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## Hyperbaric oxygen treatment suppresses withdrawal signs in morphine-dependent mice

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### Abstract

Hyperbaric oxygen (HBO<sub>2</sub>) therapy reportedly reduces opiate withdrawal in human subjects. The purpose of this research was to determine whether HBO<sub>2</sub> treatment could suppress physical signs of withdrawal in opiate-dependent mice. Male NIH Swiss mice were injected s.c. with morphine sulfate twice a day for 4 days, the daily dose gradually increasing from 50 mg/kg on day 1 to 125 mg/kg on day 4. On day 5, withdrawal was precipitated by i.p. injection of 5.0 mg/kg naloxone. Mice were observed for physical withdrawal signs, including jumping, forepaw tremor, wet-dog shakes, rearing and defecation for 30 min. Sixty min prior to the naloxone injection, different groups of mice received either a 30-min or 60-min HBO<sub>2</sub> treatment at 3.5 atmospheres absolute. HBO<sub>2</sub> treatment significantly reduced naloxone-precipitated jumping, forepaw tremor, wet-dog shakes, rearing and defecation. Based on these experimental findings, we concluded that treatment with HBO<sub>2</sub> can suppress physical signs of withdrawal syndrome in morphine-dependent mice.

### Keywords

Hyperbaric oxygen; morphine; opiate withdrawal; physical dependence; mouse

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## 1. Introduction

Hyperbaric oxygen (HBO<sub>2</sub>) is the clinical application of 100% oxygen under higher-than-normal atmospheric pressure to achieve therapeutic outcomes. On the recommendation of the Hyperbaric Oxygen Therapy Committee of the Undersea and Hyperbaric Medical Society, HBO<sub>2</sub> therapy is approved by the U.S. Food and Drug Administration (FDA) for 14 clinical conditions [Weaver, 2014] but currently not for treatment of opiate addiction. According to a published report, HBO<sub>2</sub> was beneficial in patients with narcomania (narcotic addiction) in the post-intoxication and abstinence periods [Epifanova, 1995]. However, this study remains unconfirmed.

Previously, we reported that HBO<sub>2</sub> produced an antinociceptive effect in mice that was dependent on both nitric oxide (NO) and opioid receptors [Zelinski *et al.*, 2009]. Further studies suggested that HBO<sub>2</sub> might be capable of stimulating neuronal release of endogenous opioid peptides [Heeman *et al.*, 2013]. The purpose of the present investigation was to determine in an animal model whether HBO<sub>2</sub> might be capable of reducing the morphine withdrawal syndrome.

## 2. Results

The intraclass correlation coefficients (ICCs) for two independent raters assessing the impact of HBO<sub>2</sub> treatment on physical withdrawal signs were as follow: jumps ( $\alpha = 0.995$ ,  $P = 0.000$ ); rears ( $\alpha = 0.878$ ,  $P = 0.000$ ); and fecal boli ( $\alpha = 0.826$ ,  $P = 0.000$ ). ICCs were not determined for tremors or wet-dog shakes because they were scored in real time by a single rater.

Morphine-dependent mice exhibited averages of  $24.6 \pm 8.5$  jumps,  $15.6 \pm 2.4$  tremors,  $2.9 \pm 0.7$  wet-dog shakes,  $92.2 \pm 6.8$  rears, and  $6.3 \pm 0.8$  fecal boli during a 30-min observation period following administration of naloxone. After both 30- and 60-min HBO<sub>2</sub> treatments, there were statistically significant decreases in all five endpoints: jumps [Welch's  $F(2, 15.737) = 4.510$ ,  $P = 0.028$ ]; tremors [Welch's  $F(2, 26.911) = 6.815$ ,  $P = 0.004$ ]; wet-dog shakes [Welch's  $F(2, 26.586) = 5.790$ ,  $P = 0.008$ ]; rears [ $F(2, 27) = 5.384$ ,  $P = 0.011$ ]; and fecal boli [Welch's  $F(2, 17.719) = 8.963$ ,  $P = 0.002$ ] (Figs. 2–6).

## 3. Discussion

We found that naloxone precipitated five cardinal signs of opiate withdrawal—jumping, forepaw tremor, wet-dog shakes, rearing, and defecation [Gabra *et al.*, 2008; Seyedi *et al.*, 2014; Wu *et al.*, 2014]—in morphine-dependent mice. The frequency of all five withdrawal signs was reduced by HBO<sub>2</sub> treatment, although the reduction in only four endpoints was statistically significant.

