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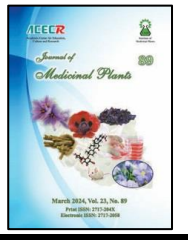

Research Article

Naringenin may prevent morphine-induced tolerance via inhibiting glycogen synthase kinase-3beta activity in mice

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ABSTRACT

Background: Opioids are essential for pain treatment, but their long-term usage results in tolerance. Naringenin, a natural flavonoid found in fruit, inhibits the enzyme that causes opioid tolerance, making it effective in treating neurodegenerative diseases. **Objective**: In this study, we evaluate the role of naringenin in morphine-induced tolerance and the glycogen synthase kinase-3beta (GSK-3β) enzyme. **Methods:** To induce tolerance to morphine in mice, repeated injections of morphine were performed for five days, and on the fifth day, a single dose of morphine was injected intraperitoneally. Pain tests (hot plate and tail flick) were performed on the first, third, and fifth days of injections. To evaluate the impact of naringenin, 45 minutes before each morphine injection, doses of 25, 50, and 100 mg/kg were administrated orally. On the last day, brain tissues were checked for biochemical factors and changes in the phosphorylation of the enzyme by the immunohistochemical method. **Results:** The results indicated that the simultaneous use of naringenin significantly increases the analgesia delay compared to the morphine group ($P < 0.001$) on the third and fifth days. Naringenin at all concentrations decreased the nitrite level caused by morphine. It showed protective effects on morphine tolerance ($P < 0.001$) in the p-GS^{Ser640} immunohistochemical assay and reduced the phosphorylation of p-GS^{Ser640} by GSK-3β, activated by chronic morphine administration. **Conclusion:** Based on the results of the present study, the beneficial effect of naringenin on the GSK enzyme in morphine-induced tolerance is confirmed, but more studies are needed to investigate its impact mechanism.

1. Introduction

Opioids are potent painkillers, but they carry risks like addiction and physical dependence. Long-term use may require higher doses due to tolerance. To manage chronic pain, opioids are used in both cancer and non-cancer patients [1].

Abbreviations: cGMP, cyclic guanosine 3′,5′-monophosphate; COX2, cyclooxygenase-2; eNOS, endothelial NOS; GFAP, glial fibrillary acidic protein; GSK3, Glycogen synthase kinase 3; iNOS, inducible NOS; NO, Nitric oxide; NOS, nitric oxide synthase; nNOS, neuronal NOS; NF-κB, nuclear factor-kappa; p-GS^{Ser640}, p-glycogen synthase

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Prolonged administration of opioids is usually defined as treatment extending more than 3 months [2]. Opioids are drugs that attach to natural opioid receptors in the body, primarily known for pain relief. Their effects vary based on composition and receptors attached [3]. Over the past 30 years, opioid use has increased dramatically in the United States [4]. More than 16 million people worldwide are opioid-dependent [5].

Prolonged opioid use causes tolerance, less effectiveness, addiction, and abuse. This involves receptor downregulation, signaling desensitization, and upregulation of drug metabolism [6]. Glycogen synthase kinase 3 (GSK3) is a serine/ threonine kinase family member. Humans have two subtypes of GSK3, GSK3α and GSK3β [7]. GSK3β regulates glycogen metabolism and cellular physiology and is involved in several cellular processes, such as embryonic development, inflammation, immune response, neuronal plasticity, apoptosis, neurodegeneration, and carcinogenesis [8]. Also, it participates in different pathological conditions, such as cancer, type 2 diabetes, Alzheimer's disease, inflammation, and bipolar disorder [9]. Chronic morphine use activates the GSK3β enzyme. While inhibiting GSK3β reduces morphine tolerance [10]. GSK3β inhibitors were found to reduce the development of morphine tolerance in a dose-dependent way [11]. Liao and his colleagues have indicated frequent morphine administration induced progressive morphine tolerance in analgesic tests such as tail flick test and naloxone withdrawal syndrome in rats which was reversed by co-administration of GSK3β inhibitors including SB216763 and SB415286 [12]. It has been shown that chronic exposure with morphine reduced Serine 9 phosphorylation of GSK3β, the inactive form of GSK3β, in the brainstems of mice and mice revealed morphine tolerance which was reversed by administration of chaperon for example 4-phenylbutyric acid (PBA) [10].

