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Effects of Three Consecutive Days of Morphine or Methadone Administration on Analgesia and Open-Field Activity in Mice with Ehrlich Carcinoma

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This study assessed the exploratory behavioral responses in BALB/c mice inoculated with Ehrlich ascitic carcinoma after 3 consecutive days of treatment with morphine or methadone. Fifty-three female mice, 60 ± 10 d old, were used. Seven days after intraperitoneal tumor inoculation (2×10^6 cells), the animals were randomized into 7 groups: morphine 5 mg/kg (MO_5), morphine 7.5 mg/kg ($MO_{7.5}$), morphine 10 mg/kg (MO_{10}), methadone 2.85 mg/kg ($ME_{2.85}$), methadone 4.3 mg/kg ($ME_{4.3}$), methadone 5.7 mg/kg ($ME_{5.7}$), and 0.9% NaCl (Saline) (n = 7). Drug treatments were administered subcutaneously every 6 h for 3 d. The animals were evaluated for analgesia using the mouse grimace scale (MGS) and for general activity using the open field test. The MGS was performed before tumor inoculation (day 0), on day 7 at 40, 90, 150, 240, and 360 min after drug injection, and on days 8 and 9 at 40, 150, 240, and 360 min after drug injection. The open field test was performed before tumor inoculation (day 0), on day 7 after inoculation at 40, 90, 150, 240, and 360 min after drug injection, and on days 8 and 9 after inoculation at 40, 150, and 360 min after drug injection. MGS results indicated that administration of morphine promoted analgesia for up to 240 min. Conversely, methadone reduced MGS scores only at 40 min. All tested doses promoted a significant dose-dependent increase in the total distance traveled and the average speed, and increase that was markedly pronounced on days 8 and 9 as compared with day 7. The frequencies of rearing and self-grooming decreased significantly after morphine or methadone administration. Despite the difference in analgesia, both drugs increased locomotion and reduced the frequency of rearing and self-grooming as compared with the untreated control animals.

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Ehrlich carcinoma is a well-known transplantable tumor in which cells from mammary adenocarcinoma are inoculated subcutaneously or intraperitoneally, and grow into either solid or ascitic tumors, respectively.[6,8] This tumor is considered to cause pain yet is widely used to determine the influence of drugs and other therapeutic substances on the inhibition of tumor growth.[23,27,33] The ascitic form of Ehrlich carcinoma is characterized by a proinflammatory response induced by tumor cells in the peritoneum and increased vascular permeability.[41] Tumor cells promote a progressive increase in the secretion of interleukin-1β (IL1β),[16] monocyte chemoattractant protein-1 (MCP-1)[54] and prostaglandin E2 (PGE-2),[28] substances all related to the phenomenon of hyperalgesia.[15]

Recognition and management of pain are an important component of international standards designed to ensure the welfare of research animals. These factors are closely related to the survival and quality of life.[34,55] Although a test to directly measure pain in animals is currently unavailable, changes in behavioral patterns can indicate pain (for example, agitation, reduced ambulation, and changes in the sequence and frequency of self-grooming and vocalization).[9] Opioid analgesics, such as morphine and methadone, although frequently regarded as the most effective treatment of cancer pain,[12,38,53] tend to alter locomotor activity and exploratory behavior in mice.[22,43,47] Although morphine alters behavioral patterns, it does not interfere with facial expression in the absence of pain.[30]

Morphine is a potent opioid that acts mainly through the occupation of pre- and postsynaptic μ-opioid receptors, which modulate the perception of pain.[52] Methadone has affinity for μ-receptors, is also an antagonist of N-methyl-D-Aspartate receptors (NMDA), and is considered an ideal treatment choice in cases of tolerance to morphine.[20] The condition of cancer pain requires long-term analgesic treatment. However, some disagreement remains regarding the optimal doses and frequency of administration of morphine and methadone in mice, and few studies have evaluated the effects of these drugs on cancer pain in mice or the effect of long-term administration.[35,39,43,46]

The current study aimed to evaluate the analgesic effect of morphine and methadone in BALB/c mice with Ehrlich ascitic carcinoma by observing the influence of these opioids on behavior. The hypothesis was that morphine and methadone would provide analgesia and mitigate pain-related behavioral changes in mice with Ehrlich ascitic carcinoma in a dose-dependent manner.

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Materials and Methods

Animals. All procedures were conducted in accordance with the Ethics Committee of the Faculty of Animal Science and Food Engineering, University of São Paulo, Brazil (3836220518). The study used 53 female BALB/cJ mice, 60 ± 10 d old and weighing 25 ± 5 g; mice were obtained from a breeding colony in the vivarium of the Department of Pathology, School of Veterinary Medicine and Animal Science, University of Sao Paulo. From this cohort of 53 animals, 4 mice were kept exclusively for the maintenance of tumor cells; the rest were assigned to experimental groups (n = 7 per group). The animals were housed under conventional conditions, randomly grouped into polypropylene cages (37 × 22 × 15 cm) housing 3 to 4 animals, under controlled temperature (22 to 23 °C), relative humidity of 55% and a 12-h light:dark cycle, with lights on at 0630. Filtered water and food (Nuvital, Quimtia, Parana, Brazil) were provided ad libitum. The daily food intake per cage was measured based on the weight of unconsumed food. After tumor inoculation, the abdominal circumference was measured at the same time each day by using a tape measure. The animals were given a 10-d minimal acclimation period before the start of the experiment.