The implications of this research are of profound importance. Current therapies used to treat heroin addiction largely employ opiate medications, including agonists, partial agonists and antagonists. While these drugs can provide relief from the opiate withdrawal syndrome, they are still prone to development of physical dependence and risk of relapse [Kleber, 2007].

Although one isolated study reported that HBO<sub>2</sub> alleviated opiate withdrawal in humans [Epifanova, 1995], the present study is, to our knowledge, the first to demonstrate this phenomenon in an animal model of opiate dependence. The withdrawal-suppressing effects of HBO<sub>2</sub> are clear from our results, but its mechanism of action is less clear.

Other studies have been able to precipitate withdrawal in morphine-dependent mice with much lower doses of naloxone [Ho *et al.*, 1972; Friedler *et al.*, 1972]. One reason for selection of 5.0 mg/kg of naloxone as our dose for precipitating withdrawal was a previous finding from our laboratory that HBO<sub>2</sub>-induced antinociception in rodents was sensitive to antagonism by both opioid antagonist drugs as well as rabbit antisera against selected endogenous opioid peptides, namely dynorphin [Zelinski *et al.*, 2009; Heeman *et al.*, 2013; Gibbons *et al.*, 2013]. This suggests that a mechanism of HBO<sub>2</sub>-induced antinociception is stimulation of neuronal release of opioid peptides. Since increased opioid peptides are a possible mechanism for relief of opiate withdrawal [Dzoljic *et al.*, 1986; Maldonado *et al.*, 1989], the higher dose of naloxone was selected to determine whether greater opioid receptor blockade might interfere with the ability of HBO<sub>2</sub> to ameliorate withdrawal signs.

There is also evidence that HBO<sub>2</sub> treatment increases levels of nitric oxide (NO) oxidation products in the rat brain [Ohgami *et al.*, 2008], increases expression of constitutive nitric oxide synthase (NOS) [Lin *et al.*, 2011], and stimulates synthesis of NO in brain tissue [Thom *et al.*, 2003]. Our own studies have consistently demonstrated that inhibition of brain NOS antagonizes HBO<sub>2</sub>-induced antinociception [Ohgami *et al.*, 2009; Zelinski *et al.*, 2009; Chung *et al.*, 2010]. These findings suggest that HBO<sub>2</sub> increases NOS activity. If an increase in NO activity is involved in suppression of opiate withdrawal by HBO<sub>2</sub>, it runs contrary to evidence that increased NOS activity is involved in the expression of opiate withdrawal itself [Machelska *et al.*, 1997; Kumar and Bhargava, 1997; Cuéllar *et al.*, 2000]. This is evidenced by suppression of the opiate withdrawal syndrome by NOS inhibitors [London *et al.*, 1995; Bhargava, 1995; Leza *et al.*, 1996; Dambisya and Lee, 1996]. This discrepancy may represent a delicate balance in the influence of NO on expression of opioid withdrawal not unlike the reported pronociceptive vs. antinociceptive roles of NO [Schmidtko *et al.*, 2009; Schmidtko, 2015], anxiogenic vs. anxiolytic functions of NO [Volke *et al.*, 1997; Vale *et al.*, 1998] as well as its involvement in neurotoxicity vs. neuroprotection [Calabrese *et al.*, 2007]. Although further research is needed to elucidate the mechanism of action of HBO<sub>2</sub>, this study provides grounds for optimism in the search for effective alternative treatments for opiate withdrawal.

## 4. Experimental procedures

### 4.1. Animals

Male NIH Swiss mice, 20–26 g, were purchased from Harlan Laboratories (Indianapolis, Indiana) for this research. All experiments were approved by the Washington State University Institutional Animal Care and Use Committee subject to post-approval review. Research was conducted in accordance with The Guide for the Care and Use of Laboratory Animals, 8<sup>th</sup> Edition (National Academies Press, Washington, DC, 2010).

Mice were housed four per cage in the Wegner Hall Vivarium at Washington State University with access to food and water ad libitum. The facility, which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), was maintained on a 12-h light:dark cycle (lights on 07:00–19:00 h) under standard conditions ( $22 \pm 1^\circ\text{C}$  room temperature, 33% humidity). Mice were kept in the holding room for at least four days after arrival in the facility for acclimation prior to use.

