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Morphine's Effects on the Gut-brain Axis, Inflammation, Behavior, and Microbiome Modulation in Rats

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ABSTRACT

Background: The interaction between the gut microbiome and neurological outcomes is increasingly recognized, including in the context of opioid use and its immunomodulatory effects. This study investigates how morphine treatment interacts with the gut-brain axis, examining its impact on gut microbiome alterations and inflammatory gene expression, along with behavioral responses in a rat model.

Materials and Methods: Wistar rats were assigned to a morphine treatment group that was administered a fixed escalating dose of morphine sulfate (30 mg/kg) or a saline control. We further evaluated the expression of the inflammatory genes *IL-6*, *TNF-α*, and *INF-γ* in the intestinal tissue using quantitative real-time polymerase chain reaction. The behavior test used included the elevated plus maze (EPM) to quantify anxiety-like behavior. Gut microbiome alteration was assessed using the quantitative real-time polymerase chain reaction technique.

Results: Morphine-treated rats showed a significant increase in anxiety-like behaviors in the EPM test compared to controls ($P<0.01$). Gut microbiome analysis revealed significant alterations, including a decrease in beneficial bacterial species, specifically *Akkermansia muciniphila* ($P=0.0008$) and *Bifidobacterium longum* ($P=0.003$), alongside a significant increase in potentially pathogenic species, such as *Bacteroides fragilis* ($P<0.001$) and *Fusobacterium nucleatum* ($P<0.001$). Inflammatory gene expression analysis demonstrated a significant increase in *INF-γ* ($P<0.001$) and *TNF-α* ($P=0.03$), while *IL-6* did not reach statistical significance ($P=0.06$).

Conclusion: This study demonstrates considerable changes in the gut microbiome and increased anxiety-like behaviors after morphine administration, corroborating a bidirectional gut-brain relationship during opioid exposure. The results bring to light promising microbiota-targeted therapies as treatment options for opioid-related behavioral dysregulation.

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Introduction

The gut microbiome has emerged as a key element in maintaining total well-being, influencing a wide range of physiological processes, from metabolism and immunity to mental well-being [1]. A crucial aspect of this influence encompasses immunoregulation, where gut microbiota can modulate inflammatory pathways that impact neurological outcomes [2]. Recent research has highlighted the complex interplay between the gut microbiome and neurological consequences, terming the relationship the gut-brain axis [3, 4].

The bidirectional signaling significantly impacts how gastrointestinal health affects brain function and behavior, particularly in response to various pharmacological medications [5]. Opioids, such as morphine, are widely utilized in managing acute and chronic pain [6]. However, their use is often accompanied by side effects, including tolerance, dependence, and a range of psychological disturbances, including depression and anxiety [7]. Evidence indicates that opioid exposure can disrupt the immune response, particularly within the gut, leading to an imbalance in regulatory and inflammatory pathways [8]. Morphine has been shown to interact with opioid receptors located on immune cells, particularly in the gastrointestinal tract, which can affect the production of cytokines and other inflammatory mediators. Through these mechanisms, morphine may suppress the production of pro-inflammatory cytokines while promoting the release of anti-inflammatory factors, leading to a complex and often context-dependent modulation of immune responses [9]. The impact of opioids on the gut microbiome has come into the spotlight, as alterations in gut microbiota composition have been found to correlate with opioid-associated side effects [10].

Dysbiosis, as the microbial imbalance is known, can exacerbate these effects and may contribute to negative psychological outcomes associated with opioid use. For instance, morphine therapy induces significant changes in the gut microbiome, affecting the abundance of specific bacterial taxa. This dysbiosis can influence the production of metabolites, such as short-chain fatty acids, which play a crucial role in maintaining gut health and modulating the immune response [10, 11]. Some bacterial species are involved in the synthesis of neurotransmitters and metabolites that may control mood and anxiety levels, providing a potential mechanism by which opioids may exert an indirect effect on mental health through microbial control [11]. Notably, beneficial bacterial species have been associated with improved gut health and mental well-being, while expansions in pathogenic species have been associated with negative health outcomes [12]. Furthermore, inflammatory markers may rise in response to an increase in pathogenic bacteria, contributing to anxiety and other psychological disorders [13]. While there are intriguing connections between opioid exposure, gut microbiota, inflammatory responses, and mental health, there is a considerable gap in the literature regarding the specific effect of morphine on the gut microbiome composition and the resultant behavioral outcomes. It is necessary to explore how morphine treatment influences the gut-brain axis through its impacts on gut microbiome diversity, anxiety-like behavior, and immune modulation in a controlled experimental setting. The present study attempts to fill this gap by systematically studying morphine's impact on the gut microbiome and the resultant behavioral changes in a rat model.