Treatment protocol. Initially, a single mouse was inoculated with Ehrlich carcinoma cells maintained in a cell bank at -80 °C. Fourteen days after inoculation, the donor mouse was euthanized in a CO_2 chamber, followed by cervical dislocation. The peritoneal fluid was collected (0.1 mL) and used to inoculate a second cell-carrier/donor mouse to maintain the tumor cells in vivo.[17] This procedure was repeated 3 times until the cell viability was greater than 95%, which was appropriate for experimental inoculations.

In the experimental groups, mice were inoculated intraperitoneally with 2 × 10^6 Ehrlich tumor cells from the donor mouse. Drug treatments were morphine sulphate (Dimorf, Cristalia, Sao Paulo, Brazil) and methadone hydrochloride (Mytedon, Cristalia, Sao Paulo, Brazil). Seven days after tumor inoculation, the mice were sorted into 7 groups using balanced randomization: morphine 5 mg/kg (MO_5), morphine 7.5 mg/kg ($MO_{7.5}$), morphine 10 mg/kg (MO_{10}), methadone 2.85 mg/kg ($ME_{2.85}$), methadone 4.3 mg/kg ($ME_{4.3}$), methadone 5.7 mg/kg ($ME_{5.7}$), and 0.9% saline (Saline) (n = 7). Drug treatments were administered subcutaneously every 6 h for 3 d, by individuals blind to the treatments (Figure 1). In this study, the morphine doses were chosen based on previous literature,[6,24] while the methadone doses were chosen to be equipotent to morphine, which was determined to be 1.75 times the doses of morphine.[14] The final volume of each injection was standardized in 0.3 mL, adjusted with 0.9% NaCl.

Mouse Grimace Scale. To assess the Mouse Grimace Scale (MGS), the mice were placed individually in an acrylic box (9 × 5 x 10 cm high) with 3 opaque sides, with a high-resolution camera (Canon EOS Rebel T5) positioned in front of the transparent side. Mice were recorded for 3 to 5 min in a brightly lit room without human presence, on day 0 (before tumor inoculation) and after tumor inoculation on days 7 (0, 40, 90, 150, 240, 360 min after injection), 8 and 9 (40, 150, 240 and 360 min after injection). Afterward, the full-time videos were analyzed in random order by an evaluator blind to the treatments. For each timepoint, orbital tightening, cheek bulge, nose bulge, ear position and whisker position were scored in accordance with a validated scale for pain assessment in mice.[30,36] Each facial unit was scored separately on a 3-point scale (0 = not present, 1 = moderate, 2 = severe), and the sum of all 5 facial action units were analyzed. The scores were then submitted to statistical analysis.

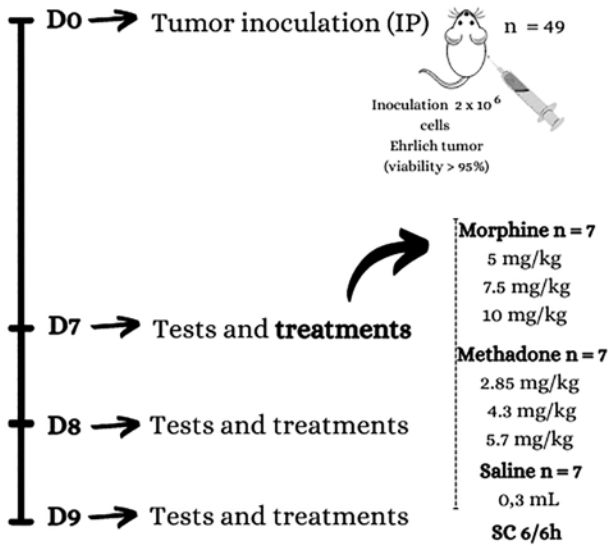

Figure 1. Schematic timeline of the experimental period. Mice were inoculated intraperitoneally with 2 × 10^6 Ehrlich tumor cells with > 95% viability. On day 7, the animals were sorted into 7 groups, and given repeated administration (every 6 h) of morphine (MO), methadone (ME) or Saline, for 3 d (day 7, 8 and 9 after tumor inoculation).

Behavioral tests. The behavioral influence of opioids was evaluated by assessing the total distance traveled (cm/5 min), the average speed (cm/s), and the frequency of the animals rearing and self-grooming behaviors using the open field test. The mice were individually placed in the center of the circular arena (40 cm in diameter and 50 cm in height) and recorded for 5 min in the absence of humans, using a video camera positioned vertically above the open field. To prevent lingering olfactory cues from affecting behavior, the arena was sanitized with a 5% alcohol solution between individuals. The animals were tested at baseline, defined as the moment before tumor inoculation (day 0), then subsequently on day 7 at 40, 90, 150, 240 and 360 min after drug injection and on days 8 and 9 after inoculation at 40, 150 and 360 min after drug injection.

The total distance traveled and the average speed of travel were evaluated from the video-recorded data obtained in the open field test using the Ethovision software (EthoVision XT, Noldus, Version 7.1). The rearing frequency was determined as the number of times the animal stood on its hind limbs. The frequency of self-grooming was determined based on the number of times the animal cleaned its limbs and body during a 5 min period. Both were assessed manually during the retrospective evaluation of the recorded videos by evaluators blind to the treatments.

