Low Concentrations of Dimethyl Sulphoxide Mask the Antinociceptive Activity of Paracetamol in the Mouse Formalin Test

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Abstract: The frequently used vehicle, dimethyl sulphoxide (DMSO), has anti-inflammatory and analgesic effects. The aim of this study was to determine the concentration of DMSO that is devoid of significant antinociceptive effect in the mouse formalin test, when administered intraperitoneally (i.p.) or intrathecally (i.t.) and to evaluate the effect of these concentrations on the antinociceptive activity of paracetamol. DMSO at concentrations of 20%, 10%, 5% and 1% were injected i.p. in Swiss albino mice, 60 minutes before the formalin test. For i.t. route, DMSO was injected at concentrations of 100%, 20%, 5% and 1%. The concentration of DMSO that was devoid of antinociception effect was used as a vehicle for paracetamol. DMSO resulted in a significant antinociceptive effect at concentrations of 20% and 10%, i.p. and at concentrations of 100% and 20%, i.t. However, DMSO 5% (i.p.) and 1% (i.t.) did not have a significant antinociceptive effect. When these concentrations were used as a vehicle for paracetamol (39.7 µmole i.p. or 1.3 µmole i.t.), they masked its antinociceptive effect, in the formalin test. These results show that low concentrations of DMSO that are devoid of significant antinociceptive effect, may mask the antinociceptive activity of paracetamol, when used as a vehicle.

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1. Introduction

Understanding the molecular mechanisms involved in the pathophysiology of pain is mandatory to discover new analgesics, the evaluation of which, would necessarily require the use of vehicles. Dimethyl sulphoxide (DMSO), being an amphipathic molecule, is soluble in both aqueous and nonpolar organic media, and is therefore, frequently used in pharmacology as a vehicle. However, DMSO is not devoid of primary pharmacological actions, including anti-inflammatory and analgesic effects (Jacob and Herschler, 1983).

In a study of the antinociceptive effect of DMSO in mice, Colucci *et al*. (2008) found that oral or intracerebroventericular injection of 100% DMSO displayed anti-nociceptive effects in the hot plate, tail-flick and formalin tests. On the other hand, a solution of DMSO-saline 1:3 (v/v) did not produce any difference in the animals' behavioral responses compared to saline. These high concentrations of DMSO are seldom used. Indeed, researchers use a wide range of DMSO concentrations as vehicles, ranging from as low as less than 0.1% (Duttaroy *et al*., 2002) to as high as 100% (Matsunaga *et al*., 2007).

Colucci *et al*. (2008) did not use the intraperitoneal (i.p.) or intrathecal (i.t.) routes, though both routes are frequently used. The i.p. administration of drugs is frequently used in mice because it is an easy procedure that can be performed by one person and does not require restraining. Although i.t. application of drugs is not as easy, it is frequently used in pain studies since it provides important information regarding the mechanisms of analgesic action of drugs and those of pain transmission, which could guide pharmaceutical development of new analgesics (Brocks and Jamali, 1992). Further, the use of DMSO as a vehicle for drugs injected i.t. or i.p. is not an unusual practice.

The formalin test -as a model of tonic pain- is particularly useful for the screening of novel compounds, since it encompasses inflammatory, neurogenic, and central mechanisms of nociception (Tjølsen *et al*., 1992). Previous work in our laboratory suggests that DMSO concentrations higher than 5% injected i.p. influences the nociceptive response to formalin in Swiss albino mice (Hamza, 2007).

The aim of the present work was to determine the concentration of DMSO that is devoid of significant antinociceptive effect in the mouse formalin test, when administered i.p. or i.t. and to determine the effect of this concentration on the antinociceptive activity of paracetamol as an example of a weak analgesic, in the same test.

2. Materials and methods

Experimental Animals:

A total of 102 male Swiss albino mice (20-25 g) bred at the medical research center, Faculty of Medicine, Ain Shams University were used for the study. Animals were housed in groups and were allowed free access to food and water until the start of experiments. The ethical guidelines for investigations of experimental pain in conscious animals (Zimmermann, 1983) were followed and experiments were approved by the Dept. of Pharmacology review board.