#### 4.2. Treatment with HBO<sub>2</sub>

Mice were placed in a B-11 research hyperbaric chamber (Reimers Systems, Inc., Lorton, VA). The chamber was ventilated with 100% O<sub>2</sub>, U.S.P. (A-L Compressed Gases Inc., Spokane, WA) at a flow rate of 20 L/min to minimize accumulation of nitrogen and carbon dioxide. The pressure within the cylindrical clear acrylic chamber (27.9 cm diameter  $\times$  55.9 cm L) was increased from 1.0 to 3.5 ATA over a period of 2 min. Animals breathed spontaneously during the HBO<sub>2</sub> treatment. After conclusion of the HBO<sub>2</sub> treatment, mice were then decompressed over 2–3 min. Different groups of mice were treated for 30 or 60 min with HBO<sub>2</sub> at 3.5 atmospheres absolute (ATA). Control mice were placed in the open hyperbaric chamber and allowed to breathe room air.

#### 4.3. Drugs

The following drugs were used in this research: morphine sulfate, U.S.P. (West-Ward, Eatontown, NJ); and naloxone hydrochloride (Tocris Bioscience, Minneapolis, MN). Both drugs were freshly prepared in 0.9% physiological saline.

For induction of physical dependence, morphine sulfate was administered s.c. twice daily at 0830 and 1630 h for four consecutive days (Fig. 1). The daily dose of morphine sulfate was 50 mg/kg on day 1, 75 mg/kg on day 2, 100 mg/kg on day 3 and 125 mg/kg on day 4. To precipitate opiate withdrawal, 5.0 mg/kg naloxone was administered i.p. on day 5. The dose schedule was derived from the scientific literature [Seyedi et al., 2014], but the t.i.d. schedule for three days was lengthened to b.i.d. for four days.

#### 4.4. Assessment of withdrawal symptoms

Immediately following the naloxone injection, mice were placed in a Plexiglas® test chamber (40 cm L  $\times$  20 cm W  $\times$  25 cm H) in groups of four and video-recorded for 30 min. The frequency of distinct physical signs of withdrawal—jumping, forepaw tremor, and wet-dog shakes (whole-body shakes), rearing and defecation—were determined for each mouse. Vehicle-treated mice served as control. Multiple raters were used for some but not all experiments; at least one of the raters was blinded to the drug treatment.

#### 4.5. Statistical analysis of data

Inter-rater reliability was estimated using intraclass correlation coefficients (ICCs) (SPSS v. 23.0, SPSS Inc., Chicago, IL). Following determination of reliability between raters, each individual endpoint was investigated for outliers using Grubb's maximum normed residual test. Outliers identified by the Grubb's test caused all endpoint data from that animal to be removed from further analysis. Grubb's test identified outliers only in the jumping and wet dog shake endpoints. A one-way between groups analysis of variance (ANOVA) was run

along with Levene's test for homogeneity of variance. Where a violation of Levene's test was found, we used Welch's F test to analyze the differences in frequency of withdrawal signs between groups with unequal variances [Welch, 1951]. A Dunnett's post hoc test was applied to identify statistically significant differences between groups

## 5. Conclusion

A 30- or 60-min treatment with HBO<sub>2</sub> can suppress multiple naloxone-precipitated physical signs of withdrawal in morphine-dependent mice. These results support an earlier report that HBO<sub>2</sub> therapy can be beneficial in treatment of heroin addicts.