Statistical analysis. Data were analyzed using GraphPad Prism version 7.0 (GraphPad Software, La Jolla, CA). Variables were considered parametric (mean ± SD) when showing a normal distribution in the Shapiro–Wilk test and coefficient of variation below 0.2; otherwise, they were considered nonparametric data (median [minimum; maximum]). The abdominal circumference, MGS, rearing frequency and frequency of self-grooming were analyzed using the Kruskal–Wallis test and Dunn posthoc test for comparison between groups at each time point, as compared with the saline group; the Friedman test and Dunn posthoc test were used for intragroup comparisons against their respective baseline. The total distance traveled and the average speed were analyzed by analysis of variance (2-way-ANOVA), followed by Dunnet posthoc test for group comparisons over time, and as compared with the saline group. The correlations between

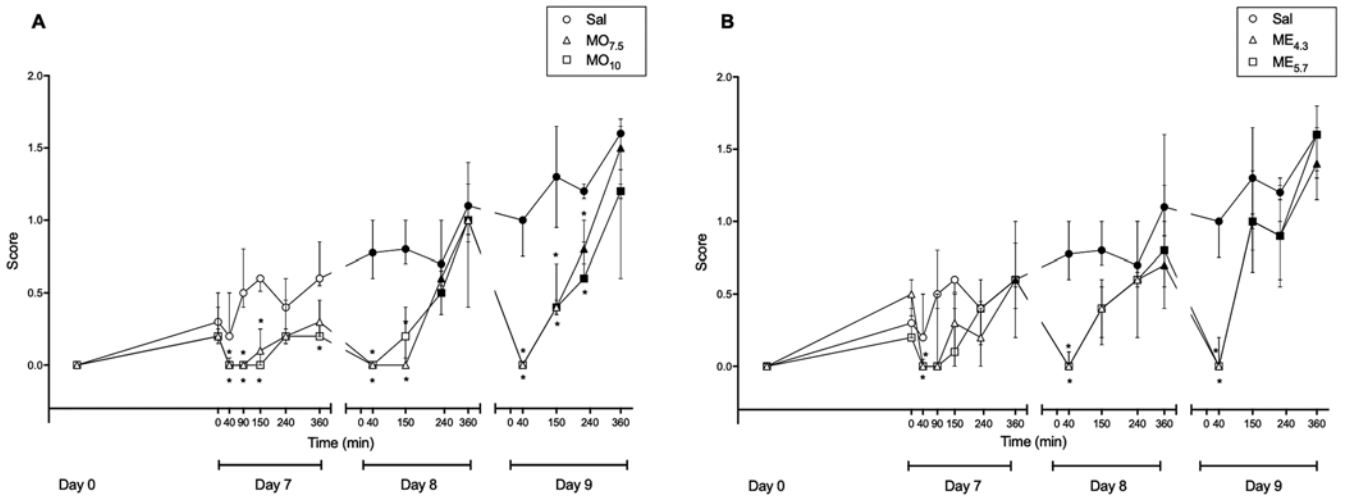

Figure 2. (A) MGS scores over time after repeated administration (every 6 h) of morphine (Mo) or (B) methadone (Me) in different dosages, for 3 d (day 7, 8 and 9 after tumor inoculation). Values are expressed as median (interquartile range) (n = 7). Black symbols express difference to the baseline values and * express differences to the Saline group (Kruskal–Wallis and Dunn posthoc test $P < 0.05$).

parameters (distance traveled, average speed, rearing frequency, and self-grooming) and drug doses were assessed using Pearson correlation. The level of significance was set as 5% ($P < 0.05$).

Results

After tumor inoculation, the animals were observed daily to assess daily water and food consumption and overall body condition. All mice developed Ehrlich ascitic carcinoma during the 7 d of incubation, and no animals died before the end of the experimental period; none of the mice were excluded from the study. In general, the abdominal circumference increased from day 6 (7.9 ± 0.3 cm) as compared with day 0 (7.1 ± 0.3 cm) ($P < 0.04$). Lower locomotor activity and grooming began at day 7 and worsened as the experimental period progressed. Feed intake (g) was significantly lower on days 8 (5.8 ± 1.6) and 9 (7.5 ± 1.6) as compared with day 1 (13 ± 2.4) ($P < 0.01$).

Mouse Grimace Scale. In the saline group, the mice had higher MGS scores, consistent with pain, from 40 min of day 8 (0.7 [0.6 to 1]) as compared with the baseline values (0 [0-0]) ($P < 0.01$). In the $MO_{7.5}$ and MO_{10} groups, a higher pain score occurred after 240 min on day 8 (0.6 [0.5–0.6]) ($P < 0.01$); on day 9, the $MO_{7.5}$ group had a higher score after 150 min (0.4 [0.3–0.7]) and in the MO_{10} group, after 240 min (0.6 [0.6–0.8]). In the $ME_{4.3}$ and $ME_{5.7}$ groups, the mice had higher scores at 360 min on day 8 (0.7 [0.5 –1]) and after 150 min (1 [0.8–1]) on day 9, as compared with their baseline values (0 [0–0]) ($P < 0.01$) (Figure 2).

In comparison to the saline group, animals from the $MO_{7.5}$ group had lower scores from 40 (0 [0–0.05]) to 150 min (0.1 [0–0.2]) on days 7 and 8 and up to 240 min (0.6 [0.5–0.6]) on day 9 ($P < 0.01$). In the MO_{10} group, lower scores occurred from 40 (0 [0–0.05]) to 360 (0.2 [0.2–0.4]) min on day 7, up to 150 min (0.2 [0–0.4]) on day 8 and up to 240 min (0.6 [0.6–0.8]) on day 9 ($P < 0.01$). In the $ME_{4.3}$ and $ME_{5.7}$ groups, a lower score occurred only at 40 min on days 7 (0 [0–0]), 8 (0 [0–0.1]) and 9 (0 [0–0.2]) ($P < 0.01$) (Figure 2).