Drug and Treatment Procedure:

Pure DMSO was purchased from BDH Laboratory supplies (England). For i.t. injection, DMSO was used as a 100% solution or freshly diluted in artificial cerebrospinal fluid (ACSF) at concentrations of 20%, 5% or 1% (v/v). For i.p. injection, DMSO was freshly diluted in normal saline at concentrations of 20%, 10%, 5% or 1% (v/v). Paracetamol was purchased from Sigma – Aldrich (US) and was dissolved in normal saline or ACSF for i.p. and i.t. injection, respectively. Heating was used to facilitate the dissolving of paracetamol (Crawley *et al*., 2008). ACSF was freshly prepared using the following composition: 124 mM NaCl, 26 mM $NaHCO_3$, 2 mM KCl, 2 mM $CaCl_2$, 2 mM $MgSO_4$, 1.25 mM KH_2PO_4, and 3.5 mM glucose (Nilsberth *et al*., 2008).

Intrathecal Injection

Direct intrathecal injection was performed as described earlier (Hylden and Wilcox, 1980). Briefly, 5 µl were injected i.t. at the thoracolumbar junction (corresponds to L5 and L6 of the spinal cord) under ether anesthesia, 60 min before the formalin test. 1% black ink was added to verify the site of injection.

Formalin Test

The formalin test was always conducted between 8 am and 7 pm i.e. (during the light phase). The mice were placed in the observation chamber; a glass cage (30 X 20 X 20 cm). A mirror was fixed behind the observation chamber to allow for an unobstructed view of the paws. To reduce variability mice were habituated to this environment for at least 30 min prior to injection of formalin, after which, the mouse was taken out of the chamber, and gently restrained. 50µl of formalin 3% was injected subcutaneously into the dorsal aspect of the hind paw using a 27-gauge needle. Immediately after s.c. injection of formalin, mice were placed back in the observation chamber and their behavior was continuously observed for the next 60 min. The number of flinches (rapid shaking of the injected paw) was counted and recorded every min. The time spent licking, biting and or holding the injected paw was estimated and recorded every min as well. The nociceptive response was expressed in all experiments as "pain related behavior", which was calculated as the total number of flinches summed to 1/10 of the time spent in licking, biting and or holding the injected paw (Gühring *et al*., 2002; Hamza, 2007). Pain related behavior was calculated for phase 1 (first 15 min following formalin injection), phase 2 (16-60 min following formalin injection) and the sum of both phases.

Statistical Analysis:

All results were expressed as mean ± S.E.M. Statistical analysis was carried out using Graphpad prism, version 3.02 for Windows, GraphPad Software, San Diego, California, USA. Comparison between groups was done using one way analysis of variance; ANOVA followed by Dunnett's or Bonferroni's Multiple Comparison Test. $P < 0.05$ was considered statistically significant. The DMSO concentration was considered devoid of antinociceptive activity, if the sum of pain related behavior throughout the 60 min observation period was not significantly different from that of the control group, using unpaired two-tailed Student's t-test.

3. Results:

DMSO induces a concentration dependent decrease in formalin induced pain related behavior

As expected s.c. formalin injection in the hind paw resulted in two distinct phases of pain related behavior in the form of rapid shaking, licking, biting and holding of the injected paw. DMSO diluted in saline and injected i.p. 60 min before the formalin test resulted in a significant antiniociceptive effect at 20% and 10% concentrations ($p < 0.001$; one way ANOVA, followed by Dunnett's multiple comparison test). This antinociceptive effect was significant in both phases of the formalin test at 20% concentrations ($p < 0.001$) and in phase 1 only at 10% concentrations ($p < 0.001$; **Figures 1a&b)**. Dunnett's multiple comparison test did not detect a significant antinociceptive effect at 5% and 1% concentrations. Unpaired Student's t test, did not show a significant difference between DMSO 5% and saline groups ($p = 0.093$) and thus this concentration was selected to be used as a vehicle for paracetamol.

Similarly, DMSO diluted in ACSF and injected i.t. 60 min before the formalin test resulted in a significant antiniociceptive effect at 100% ($p < 0.001$) and 20% concentrations ($p < 0.05$). This antinociceptive effect was significant in phase 1 only ($p < 0.001$ at 100% and 20%; **Figures 1c&d**). DMSO also showed a significant antinociceptive effect in phase 1 only, at 5% concentration ($p < 0.05$).