By quantifying inflammatory gene expression alongside behavioral testing, this study elucidates the intricate interaction between morphine treatment, gut microbial modulation, and immune response. The findings may pave the way for the creation of microbiome-targeted therapeutic strategies to prevent opioid-associated behavioral disruption and enhance patient care in the management of pain.

Materials and Methods

Study design

This study utilized a randomized controlled experimental study design to investigate morphine administration impacts on gut microbiome structure and anxiety-like behavior in a rat model. Male Wistar rats aged 8–10 weeks (body weight 220–250 g) were obtained from the Pasteur Institute of Iran and maintained under controlled environmental conditions: 12-h light/dark cycle, temperature 22±2 ˚C, and humidity 55±5%.

Animal grouping

A total of 40 adult male Wistar rats were randomly assigned to two treatment groups, namely the morphine and control groups. The rats in the morphine treatment group received rising doses of morphine sulfate (30 mg/kg) via intraperitoneal injection. Rats in the control group received the same amount of saline. The morphine group received an increasing dosage schedule, administered twice daily for two weeks. The morphine group's dosing regimen was as follows: The rats received escalating doses of 7 mg/kg and 10 mg/kg on day 1 and day 2, respectively. On day 3, the dose was increased further to

15 mg/kg and 20 mg/kg. On day 4, the rats were administered 25 mg/kg and 30 mg/kg. on days 5-14, morphine dosing was maintained at a constant 30 mg/kg level [14]. Saline controls were administered equal volumes of sterile 0.9% saline on the same administration schedule.

Behavioral assessments

Behavioral assays were conducted pre- and post-treatment to assess anxiety-like behavior. The elevated plus maze (EPM) consisted of two open arms and two closed arms on a plus-shaped platform elevated above the ground. The inflammatory pathways also interact with stress responses, so behavioral assessments will reflect the interaction of these systems. Anxiety-like behavior was assessed by monitoring time spent in open arms as opposed to closed arms for 5 min. Increased time in closed arms indicated higher anxiety levels.

Collection of fecal samples and gut microbiome analysis

We collected fecal samples from every rat at baseline (before treatment) and upon completion of the treatment. The samples were frozen in liquid nitrogen and stored at -80 ˚C until analysis could be performed. DNA was extracted from 0.2 g of fecal material by the QIAamp DNA mini kit (Qiagen, Valencia, CA, USA), following the manufacturer's guidelines. The quality and quantity of the DNA extracts were assessed by gel electrophoresis and with a nanodrop spectrophotometer (ND-1000, NanoDrop Technologies). Quantitative real-time polymerase chain reaction (qRT-PCR) was used to quantify the bacterial genomic abundances in the fecal samples. The targeted species for analysis, according to microbiome databases like Disbiome [15] and the literature, were *Akkermansia muciniphila*, *Bifidobacterium longum, Lactobacillus rhamnosus, Fusobacterium nucleatum, Bacteroides fragilis*, and *Clostridium difficile*. Specific primer amplification was normalized against the *16S rRNA* gene, and nucleotide BLAST was performed to confirm primer specificity. The polymerase chain reaction reactions was performed in duplicate using the Roche LightCycler® 96 system (Roche, Switzerland). The 20-μL reaction mixture contained distilled water, SYBR Green master mix (Takara, Japan), DNA template, and forward and reverse primers. Primer sequences used for quantitative RT-PCR reaction are presented in Table 1. Amplification included an initial heating step at 95 ˚C for 1 min, followed by 40 cycles of denaturation, annealing, and extension. Melting curve analysis was performed after amplification. For bacterial loads, serial dilutions of DNA from the standard strains of *Escherichia coli* were prepared, and DNA concentrations for all bacteria in the fecal samples were extrapolated from the standard curve.