Distance traveled. In the saline group, the total distance traveled (cm/5min) decreased significantly at all evaluated time points as compared with the baseline ($P < 0.0225$). A positive correlation was observed between the morphine doses evaluated and the distance traveled (r = 0.86; $P < 0.01$). In the MO_5 group, the distance traveled increased on days 8 and 9 at 40 min (3995 ± 1153) as compared with the baseline (2105 ± 231) ($P < 0.01$); a decrease also occurred on days 7 (1521 ± 340), 8 (1083 ± 278) and 9 (1166 ± 263) at 360 min ($P < 0.0490$). In the $MO_{7.5}$ group, the distance traveled increased as compared with baseline values at 40 min on day 7 (3475 ± 328), 8 (4535 ± 379) and 9 (4910 ± 304), and at 90 min on day 7 (3249 ± 305) ($P < 0.01$). A decrease occurred at 360 min on days 7 (1473 ± 152), 8 (1239 ± 150) and 9 (990 ± 108) ($P < 0.0101$); at 0 min on days 8 and 9 (1239 ± 150) and at 150 min on day 9 (1517 ± 399). In the MO_{10} group, the distance traveled was higher at 40 min on days 7 (3610 ± 819), 8 (5370 ± 1985) and 9 (5492 ± 2247), and at 90 min on day 7 (3212 ± 1369) as compared with the baseline ($P < 0.01$). A decrease occurred at 360 min on days 8 (830 ± 321) and 9 (856 ± 302) ($P < 0.01$) and at 0 min on days 8 and 9 (830 ± 321).

Compared with the saline group (1613 ± 216), the distance traveled was greater in the MO_5 (2594 ± 360), $MO_{7.5}$ (3475 ± 328) and MO_{10} (3610 ± 819) groups at 40 min on day 7 ($P < 0.01$); at 40 min on day 8 in the MO_5 (3994 ± 1152), $MO_{7.5}$ (4535 ± 379) and MO_{10} (4910 ± 304) groups ($P < 0.01$); and day 9 in the MO_5 (3817 ± 1614), $MO_{7.5}$ (4910 ± 304) and MO_{10} (5492 ± 2247) groups ($P < 0.01$). An increase occurred at 90 min in the MO_5 (2728 ± 797), $MO_{7.5}$ (3249 ± 505) and MO_{10} (3512 ± 1369) groups ($P < 0.01$) on day 7; and at 150 min in the MO_{10} group on days 7 (2243 ± 810) and 8 (2161 ± 275) ($P < 0.01$).

With regard to the methadone treatments, a correlation was observed between the tested doses and the distance traveled (r = 0.59; $P < 0.01$). In the $ME_{2.85}$ group, an increase in the distance traveled occurred at 40 min on days 8 (3423 ± 1240) and 9 (3938 ±1427) as compared with baseline (2185 ± 293) ($P < 0.01$). A decrease occurred at 240 (1437 ± 391) and 360 min (1171 ± 475) on day 7; 150 to 360 min on days 8 and 9 (1143 ± 337) ($P < 0.0333$) and on days 8 and 9 at 0 min (1171 ± 475). In the $ME_{4.3}$ group, an increase in the distance traveled occurred at 40 min on days 7 (2933 ± 700), 8 (4464 ± 973), and 9 (5505 ± 445), as compared with baseline (2056 ± 261) ($P < 0.0209$). A decrease occurred from 150 to 360 min on days 8 and 9 (948 ± 282) ($P < 0.01$) and on days 8 and 9 at 0 min (880 ± 248). In the $ME_{5.7}$ group, an increase in the distance traveled occurred at 40 min on days 7 (3203 ± 447), 8 (4398 ± 1327), and 9 (5037 ± 1300), as compared with baseline (2069 ± 369) ($P < 0.01$). A decrease occurred at 240 (1291 ± 533) and 360 min (1244 ± 443) on day 7 ($P < 0.0313$); 150 to 360 min