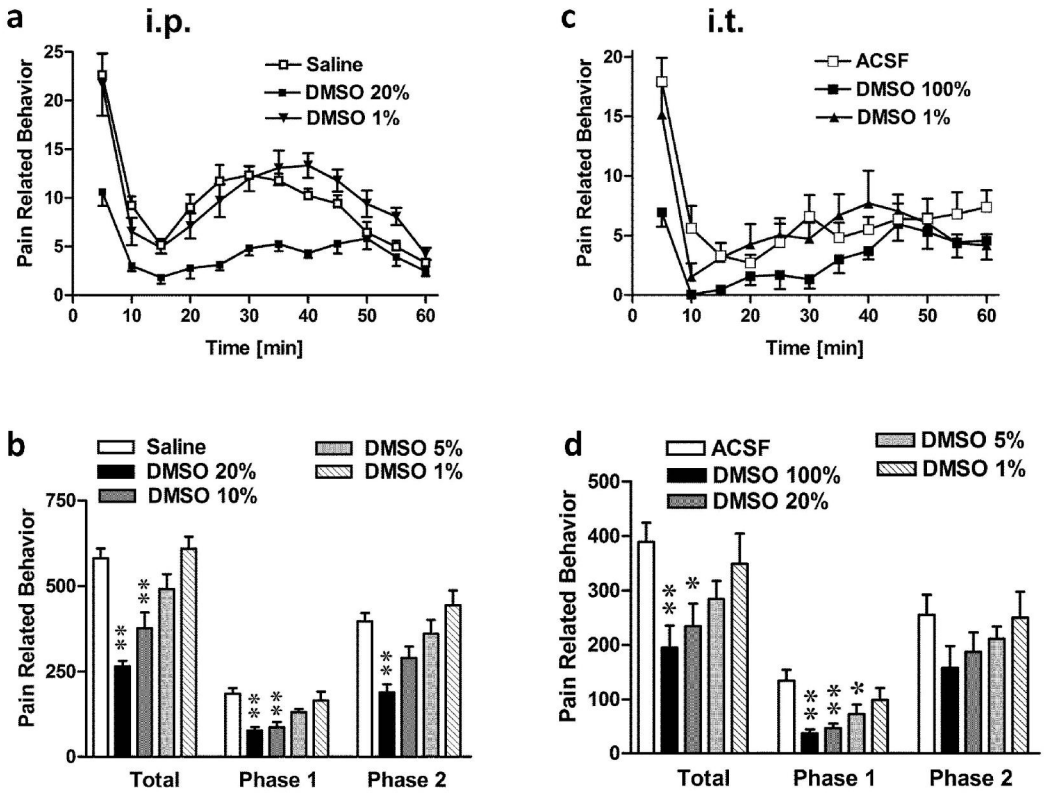

Figure 1: Effect of DMSO. on the formalin induced pain related behavior in mice. Line Graph: Each point represents the mean of pain related behavior over 5 minutes. Bar Graph: Each column represent the sum of pain related behavior over the 60 minutes observation time (sum of phase 1 and phase 2); phase 1 represents the first 15 min. and phase 2 represents 16-60 min. Data are presented as mean ± S.E.M. (a&b) Saline or DMSO administered i.p. at concentrations of 20%, 10%, 5% or 1%. Number of animals = 6-8 per group. (c&d) ACSF or DMSO administered i.t. at concentrations of 100%, 20%, 5% or 1%. Number of animals = 8-10 per group.**indicates $p < 0.001$, *indicates $p < 0.05$; one way ANOVA, followed by Dunnet's Multiple Comparison Test.

DMSO 1% on the other hand, did not show any antinociceptive effect in either phase. Though Dunnett's multiple comparison test did not detect a significant antinociceptive effect at the 5% concentrations, unpaired Student's t test showed a significant difference between DMSO 5% and ACSF groups ($p = 0.049$), so the 1% concentration was selected to be used as a vehicle for i.t. paracetamol.

Low concentrations of DMSO mask the antinociceptive effect of paracetamol when used as a vehicle

Paracetamol (39.7 µmole), dissolved in saline and injected i.p. 60 min before the formalin test, induced a significant antinociceptive effect ($p< 0.01$; one way ANOVA, followed by Bonferroni's multiple comparison test). This antinociceptive effect was evident in phase 2 ($p< 0.05$), but not phase 1.

When paracetamol was dissolved in 5% DMSO, there was no significant difference between paracetamol and its vehicle (DMSO 5%) in pain related behavior recorded all through the 60 min observation period, indicating that it masked its antinociceptive effect. However, paracetamol dissolved in DMSO 5% was not significantly different from paracetamol dissolved in saline. Further, DMSO 5% did not mask the antinociceptive effect of paracetamol in phase 2 **(Figures 2a&b)**.

