, respectively) can greatly affect the results. With specific regard to formalin concentrations, in a series of experiments in which two different concentrations were used (0.1 and 10%) in both male and female rats [41, 43], the higher concentration induced greater levels of licking and flexing in females, whereas the lower concentration tended to induce greater levels of these behaviours in males.

Another variable that can greatly affect pain behaviour is the presence of food in the experimental apparatus when food-restricted animals are used. In the study by Aloisi and colleagues [26], the animals were familiarised with the experimental apparatus, and on the day of testing the formalin was injected before exposing the animal to this apparatus. Of interest is the finding that the presence of food affected only the duration of licking, which can be explained by the competition between the drive to relieve hunger and the urge to lick the site of injection, as opposed to the presence of analgesia. This has been confirmed by other observations. Even before opening the alley at the end of which food was available, all of the animals that were allowed to hoard food pellets (both sham- and formalin-injected) showed higher levels of exploratory activity and rearing than the group not allowed to hoard, which Jones and Robbins [44] have attributed to the expectation of food. Moreover, the time spent eating was similar when comparing the animals injected with formalin to those that were sham-injected [26].

**Behavioural and hormonal analysis**

The influence of nociceptive stimuli upon spontaneous behaviours such as locomotion, exploration, rearing, head-dipping, grooming, sleeping, and inactivity, has also been evaluated. Researchers have been prompted to more thoroughly characterise the behavioural response to different formalin concentrations, following the casual observation of “a different behavioural response” in rats treated with different formalin concentrations, together with the well-known effect of pain in activating opioid systems and the data on the biphasic effects of opioids on behaviour [45]. First in rabbits and then in rats, it became evident that behaviour differed according to the concentration of formalin [45-47]. The level of activity was generally decreased in the group treated with the higher formalin concentration (10%), whereas it was not affected at all, or showed only a slight increase, in the group injected with the lower concentration (0.1%). The latter group, in addition to a lack of motor inhibition, showed evidence of behavioural activation, specifically, a lack of sleeping-like episodes and an increase in the duration of pendulum, a behaviour considered to be a symptom of a predisposition to a hyperkinetic mode of reaction [48]. The differences in the arousal state between the two groups was also confirmed by the behavioural response to a new object (i.e., a small plastic cylinder) placed in the open-field at the end of the formalin test: the recipients of the lower concentration displayed a higher number of approaches (i.e., moving and touching the object) [45]. The differences in behaviour were then confirmed by the hormonal determination (i.e., ACTH, β-endorphin, and corticosterone) [49]. The first and most important finding was the clear lack of an increase in corticosterone in both groups, suggesting that neither the experimental procedures nor the test stressed the animals. In animals treated with a 0.1% formalin concentration, ACTH and β-endorphin showed a trend of decrease with respect to the sham-injected group. On the whole, these results indicate that the specific concentration of formalin is a very important parameter, given that the behavioural and hormonal modifications constitute the higher expression of the integration carried out by the CNS in response to peripheral input.
The analysis of spontaneous behaviour is very important in drug-treated animals, whose level of alertness could be greatly affected. Systems have been devised to selectively test motor functions (e.g., the placing/stepping reflex or the bar tests). However, the evaluation of spontaneous behaviour is generally limited to the posture of the animals, without quantifying the responses to these tests.

Conclusions

Pain research has reached the point at which the need to decrease the number of animals and that of providing clinicians with concrete indications for managing pain can no longer be considered as marginal aspects of the research. In addition to standardising the procedures for the actual performance of the formalin test, it is fundamental to reduce the variability related to the conditions and manipulation to which the animals are subjected before the test. The information provided herein indicate that, in studies on pain-control mechanisms, it is important that any situation potentially influencing the animal’s internal state be avoided or controlled to the greatest possible extent and that researchers must make all attempts to distinguish between the effects of formalin itself and those of collateral experimental manipulation.

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References

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