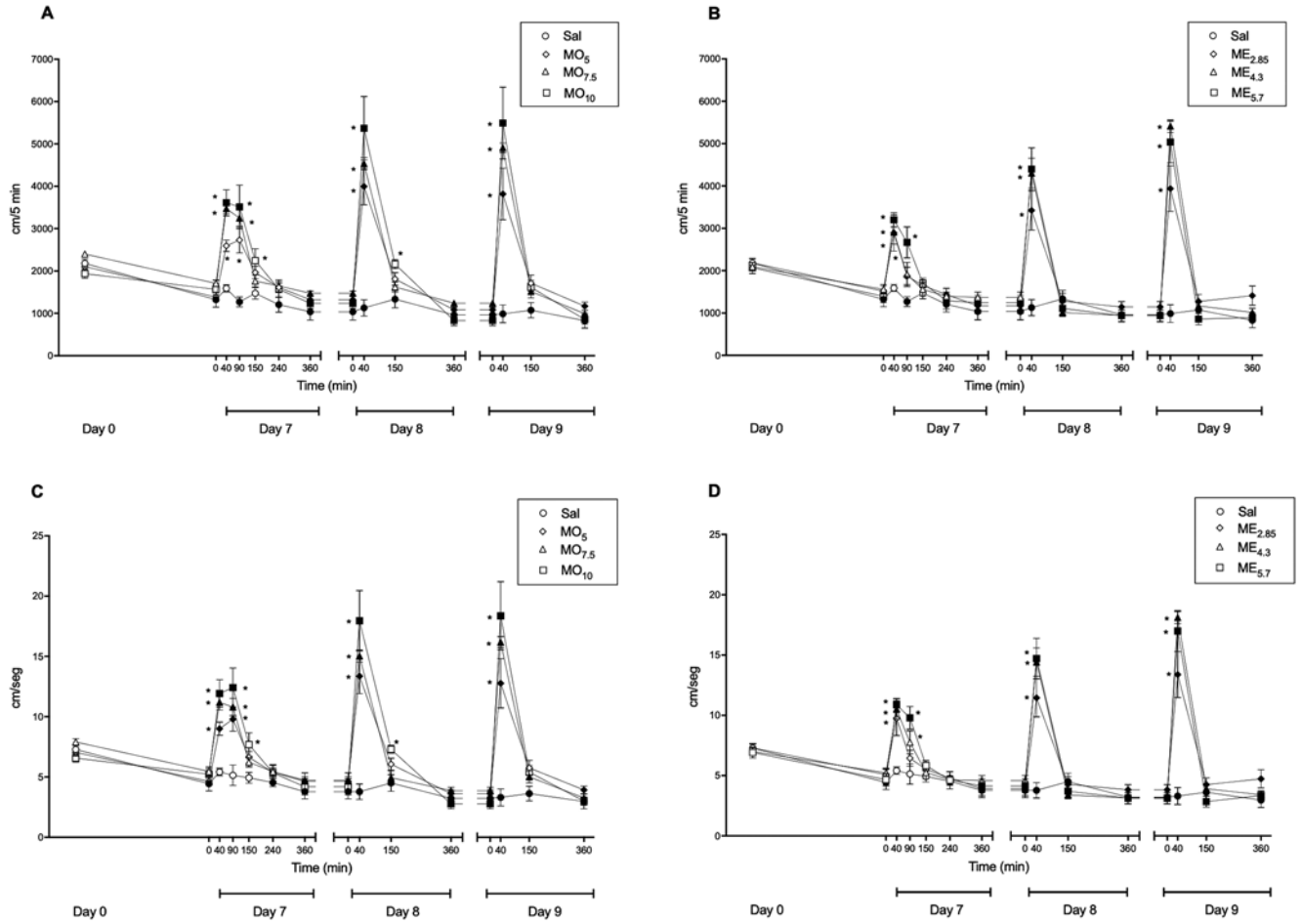

Figure 3. Locomotor activity (distance traveled and average speed) over time after repeated administration (every 6 h) of morphine (MO) or methadone (ME) in different dosages, for 3 d (day 7, 8 and 9 after tumor inoculation). Values are expressed as mean ± error deviation (n = 7). Black symbols express difference to the baseline values and * express differences to the Saline group (Two-way ANOVA and Dunnet posthoc test, $P < 0.05$). (A) Morphine distance traveled, (B) methadone distance traveled, (C) average speed morphine, (D) average speed methadone.

on days 8 and 9 (1111 ± 384) ($P < 0.01$) and on days 8 and 9 at 0 min (938 ± 359).

As compared with the saline group (1613 ± 216), the distance traveled by the $ME_{2.85}$, $ME_{4.3}$ and $ME_{5.7}$ groups was greater at 40 min on days 7 (2933 ± 701) ($P < 0.01$), 8 (4465 ± 973) ($P < 0.01$), and 9 (5505 ± 445) ($P < 0.01$). An increase was detected at 90 min on day 7 in the $ME_{2.85}$ (2891 ± 1142), $ME_{4.3}$ (2933 ± 700) and $ME_{5.7}$ (3203 ± 447) groups ($P < 0.0350$); and at 360 min on day 9 in the $ME_{2.85}$ group ($P < 0.0443$) (Figure 3).

Average speed. In the saline group, the average speed (cm/sec) decreased as compared with the baseline speed (7 ± 1) ($P < 0.0259$), except at 40, 90, and 150 min of day 7. A correlation was found between morphine dose and the average speed (r = 0.81; $P < 0.01$). In the MO_5 group, the average speed was increased at 40 min on days 8 (13 ± 4) and 9 (13 ± 5) as compared with baseline (7 ± 1) ($P < 0.01$). Moreover, an increase occurred at 90 min on day 7 (10 ± 3) ($P < 0.0342$) and a decrease at 360 min on days 8 (4 ± 1) and 9 (4 ± 1) ($P < 0.0105$). In the $MO_{7.5}$ group, the average speed was higher at 40 min on days 7 (11 ± 2), 8 (15 ± 1) and 9 (16 ± 1), and at 90 min on day 7 (11 ± 2) as compared with baseline ($P < 0.01$). A decrease occurred at 150 min on days 8 (5 ± 1) and 9 (5 ± 1); 360 min on days 7 (5 ± 1), 8 (4 ± 1) and 9 (3 ± 0) ($P < 0.0218$); and at 0 min on days 8 and 9 (5 ± 1) ($P < 0.01$). In the MO_{10} group, the average speed was higher at 40 min on days 7 (12 ± 3), 8 (18 ± 7) and 9 (18 ± 7) and at 90 min on day 7 (12 ± 4) as compared with baseline ($P < 0.01$). A decrease occurred at 360 min on days 8 (3 ± 1) and 9 (3 ± 1) ($P < 0.01$) and at 0 min on days 8 and 9 (3 ± 1).

As compared with the saline group, the average speed was higher in the MO_5, $MO_{7.5}$ and MO_{10} groups at 40 to 90 min on day 7 and at 40 min on days 8 and 9 ($P < 0.01$). Moreover, a difference between the MO_{10} and Saline groups occurred at 150 min on days 7 (8 ± 2), 8 (7 ± 1) and 9 (18 ± 7) ($P < 0.01$) (Figure 3).