Similarly, when 1% DMSO was used as a vehicle for paracetamol (1.3 µmole; i.t.), it masked its antinociceptive effect. Paracetamol dissolved in ACSF and injected i.t. 60 min before the formalin test, induced a significant antinociceptive effect ($p< 0.01$; one way ANOVA, followed by Bonferroni's multiple comparison test). This antinociceptive effect was evident in both phases of the formalin test ($p< 0.05$). However, when 1% DMSO was used as a vehicle for paracetamol there was no significant difference between the two groups. Meanwhile, paracetamol dissolved in DMSO 1% was not significantly different from paracetamol dissolved in ACSF **(Figures 2c&d)**.

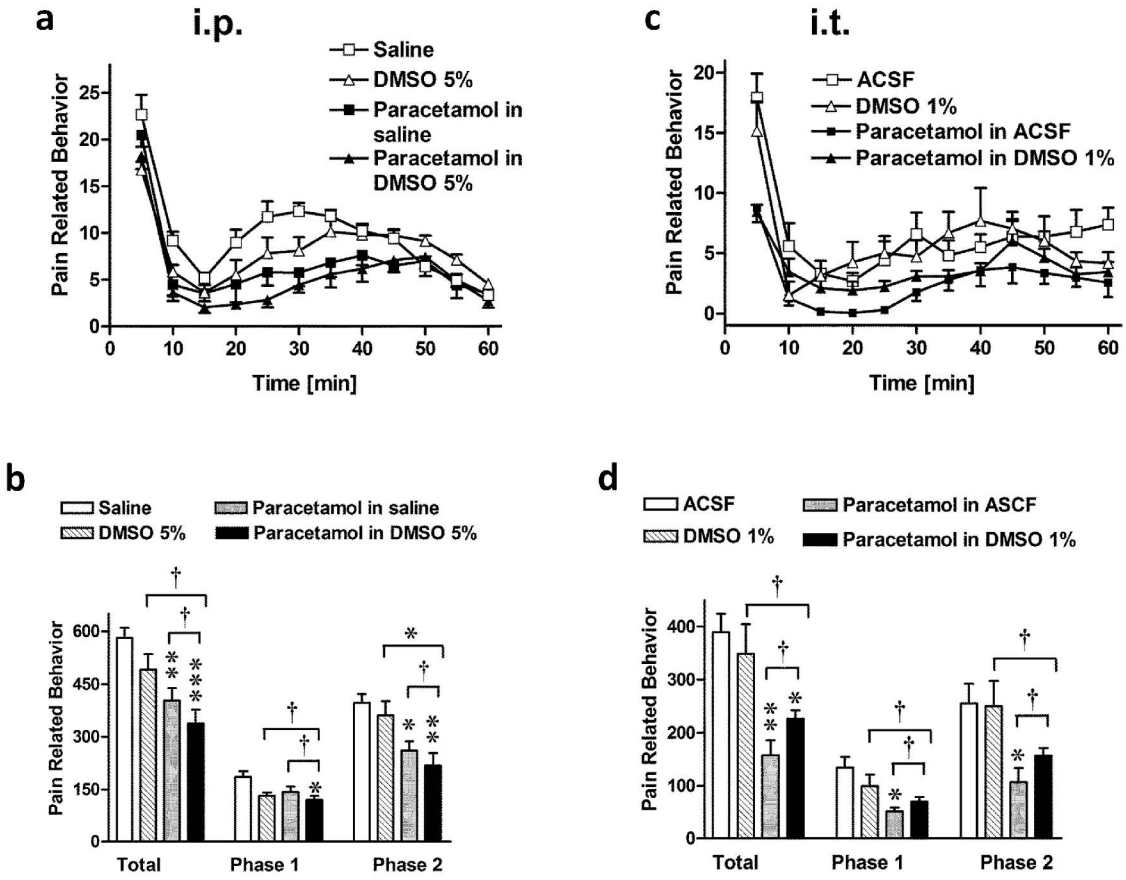

Figure 2:Effect of DMSO 5% (i.p.) and 1% (i.t.) on paracetamol-induced antinociceptive effect in the mouse formalin test. Line Graph: Each point represents the mean of pain related behavior over 5 minutes. Bar Graph: Each column represent the sum of pain related behavior over the 60 minutes observation time (sum of phase 1 and phase 2); phase 1 represents the first 15 min. and phase 2 represents 16-60 min. Data are presented as mean ± S.E.M. (a&b) paracetamol (39.7 µmole; i.p.) dissolved in saline or DMSO 5%. Number of animals = 6-8 per group. (c&d) paracetamol (1.3 µmole; i.t.) dissolved in ACSF or DMSO 1%. Number of animals = 8-10 per group. **indicates $p<0.01$, *indicates $p<0.05$ in comparison to saline or ACSF group; † indicates $p > 0.05$; one way ANOVA, followed by Bonferroni's Multiple Comparison Test.