## Acknowledgments

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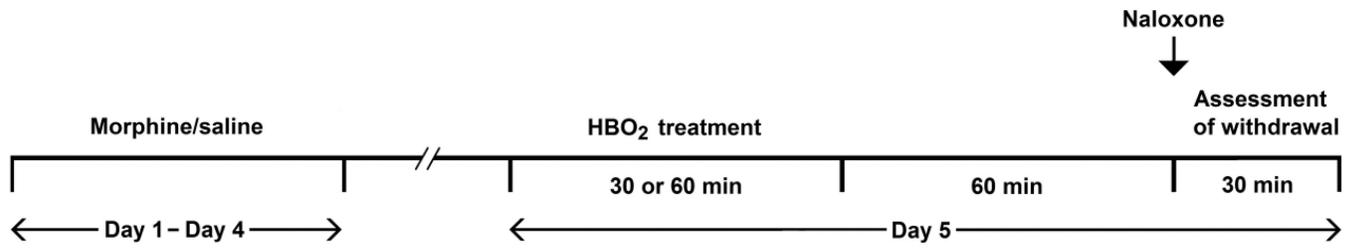
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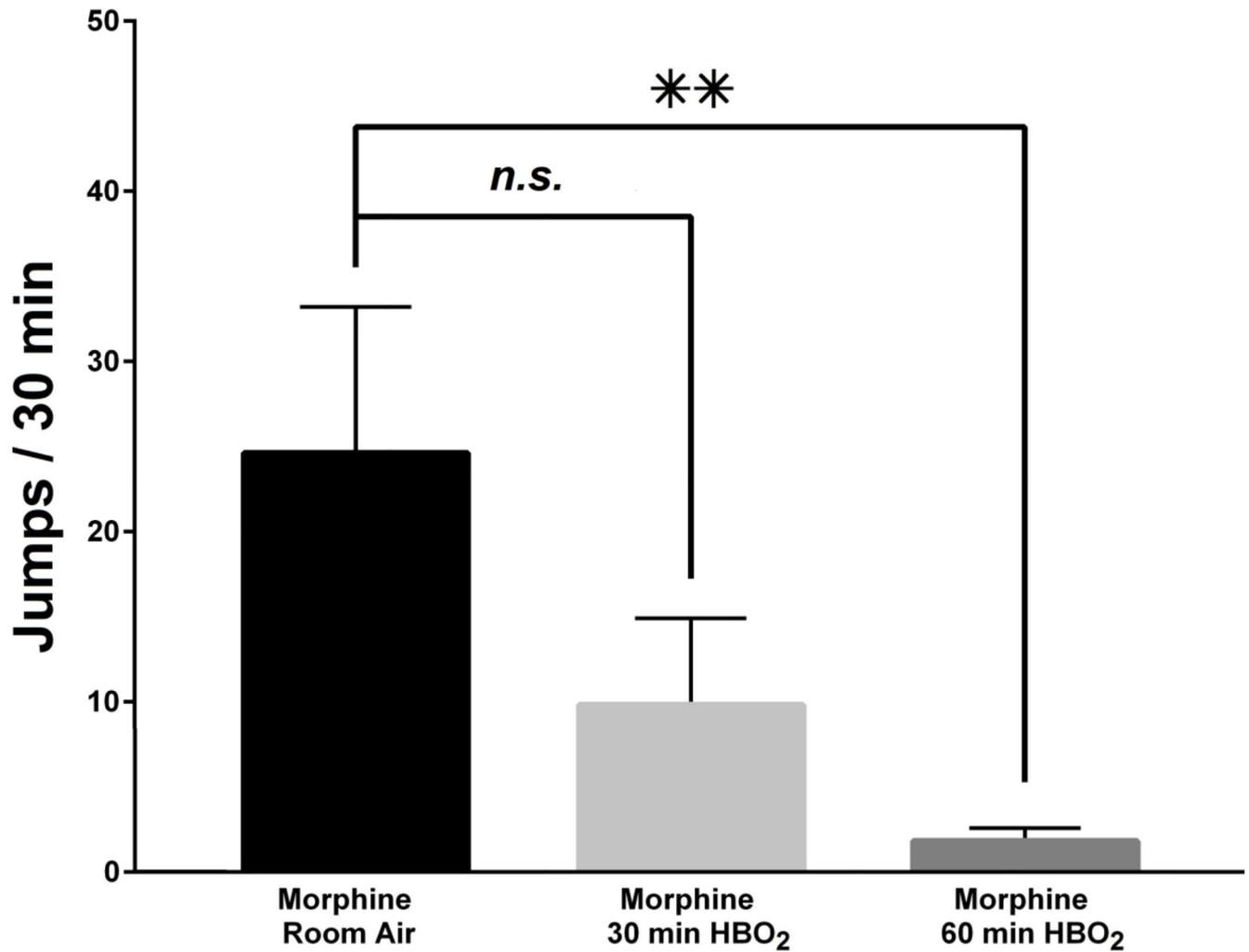
**Highlights**

- Physical dependence was induced by twice-daily morphine injections over 4 days.
- Naloxone precipitated jumping, tremors, wet-dog shakes, rearing and defecation.
- Withdrawal signs were suppressed by treatment with hyperbaric oxygen (HBO<sub>2</sub>).