A positive correlation was found between methadone doses and the average speed (r = 0.63; $P < 0.01$). In the $ME_{2.85}$ the average speed was higher at 40 min on days 8 (11 ± 4) and 9 (13 ± 5) as compared with baseline ($P < 0.01$). A decrease occurred at 240 and 360 min on day 7 ($P < 0.0443$), and at 150 to 360 min on days 8 and 9 ($P < 0.0345$) (Figure 3). In the $ME_{4.3}$ the average speed was higher at 40 min on days 7 (11 ± 2), 8 (11 ± 4), and 9 (13 ± 5) as compared with baseline ($P < 0.01$). A decrease occurred at 150 to 360 min on days 8 and 9 ($P < 0.01$) (Figure 3). The highest methadone dose ($ME_{5.7}$) increased the average speed at 40 (11 ± 1) to 90 (10 ± 2) min on day 7 and at 40 min on days 8 (15 ± 4) and 9 (17 ± 4) ($P = 0.0333$) (Figure 3).

As compared with the saline group (5 ± 1), mice from the $ME_{2.85}$ and $ME_{4.3}$ groups showed a significantly higher average speed at 40 min (10 ± 4) ($P < 0.01$) on all test days. In the $ME_{5.7}$ group, an

Table 1. Rearing frequency over time after repeated administration (every 6 h) of morphine (MO) or methadone (ME) in different dosages, for 3 d (day 7, 8 and 9 after tumor inoculation).

		Rearing frequency											
		Baseline	D7 40	D7 90	D7 150	D7 240	D7 360	D8 40	D8 150	D8 360	D9 40	D9 150	D9 360
Treatment	Saline	27 (19–37)	15 (9–20)	13 (7–16)	16 (8–21)	14 (4–18)	11 (1–17)*	9 (3–17)	16 (13–21)	11 (1–20)	6 (1–14)*	5 (2–12)*	7 (1–11)*
	MO5	32 (26–34)	0 (0–0)*#	0 (0–0)*#	3 (0–13)	9 (5–25)	7 (2–25)	0 (0–0)*#	3 (0–4)*#	6 (6–6)	0 (0–0)*#	2 (0–6)*	4 (1–6)
	MO7.5	27 (14–31)	0 (0–0)*#	0 (0–0)*#	0 (0–2)*#	11 (2–24)	7 (4–15)	0 (0–0)*#	0 (0–7)*#	6 (2–12)	0 (0–0)*#	0 (0–5)*	4 (1–7)
	MO10	29 (20–38)	0 (0–0)*#	0 (0–0)*#	3 (0–9)#	8 (4–22)	7 (1–14)	0 (0–0)*#	0 (0–3)*#	4 (2–6)	0 (0–0)*#	1 (0–2)*	3 (0–4)
	ME2.85	36 (29–38)	0 (0–0)*#	0 (0–1)*#	10 (2–18)	10 (5–24)	7 (1–22)	0 (0–0)*#	10 (7–14)	12 (8–16)	0 (0–0)*#	6 (4–12)	4 (2–12)
	ME4.3	30 (24–37)	0 (0–0)*#	0 (0–0)*#	0 (0–9)*#	11 (4–16)	11 (5–23)	0 (0–0)*#	2 (0–7)*#	6 (4–13)	0 (0–0)*#	2 (0–4)*	4 (2–10)
	ME5.7	25 (16–38)	0 (0–0)*#	0 (0–0)*#	0 (0–1)*#	4 (4–9)	5 (5–10)	0 (0–0)*#	1 (0–3)*#	4 (2–7)	0 (0–0)*#	1 (0–2)*	0 (0–1)*

Data are shown as median (interquartile range) (n = 7). * express difference to the baseline values (Friedman and Dunn posthoc test, P < 0.05) and # express differences to the Saline group (Kruskal–Wallis and Dunn posthoc test, P < 0.05).

increase in the average speed occurred between 40 (11 ± 1) (P < 0.01) and 90 (10 ± 2) (P < 0.01) min on day 7 and only at 40 min (P = 0.0001) on days 8 (15 ± 1) and 9 (17 ± 4) (P < 0.01) (Figure 3).

Rearing frequency. Mice in the saline group demonstrated a significant decrease in rearing frequency at 360 min on day 7 (11 [0.5–17.5]), and at every evaluated time point on day 9 (6 [1 to 14]) (P < 0.02). A negative correlation was found between the morphine dose and the rearing frequency (r = -0.8; P < 0.01). In addition, the rearing frequency was significantly lower in the MO_5 animals between 40 (0 [0–0]) and 90 min (0 [0–0]) on day 7 (P < 0.01) and between 40 (0 [0–0]) and 150 (0 [0–4]) min on days 8 and 9 (P < 0.0204). The $MO_{7.5}$ and MO_{10} groups had a lower earing frequency at all evaluated time points (P < 0.0231). As compared with the saline group, the MO_5 mice reared less on day 7 between 40 and 90 min (0 [0–0]) (P < 0.01). On day 8, a significant decrease occurred between 40 (0 [0–0]) (P < 0.01) and 150 (3 [0–4]) min (P = 0.0165); on day 9, a decrease occurred only at 40 min (0 [0–0]) (P = 0.0001). In the $MO_{7.5}$ and MO_{10} groups, rearing was less frequent from 40 (0 [0–0]) to 150 min on days 7 (0 [0–2]) and 8 (0 [0– 7]) (P < 0.0103) and at 40 min on day 9 (0 [0–0]) (P < 0.01) (Table 1).