4. Discussion:

In light of the previously reported analgesic effects of DMSO (Colucci *et al*., 2008; Păunescu *et al*., 2009), and its occasional use as vehicle in experimental pain studies, it becomes necessary to further characterize this analgesic effect.

We show here that DMSO concentrations as low as 10% (i.p.) and 5% (i.t.) do have an antinociceptive activity in the mouse formalin test. This is in contrast to the earlier report that DMSO concentrations of 25%, administered orally or by intracerebroventricular injection did not show an antinociceptive effect in the formalin test or in the tail flick test (Colucci *et al*., 2008). The different route may contribute to the discrepancy of results seen here, particularly that DMSO was administered orally, 10 or 30 minutes before the formalin test. Thus it is possible that the peak serum concentration was not achieved by the time formalin was injected. In this regard, i.p. administered DMSO results in a higher plasma level in the rat compared to the oral route. Further the peak plasma level after oral administration is reached after 1 hour (Hucker *et al*., 1966). As for the central administration, it is known that intrathecal administration is of particular importance in pain studies, targeting the first relay station in pain signaling. A different response in this case is not unexpected. Strain differences may also account for this difference, as genetic based differences in pain inhibition in laboratory rodents is well established (Mogil *et al*., 1996).

Păunescu *et al*. (2009) also reported that DMSO at 50% concentrations administered i.p. did not have an analgesic effect. However, this study used the mouse writhing test as a pain model. Colucci *et al*. (2008) reported that intraplanter administration of DMSO 10 min before the formalin test, potentiated the nociceptive effect of formalin. Further, local

injection of DMSO before zymosan potentiated its inflammatory effect. This was significant as early as 2 h following zymosan injection and lasted for 3 days. Taken together, this suggests that DMSO by itself has a local proinflammatory effect. Therefore, DMSO injected i.p. would have a local effect on the writhing test, besides its systemic effect, which in this case may mask the antinociceptive effect of systemic DMSO. It is worth mentioning, however, that when DMSO was locally injected 30 min before the formalin test in the Colucci study, its analgesic effect was not masked by this proinflammatory effect.

In the present study, DMSO administered i.t. as well as the lower concentration administered i.p. (10%) showed an antinociceptive effect at phase 1 only. On the other hand, 20 % DMSO administered i.p. affected phase 2. This suggests a different antinociceptive mechanism at the central and peripheral levels. This is consistent with the Colucci study, where systemic administration of DMSO resulted in a more pronounced effect on the second phase of the formalin test than central administration.

The effect of DMSO on the antinociceptive activity of analgesics becomes of interest here. We show in the present work, masking of paracetamol antinociceptive effect in the formalin test, when DMSO was used as a vehicle. Even though the concentration used was devoid of antinociceptive activity by itself. This is consistent with the reduction in the antinociceptive potency of morphine administered into the ventrolateral periaqueductal gray following 5% DMSO and 20% DMSO twice daily for two days (Fossum *et al*., 2008). .DMSO, however, did not mask the antinociceptive activity of paracetamol injected i.p. during the second phase of the formalin test, in the present study. Consistent with this finding, DMSO 50% used as a vehicle for metamizole did not significantly affect its analgesic effect in the mouse writhing test (Păunescu *et al*., 2009). Difference between these results may be due to different mechanisms of drug action, different route of administration and different pain models used.

Looking into the mechanism of antinociceptive activity is beyond the scope of the present work. However, given its effect on phase 1 only, when administered i.t., the antinociceptive effect of DMSO at the spinal level may be due to an antagonistic effect to substance P (Ohkubo *et al*., 1990). In this regard, Chassaing *et al*. (1986) suggested that substance P shows conformational change in DMSO, though this was tested at supraphysiological concentrations. Another possibility is the contribution of COX-2 downregulation, to the antinociceptive effect of DMSO, given that DMSO dose-dependently reduced COX-2-derived PGE_2 in IL-1β-stimulated Caco-2 cells. Furthermore, low concentration of DMSO (0.5%) reduced COX-2 gene expression in the same cells (Hollebeeck *et al*., 2011). Further research is needed to reveal the underlying mechanism of antinociceptive effect of DMSO. Meanwhile, DMSO should be dealt with cautiously if it is to be used as a vehicle in pain studies.

Declaration of interests
The authors declare no conflict of interest.
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