Inflammatory gene expression analysis

After the treatment period and behavioral tests, we collected intestinal tissue samples from a subset of euthanized rats for quantitative real-time polymerase chain reaction analysis to evaluate the expression levels of important genes that mediate immune regulation and inflammation. We specifically aimed to measure the expression levels of *IL-6*, *TNF-α*, and *INF-γ*, as these genes are pivotal in mediating the inflammatory response and are known to be influenced by changes in gut microbiome composition. A heightened expression of these genes may indicate an overall inflammatory state in the intestinal environment following morphine administration. Total ribonucleic acid was extracted from the intestinal tissues using the TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), following the manufacturer's protocol to ensure high-quality ribonucleic acid for downstream applications. A reverse transcription (RT) reaction was carried out to obtain complementary DNA (cDNA), using 5 μg of the total RNA, an oligo(dT) primer, and ReverTra Ace (Toyobo, Osaka, Japan) as a reverse transcriptase, following the manufacturer's instructions. For the quantitative PCR (qPCR) phase, the specific genes associated with inflammation were examined for their expression levels using designated primers tailored for each gene. All primers and probes were designed using Oligo7 Primer Analysis Software, version 7.60 (Molecular Biology Insights, Inc., Colorado Springs, CO) and are listed in Table 2. The relative expression of these genes was determined using the ΔΔCT method, which allows for the comparison of gene expression levels between different samples, providing insights into the inflammatory response in the intestinal tissues. This comprehensive approach helps elucidate the role of these inflammatory markers in the context of the study.

Statistical analysis

The data were analyzed using the SPSS software, version 26, and graph plotting was conducted using GraphPad Prism software, version 6. For the quantitation of microbiome load in fecal samples, a series of standard dilutions was prepared for each real-time polymerase chain reaction run. The threshold cycle (Ct) was determined when sample fluorescence achieved a pre-set threshold and was calibrated against the standard curve. Normality of data distribution was assessed using the

Table 1. Primer sequences used for specific bacterial species analysis

Bacteria	Forward	Reverse	Tm (˚C)
E. coli	CATTGACGTTACCCGCAGAAGAAGC	CTCTACGAGACTCAAGCTTGC	55
A. muciniphila	CAGCACGTGAAGGTGGGGAC	CCTTGCGGTTGGCTTCAGAT	55
B. longum	TTCCAGTTGATCGCATGGTCTTCT	GGCTACCCGTCGAAGCCACG	56
L. rhamnosus	AGCAGTAGGGAATCTTCCA	CACCGCTACACATGGAG	58
F. nucleatum	AGAGTTTGATCCTGGCTCAG	GTCATCGTGCACACAGAATTGCTG	56
B. fragilis	CTGAACCAGCCAAGTAGCG	CCGCAAACTTTCACAACTGACTTA	56
C.difficile	TTGAGCGATTTACTTCGGTAAAGA	CCATCCTGTACTGGCTCACCT	55
16SrRNA	ACTCCTACGGGAGGCAGCAG	ATTACCGCGGCTGCTGG	62

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Table 2. Primer sequences used for intestinal inflammation analysis

Gene		Primer Sequence	Amplicon Size (Base Pairs)	Accession Number
Cytokine genes	*TNF-α*	F- ACCATGAGCACGGAAAGCAT R- AACTGATGAGAGGGAGCCCA	220	NM_012675.3
	INF-γ	F- GGCAAAAGGACGGTAACACG R- GTGCTGGATCTGTGGGTTGT	215	NM_138880.2
	IL-6	F- CACTTCACAAGTCGGAGGCT R- AGCACACTAGGTTTGCCGAG	502	NM_012589.2
Housekeeping gene	*GAPDH*	F- ACGGGAAACCCATCACCATC R- CTCGTGGTTCACACCCATCA	206	NM_017008

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Shapiro-Wilk test to determine if the data followed a normal distribution. For parametric tests, those that met the assumption of normality were analyzed using one-way analysis of variance. When significant differences were found among groups, post-hoc analyses were conducted using the Tukey test to determine pairwise comparisons. Non-parametric tests, such as the Kruskal-Wallis test, were used for data that did not meet the assumption of normality. For multiple comparisons, the Bonferroni correction was applied to control the family-wise error rate. Inflammatory gene expression data were analyzed similarly, with duplicate loading of samples and mean values calculated for each experimental group. The results are presented as Mean±SD, and a $P<0.05$ was considered statistically significant.

Results

Behavioral test

The effect of morphine treatment on anxiety-like behavior was evaluated through the EPM. Before the treatment, baseline scores showed no differences in anxiety between morphine and saline control groups ($P>0.05$).

Yet after the two-week treatment, rats treated with morphine showed increased anxiety-like behaviors relative to controls. Specifically, morphine-treated rats spent less time in the open arms of the EPM, with a mean of 19.55±5.92 s, compared to 55.30±10.17 s in the saline controls ($P<0.01$).