The rearing frequency of the $ME_{2.85}$ mice was significantly lower between 40 (0 [0–0]) and 90 min (0 [0–1]) on day 7 (P < 0.01) and between 40 (0 [0–0]) and 150 (10 [7–14]) min on days 8 and 9 (P < 0.0204). In the $ME_{4.3}$ and $ME_{5.7}$ groups, a decrease in rearing frequency occurred at all evaluated time points (P < 0.0231). As compared with the saline group, the $ME_{2.85}$ mice reared less on day 7 between 40 (0 [0–0]) and 90 min (0 [0–1]) (P < 0.01). On day 8, a significant decrease occurred from 40 (0 [0–0]) (P < 0.01) to 150 (3 [0–4]) min (P = 0.0165); on day 9, a decrease occurred from 40 (0 [0–0]) (P < 0.01) to 150 min. In the $ME_{4.3}$ and $ME_{5.7}$ groups, the rearing frequency was lower from 40 (0 [0–0]) to 150 min on days 7 (0 [0–9]) and 8 (0 [0–7]) (P < 0.0103) and at 40 min on day 9 (0 [0–0]) to 360 min in the $ME_{5.7}$ group (4 [2–10]) (P = 0.0001) (Table 1).

Frequency of self-grooming. No significant differences in self-grooming were found in values from the saline group. A negative correlation was found between the grooming frequency and the morphine dose (r = -0.8; P < 0.01). In the MO_5 group, decreased grooming occurred at 40 min on days 7 (0 [0–0]), 8 (0 [0–0]), and 9 (0 [0–0]) (P < 0.0231) as compared with baseline (2 [1–3]). In the $MO_{7.5}$ and MO_{10} groups, a difference was detected at 40 and 90 min (0 [0–0]) (P < 0.0295) on day 7 and at 40 min on days 8 and 9 (0 [0–0]) (P < 0.0258). In the MO_5 and $MO_{7.5}$ groups, grooming was less frequent between 40 (0 [0–0]) (P < 0.01) and 90 min (0 [0–0]) (P < 0.0117) on day 7, between 40 (0 [0–0]) (P < 0.01) and 150 min (1 [1–1]) (P = 0.0377) on day 8, and only at 40 min (0 [0–0]) (P < 0.01) on day 9 as compared with the saline group. In MO_{10} mice, grooming was reduced between 40 (P < 0.01) and 90 min (0 [0–0]) (P = 0.0013) on day 7 and only at 40 min (0 [0–0]) (P < 0.01) on days 8 and 9 (Table 2).

Mice treated with methadone showed a correlation between self-grooming and dose (r = -0.4; P = 0.0103). The frequency of grooming fell as compared with baseline at 40 min on all test days (P < 0.0333) (Table 2). In the $ME_{2.85}$ group, decreased grooming occurred at 40 min (0 [0–0]) (P < 0.01) on day 7, between 40 (0 [0–0]) (P < 0.01) and 150 min (1 [1–1]) (P = 0.0299) on day 8, and only at 40 min (0 [0–0]) (P < 0.01) on day 9. In $ME_{4.3}$ mice, significant differences occurred only at 40 min (0 [0–0]) (P < 0.01) on all test days. In the $ME_{5.7}$ group, the frequency of grooming significantly differed between 40 (0 [0–0]) (P < 0.01) and 90 min (0 [0–1]) (P = 0.0225) on day 7, at 150 min (1 [0–2]) (P < 0.0389) on day 8, and at 40 min (0 [0–0]) (P < 0.01) on day 9 (Table 2).

Discussion

In this study, all mice showed physical and behavioral changes secondary to tumor development. The choice to use female mice of this specific strain was based of their higher sensitivity to ambulation tests after morphine and methadone administration.[5,10,26] Ehrlich tumor model is considered highly aggressive, with the induced disease having both inflammatory and compressive characteristics.[15] The mice displayed reduced activity in the cage, reduced grooming behavior from the 7th day of evaluation, and an increase in abdominal circumference that was significant from the 6th day of evaluation. Although food intake was significantly lower on days 8 and 9, we could not distinguish between experimental groups due to their random placement in cages. Although the exact onset of pain remains undefined, the signs observed in these mice are considered representative of oncological disease.[42] Locomotor activity and exploratory behavior can also reflect the presence of pain[7] and were analyzed in mice with Ehrlich carcinoma whether or not they received opioids.[9] This model was selected due to its frequent use in studies of both pro- and anti-tumor agents and the absence of an established specific protocol for pain management.[17,27,34,33]

In cancer patients, pain is commonly associated with tumor growth.[32] Based on the MGS, pain was present in mice from the saline group beginning with the first evaluation on day 8 of the study. The analgesia promoted by 10 mg/kg of morphine reduced allodynia and the MGS scores at 1 h after administration when assessed in a 4T1 breast cancer model in mice.[2] In our study, morphine reduced the MGS scores for 150 min after the first administration, correlating with another study that recommends the repeated administration of morphine every 2 to 3 h.[18] We further found that morphine could promote analgesia for

Table 2. Grooming frequency over time after repeated administration (every 6 h) of morphine (MO) or methadone (ME) in different dosages, for 3 d (day 7, 8 and 9 after tumor inoculation).