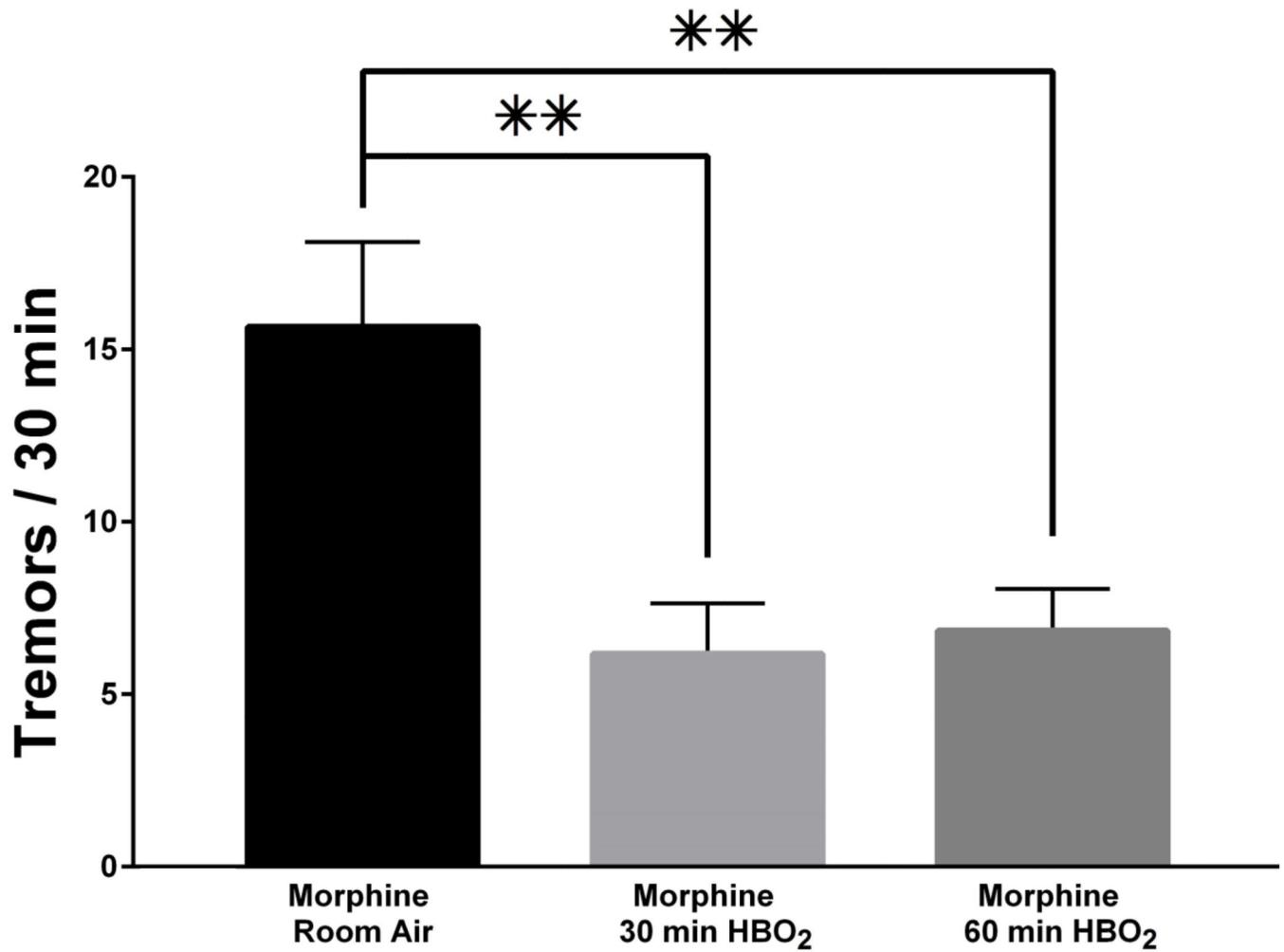


**Fig. 1.**

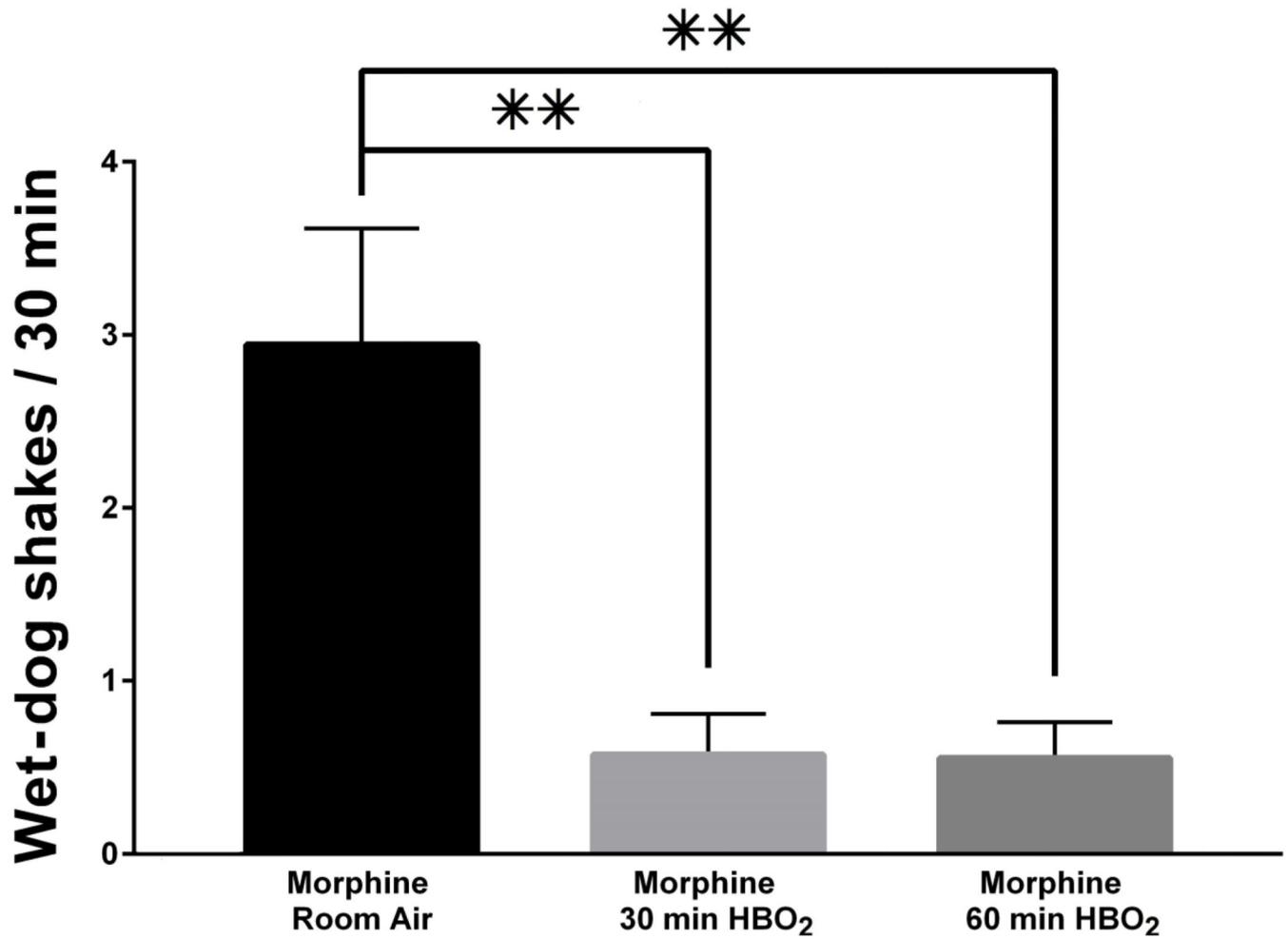
Timeline of research. Mice received s.c. injections of morphine sulfate or saline b.i.d. for four consecutive days. On day 5, all mice received HBO<sub>2</sub> or room air treatment for 30 or 60 min. One hour later, mice received an i.p. injection of naloxone to precipitate withdrawal symptoms or saline as control. Recording of withdrawal symptoms started immediately after the naloxone injection, and lasted for 30 min.