Rats treated with morphine also spent more time in the closed arms, a measure of higher anxiety levels, with a mean of 216.1±19.43 s compared to 176.6±9.84 s in controls ($P<0.05$; Figure 1).

Gut microbiome analysis

Fecal samples were assayed to determine alterations in gut microbiome composition following morphine treatment. qRT-PCR results indicated notable changes in some of the bacterial populations (Figure 2). Notably, beneficial bacteria such as *A. muciniphila* were significantly reduced in the morphine group (0.050±0.024 copies/ng DNA) compared to controls (0.28±0.10 copies/ng DNA; $P=0.0008$). Similarly, levels of *B. longum* were also significantly reduced in morphine-treated rats (*B. longum*: 0.013±0.02 vs 0.25±0.11; $P=0.003$). *L. rhamnosus* showed a non-significant decrease (0.017±0.02 vs 0.14±0.20; $P=0.4$), suggesting marginal biological relevance. Conversely, potentially pathogenic species, such as *B. fragilis*, *F. nucleatum*, and *C. difficile*, were increased in the morphine treatment group. *B. fragilis* counts increased to 0.41±0.30 copies/ng DNA in morphine-treated rats, compared with 0.04±0.03 in con-

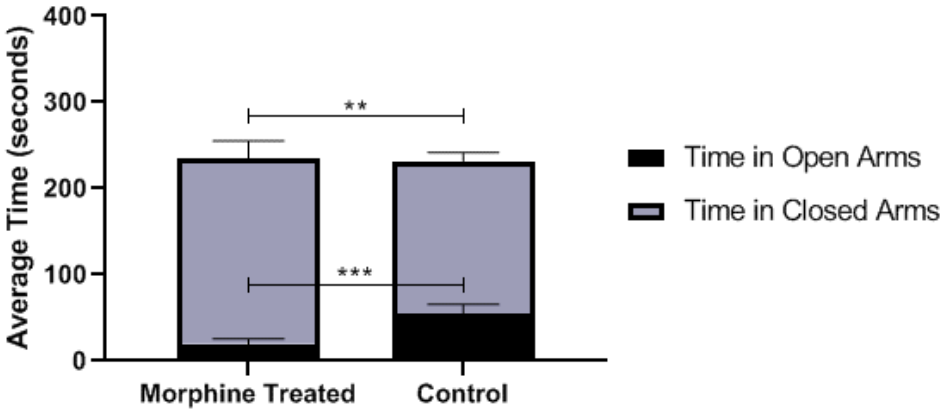

Figure 1. Impact of morphine administration on anxiety-like behavior as assessed by the EPM test
***P<0.001, **P<0.01.

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trols (P<0.001). Similarly, *F. nucleatum* was considerably higher in morphine rats (0.44±0.26 vs 0.17±0.10; P<0.001), and *C. difficile* was at higher levels (0.25±0.13 vs 0.1±0.04; P=0.01).

Intestinal inflammation analysis

The analysis of inflammatory gene expression in intestinal tissues revealed that morphine treatment was associated with alterations in the expression levels of key cytokines (Figure 3). Specifically, the expression levels of *IL-6* (2.22±1.77; P=0.06) and *TNF-α* (1.90±1.17; P=0.03) showed upward trends in the morphine group compared to the control group; however, these differences did not reach statistical significance. In contrast, the expression of *INF-γ* (3.21±0.16) exhibited a significant increase in the morphine-treated group compared to the control group (P<0.001). This elevated expression suggests a prominent inflammatory response that may be linked to the impact of morphine administration on immune regulation within the intestinal environment.

Discussion

The current study contributes valuable evidence about the effect of morphine injection on anxiety-like behaviors and gut microbiome changes in a rat model. The observed increase in anxiety-like behaviors following