		Grooming frequency											
		Baseline	D7 40	D7 90	D7 150	D7 240	D7 360	D8 40	D8 150	D8 360	D9 40	D9 150	D9 360
Treatment	Saline	2 (1–3)	2 (2–4)	2 (1–2)	2 (1–4)	2 (1–2)	2 (1–2)	2 (2–3)	3 (2–3)	2 (1–3)	2 (1–2)	2 (2–2)	1 (1–1)
	MO5	2 (1–3)	0 (0–0)*#	0 (0–0)#	1 (1–2)	2 (1–2)	3 (1–4)	0 (0–0)*#	1 (1–1)#	3 (2–3)	0 (0–0)*#	3 (2–3)	2 (1–2)
	MO7.5	2 (1–4)	0 (0–0)*#	0 (0–0)*#	2 (1–2)	2 (1–2)	2 (2–5)	0 (0–0)*#	1 (1–2)#	2 (2–2)	0 (0–0)*#	1 (0–1)	2 (1–3)
	MO10	2 (1–3)	0 (0–0)*#	0 (0–0)*#	1 (0–2)	2 (1–3)	1 (1–4)	0 (0–0)*#	1 (1–2)	3 (1–4)	0 (0–0)*#	1 (1–2)	2 (1–4)
	ME2.85	3 (2–3)	0 (0–0)*#	1 (1–2)	1 (1–2)	1 (1–3)	2 (1–5)	0 (0–0)*#	1 (1–1)	1 (1–2)	0 (0–0)*#	2 (2–3)	1 (1–3)
	ME4.3	2 (1–3)	0 (0–0)*#	1 (0–2)	2 (1–2)	1 (1–3)	4 (2–5)	0 (0–0)*#	1 (1–2)	1 (1–3)	0 (0–0)*#	2 (1–3)	2 (2–3)
	ME5.7	2 (1–2)	0 (0–0)*#	0 (0–1)#	1 (1–2)	2 (1–4)	2 (1–4)	0 (0–0)*#	1 (0–2)#	2 (1–3)	0 (0–0)*#	2 (1–2)	1 (1–3)

Data are shown as median (interquartile range) (n = 7). * express difference to the baseline values (Friedman and Dunn posthoc test, $P < 0.05$) and # express differences to the Saline group (Kruskal–Wallis and Dunn posthoc test, $P < 0.05$).

up to 240 min after serial administration at doses of 7.5 and 10 mg/kg. We tested methadone in our study due to its analgesic properties and the possibility of reversing the tolerance caused by morphine in mice.[45] However, although the dose of methadone was chosen based on literature[13,35,46] to be equipotent to morphine,[31,44] equivalent analgesia was not observed.

After morphine administration, open-field locomotor activity increased in a dose-dependent manner, even at doses lower than those previously studied, to a similar degree as in other studies.[29,43,51] Although activity increased significantly after methadone administration, as previously verified,[39] the effect was not dose-dependent, perhaps due to insufficient differences among doses that were expected to be equipotent to the morphine doses.

In our study, open-field locomotor activity was higher on days 8 and 9 than on day 7 for both the intermediate and the highest morphine and methadone doses. This has not been documented in previous studies in which activity was evaluated after a single administration of drug.[5,39,43] Drug administration for 7 d has been investigated, but the locomotor activity increase over the initial days was not evaluated.[51] Some have postulated that the increased locomotor activity after repeated drug administration is related either to adequate pain control or to the excitation promoted by the opioids, secondary to the dopamine release in the nucleus accumbens.[11,25,29] In addition, a cumulative effect may be involved. To clarify this, higher doses or a longer duration of treatment would have to be tested.

A strong negative correlation was observed between the total distance traveled/average speed and the rearing frequency in morphine-treated mice, corroborating another study.[43] Rearing frequency is triggered and modulated by the hippocampus, and is enhanced in situations of uncertainty or after the administration of psychoactive agents such as caffeine,[40] or ketamine.[37] Reduced rearing occurs when the environment is deemed dangerous or unfamiliar, thus inhibiting the exploratory drive[21] in situations of stress, anxiety,[50] ataxia, or stimulating a competing increase in horizontal locomotor activity.[19] Morphine-treated mice showed a lower rearing frequency and a higher average speed, and consequently, an increased horizontal distance traveled.

Grooming behavior is considered innate in rodents and is associated with hygiene maintenance, thermoregulation, social communication, and excitement.[49] It remains one of the primary behaviors assessed in rodents and has a distinct sequence, with cephalocaudal progression.[48] Drugs that alter dopamine release,[1] as does morphine, or that have GABAergic inhibitory action[4] and NMDA antagonism, as does methadone, can modify the frequency and the sequence of the self-grooming process in rodents.[3] The frequency of self-grooming fell after the administration of both morphine and methadone. Furthermore, both increases and decreases in this behavior have been associated with the clinical manifestation of oncologic pain in both rats and mice.[42] Although the reduction in self-grooming was significant, the behavior returned to a pattern close to the saline group by 90 min after drug administration.

One of the limitations of this study was the omission of a group that received opioids in the absence of a tumor. This group not included in order to reduce the number of mice used in the study. Instead, we compared our data with preexistent literature. In addition, the use of higher doses of methadone would have tested whether the drug can provide analgesia for more than 40 min in the experimental conditions. Moreover, a pro- and anti-inflammatory cytokine profile might have been useful to assess the effects of morphine and methadone on the inflammatory response produced by mice with Ehrlich ascitic carcinoma.

In summary, we found that repeated administration of morphine at doses of 7.5 and 10 mg/kg promoted analgesia in mice with Ehrlich carcinoma for 4 h. Methadone, however, was not analgesic at the doses used in our study. Our results corroborate some findings in the literature that describe an increase in locomotor activity and a decrease in self-grooming and rearing frequency in mice after the subcutaneous administration of morphine or methadone.[39,43,51] These effects became more pronounced over the days of treatment. Thus, morphine should still be considered the drug of choice for pain management in mice with Ehrlich ascitic carcinoma.

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