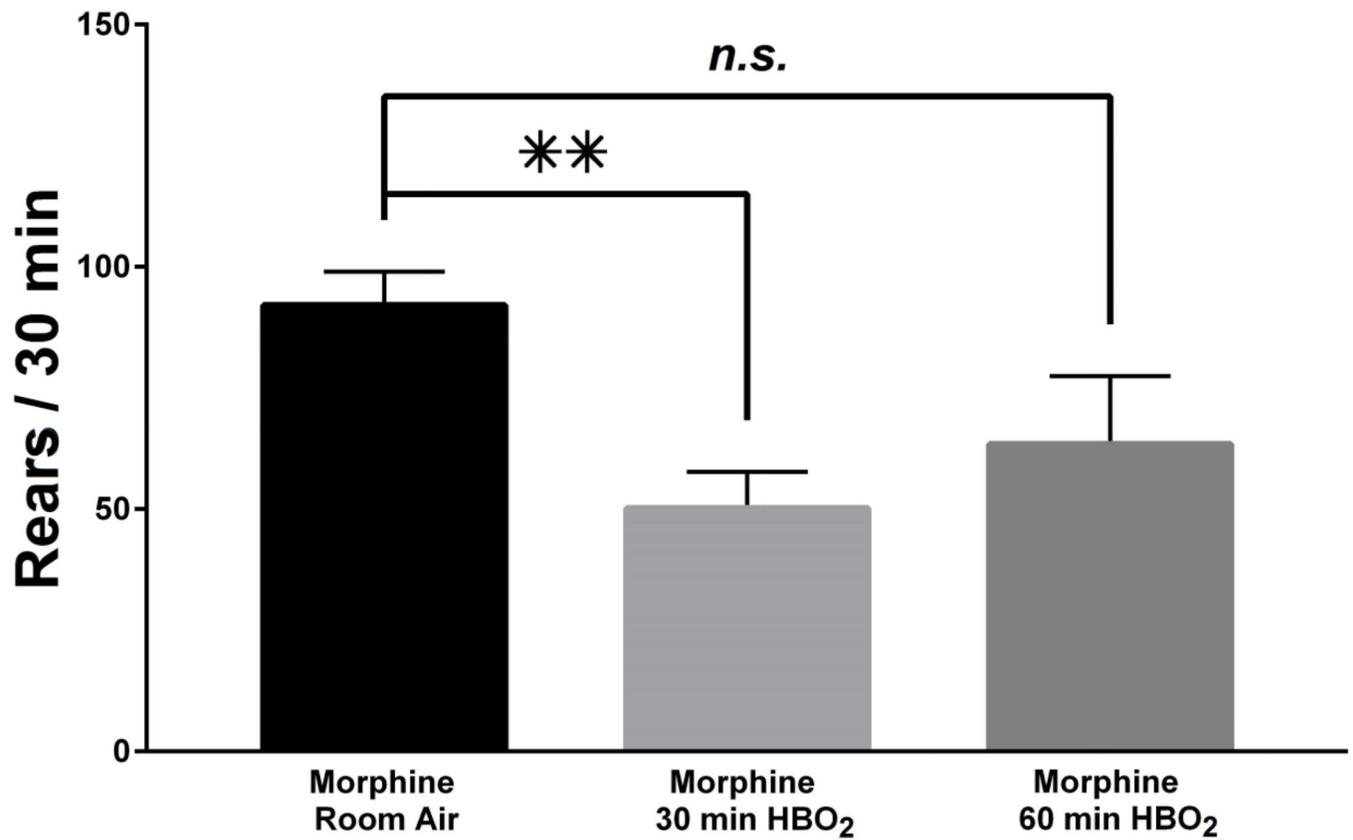
**Fig. 2.** Effect of HBO<sub>2</sub> on naloxone-induced jumping in chronic morphine-treated mice. Data are presented as the mean  $\pm$  SEM (Morphine + Room Air, N = 14; Morphine + 30-min HBO<sub>2</sub>, N = 14; and Morphine + 60-min HBO<sub>2</sub>, N = 21). Significance of difference: \*\*,  $P < 0.01$ , compared to Morphine + Room Air control group.