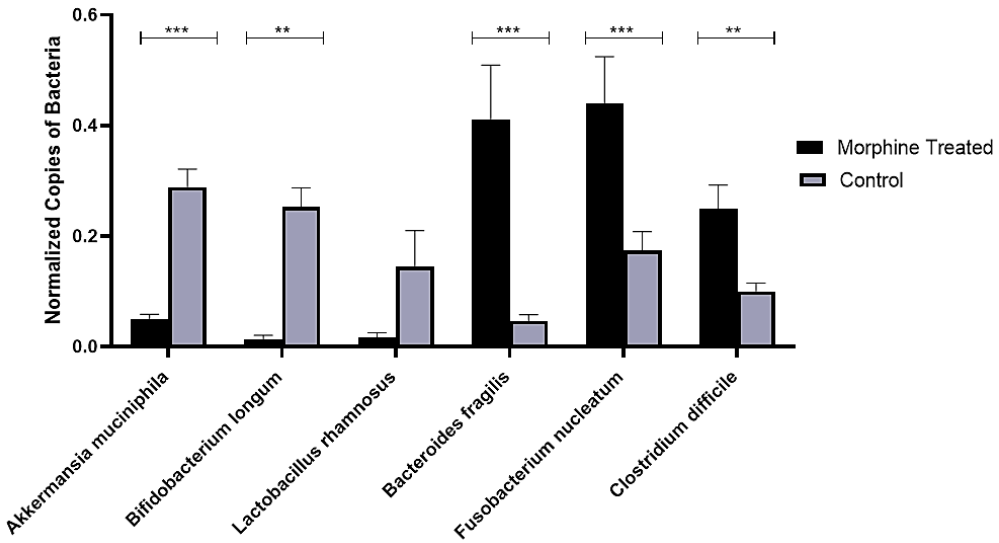

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Figure 2. Normalized bacterial abundance (*A. muciniphila*, *B. longum*, *L. rhamnosus*, *F. nucleatum*, *B. fragilis*, and *C. difficile*) in morphine-treated vs control groups

Note: Bacterial abundances normalized to *16SrRNA* copies. ***P<0.001; **P<0.01.

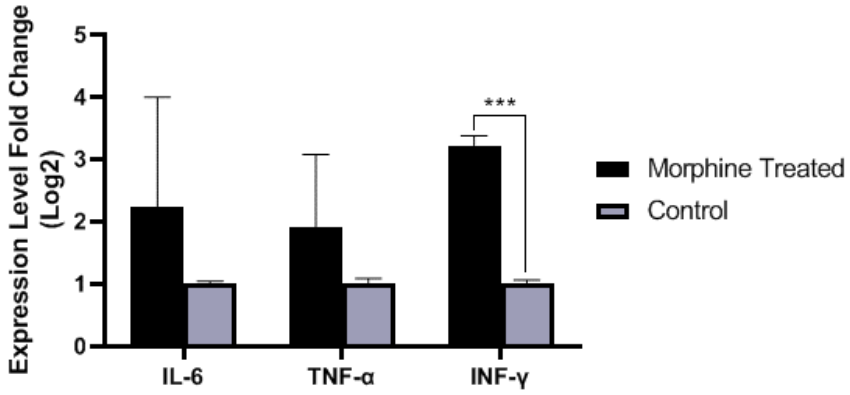

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Figure 3. Changes in selected intestinal inflammatory genes (*IL-6, TNF-a, and INF-γ)* following morphine treatment
Notes: The obtained data for each experimental group is reported as the Mean±SD (n=10). ***P<0.001.

morphine treatment supports the notion that opioid exposure may exacerbate anxiety symptoms, potentially through mechanisms involving gut microbiome dysbiosis and inflammatory responses. This behavioral effect supports earlier research showing that opioids can intensify anxiety and other mental health disorders due to their profound impacts on the central nervous system and neurobiological mechanisms that regulate anxiety [16]. This supports the theory that modifications in the immune system and inflammation play a critical role in this relationship. The significant changes in inflammatory markers (e.g. *TNF-α*, P=0.03; *INF-γ*, P<0.001) underscore how morphine may influence immune pathways related to anxiety.

qRT-PCR analysis revealed substantial changes in the gut microbiome following morphine administration. We observed significant reductions in beneficial bacterial strains, such as *A. muciniphila* (P=0.0008) and *B. longum* (P=0.003). While *L. rhamnosus* did not show statistical significance (P=0.4), a trend toward reduction was noted. The decline in short-chain fatty acid-producing bacteria may diminish anti-inflammatory signaling and exacerbate anxiety via vagal pathways, as highlighted by previous studies [17]. This finding is consistent with literature emphasizing the health-promoting effects of these bacterial species and their role in improving mood and psychological well-being [18-21]. The loss of beneficial species likely contributes to increased inflammatory markers in the gut, correlating with worsened psychiatric outcomes [22]. Other research supports that reductions in short-chain fatty acid-producing bacteria can enhance anxiety-like behaviors, given their role in producing metabolites that influence brain function [23]. Conversely, we observed significant increases in potentially pathogenic species, including *B. fragilis* (P<0.001) and *F. nucleatum* (P<0.001). These findings suggest that the proliferation of these pathogens may further amplify dysbiosis and inflammation, contributing to heightened psychological distress [24, 25]. The association between increased levels of these pathogens and elevated inflammatory markers underscores the importance of immune modulation in understanding morphine's effects [26]. However, some studies report no significant microbiome changes or behavioral effects following morphine treatment [27, 28]. These discrepancies may arise from differing treatment durations or individual variability among study subjects. In contrast, our findings align with research linking opioid-induced dysbiosis to anxiety [29, 30], suggesting that the effects of opioids on the microbiome and behavior may be dose- and time-dependent. This inconsistency in the literature emphasizes the need for further investigations to clarify the relationship between opioid use and gut-brain interactions.