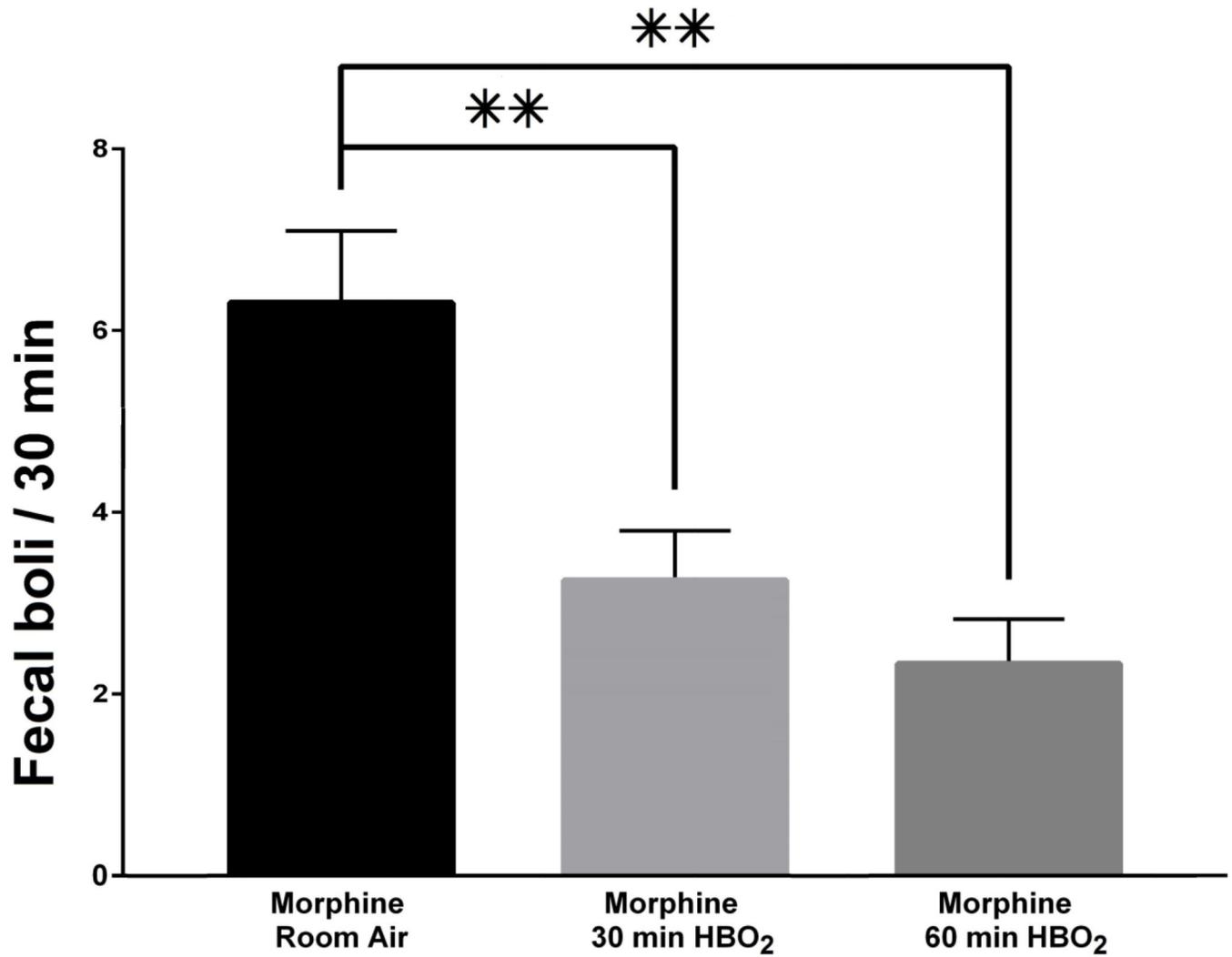
**Fig. 3.** Effect of HBO<sub>2</sub> on naloxone-induced tremor in chronic morphine-treated mice. Data are presented as the mean ± SEM (Morphine + Room Air, N = 18; Morphine + 30-min HBO<sub>2</sub>, N = 14; and Morphine + 60-min HBO<sub>2</sub>, N = 21). Significance of difference: \*\*,  $P < 0.01$ , compared to Morphine + Room Air control group.



**Fig. 4.** Effect of HBO<sub>2</sub> on naloxone-induced wet-dog shakes in chronic morphine-treated mice. Data are presented as the mean ± SEM (Morphine + Room Air, N = 18; Morphine + 30-min HBO<sub>2</sub>, N = 14; and Morphine + 60-min HBO<sub>2</sub>, N = 21). Significance of difference: \*\*,  $P < 0.01$ , compared to Morphine + Room Air control group.



**Fig. 5.** Effect of HBO<sub>2</sub> on naloxone-induced rearing in chronic morphine-treated mice. Data are presented as the mean ± SEM (Morphine + Room Air, N = 14; Morphine + 30-min HBO<sub>2</sub>, N = 10; and Morphine + 60-min HBO<sub>2</sub>, N = 12). Significance of difference: \*\*,  $P < 0.01$ , compared to Morphine + Room Air control group.



**Fig. 6.** Effect of HBO<sub>2</sub> on naloxone-induced defecation in chronic morphine-treated mice. Data are presented as the mean  $\pm$  SEM (Morphine + Room Air, N = 14; Morphine + 30-min HBO<sub>2</sub>, N = 10; and Morphine + 60-min HBO<sub>2</sub>, N = 12). Significance of difference: \*\*,  $P < 0.01$ , compared to Morphine + Room Air control group.