The findings of this study reveal the critical intersection of opioid use, the gut microbiome, inflammatory responses, and mental health, with significant implications for clinical practice. Morphine may compromise gut barrier integrity, facilitating bacterial translocation and systemic inflammation via toll-like receptor activation [31]. It also modifies gut motility and secretion, favoring pathogenic overgrowth while suppressing beneficial species, potentially through opioid receptor-mediated inhibition of probiotic growth. Given the high rates of opioid prescriptions for acute and chronic pain management, understanding the multifaceted actions of these medications, specifically their influence on gut microbiome and mental health, is essential for developing comprehensive treatment protocols. The association between morphine administration, gut microbiome alterations, and anxiety-like behaviors indicates that clinical prac-

titioners should consider not only the pharmacological aspects of opioid therapy but also its potential psychological and gastrointestinal effects. A holistic approach could integrate gut health assessments and probiotic or nutritional therapies alongside opioid treatment to mitigate adverse psychological effects [32, 33]. Such strategies could improve patient care by enhancing resilience and treatment outcomes. Furthermore, the identification of specific beneficial and harmful morphine-responsive bacterial groups presents opportunities for developing microbiome-targeted therapies as adjunct treatments for opioid patients [26]. Clinicians must adopt more judicious prescribing practices, weighing the risks and benefits of opioid use in patients with histories of anxiety or gastrointestinal disorders [34]. Recognizing that dysbiosis can exacerbate psychological issues may lead to more targeted pain management programs that incorporate both physical and psychological wellness.

Conclusion

This study explains the significant impacts of morphine treatment on anxiety-like behavior and changes in gut microbiota in a rat model. The decrease in beneficial gut microbiota and the concomitant increase in potentially harmful species, alongside elevated levels of inflammatory mediators, directly correlate with heightened anxiety behaviors, highlighting the detrimental impact opioids have on psychological and immune well-being. These findings indicate that modulation of the gut microbiome must be taken into account for treating opioid-induced side effects, as microbiome-targeting interventions may open new prospects for the creation of improved mental treatments for individuals receiving opioid treatment. Additional research focusing on the interplay of inflammation, immune pathways, and gut microbiota will be needed to further elucidate morphine's multifaceted effects on health.

Study limitations

Despite these insights, several limitations must be acknowledged. First, the rat model, while beneficial for controlled experimentation, may limit the generalizability of findings to humans due to interspecies variability in gut microbiota and opioid responses. Additionally, the study assessed morphine administration over a relatively short duration of two weeks, which does not comprehensively address the long-term effects of opioid use on psychological and gut microbiome health. Long-lasting opioid exposure may yield different implications, necessitating further longitudinal studies to evaluate the consequences of extended treatment durations. Another limitation is the exclusive focus on morphine, which may not reflect the effects of other opioids with different impacts on gut microbiome and behavior. Future investigations should encompass a broader range of opioid analgesics to provide a more complete understanding of these associations. Lastly, while changes in gut microbiome and anxiety-like behaviors were observed, the biochemical mechanisms underlying these effects remain unclear. Further research into the pathways by which morphine influences gut health and behavior, particularly the roles of inflammatory markers and neurotransmitter concentrations, will be crucial for advancing our understanding in this area.

Ethical Considerations

Compliance with ethical guidelines

This study was approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran (Code: IR.IAU.PS.REC.1403.279) and adhered to the National Institutes of Health guidelines for the care and use of laboratory animals.

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Authors' contributions

Conceptualization, Samira Tarashi; Methodology, Kianoosh Ferdosnejad; Investigation, Bahman Rahimlou; Writing the original draft: Bahman Rahimlou and Kianoosh Ferdosnejad; Review and editing: Samira Tarashi and Mohammad Saber Zamani; Supervision, Samira Tarashi.

Conflicts of interest

The authors declared no conflict of interest.

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