Morphine use can cause oxidative stress, increasing reactive oxygen species and lead to tolerance and dependence on opioids. Nitric oxide (NO) also contributes to pain pathways and regulates opioid antinociception [13].

Naringenin is an essential phytochemical that belongs to the flavanone group of polyphenols and is mainly found in citrus fruits such as grapefruits, tomatoes, and cherries [14]. Analgesic effects of naringenin have been indicated via induction of anti-inflammatory effects and stimulating antioxidant enzymes [15]. Previous studies have pointed out that naringenin has neuroprotective and analgesic properties in neurodegenerative disorders and neuropathic pain models. It also has therapeutic potential in inflammatory pain models [16]. It has been demonstrated that naringenin improved cognitive functions in ICV-STZ (intracerebroventricular injection of streptozotocin) induced dementia via reducing the activity of GSK3β IN both cerebral and hippocampus cortex of rats [17].

Therefore, this study examines the impact of naringenin, a citrus flavonoid with antioxidant, anti-inflammatory, and analgesic impacts, on chronic morphine-induced tolerance in mice as a GSK3β inhibitor.

2. Materials and methods

2.1. Animal preparation

The study was conducted on male mice kept in standard cages one week before the experiment based on Dambisya et al. protocol [18]. The temperature was 22 ± 2 degrees Celsius, and there was a 12-hour light-dark period in the Faculty of Pharmacy, University of Medical Sciences, Kermanshah laboratory. During this period, the animals had free access to food and water (IR.KUMS.REC.1398.1108).

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2.2. Drug injections

Morphine HCl and naringenin were purchased from Sigma-Aldrich (Buchs, Switzerland). They both were dissolved in normal saline.

Animals were divided into eight groups (7 mice in each group): (1) control group, which only received normal saline; (2) morphine group; (3) naringenin group (25 mg/kg); (4) naringenin group (50 mg/kg); (5) naringenin group (100 mg/kg); (6) morphine + naringenin group (25 mg/kg); (7) morphine + naringenin group (50 mg/kg); (8) morphine + naringenin group (100 mg/kg). The administration of morphine was done as following:

Three injections per day were done intraperitoneally for four days, including 50 mg/kg at 8:00 and 12:00 o'clock and the third injection75 mg/kg at 17:00 o'clock. Also, a single dose of 50 mg/kg was administered on the fifth day at 8:00 am [19].

To evaluate the impact of naringenin, 45 minutes before each morphine injection, different doses of naringenin (25, 50, 100 mg/kg dissolved in normal saline) were fed to the animals by gavage. The pain tests were done on the days 1,3 and 5. At the end of the experiment on the fifth day after pain tests, animals were euthanized, and their brains were examined for biochemical factors. Using the Griess test the amount of nitric oxide metabolites was measured to indicate the opioid tolerance. The number of changes in the downstream target protein of GSK-3β phosphorylation, p-GS, was also collected via immunohistochemical.

2.3. Nociceptive threshold tests

Pain-related tests (hot-plate and tail-flick) were conducted on the first, third, and fifth days of injections. In the tail-flick test, a timer is started 45 minutes after the first daily morphine injection while an intense light beam is focused on the animal's tail [20]. When the animal wags its tail, the timer stops. The measured delay time is an indicator of the pain threshold. The cut-off in this test was 10 seconds. The hot-plate device is a plate heated by electric current [21]. When the plate temperature reached 55 degrees, the mice were placed on it. The end time of the test was measured when they licked their hind legs or jumped. The test cutoff was 90 seconds.

2.4. Griess assay

At the end of study, the whole brain of 4 animals of each group were collected and after the homogenization of that in normal saline and centrifugation, the supernatant solution was used for the Griess test [22]. For protein removal with zinc sulfate, 400 µl of the sample were mixed with 6 mg of zinc sulfate powder. For measuring the concentration of total nitrite and nitrate (Nox) in an ELISA [23] microplate, first 100 µl of deproteinized serum, then 100 µl of a mixture (1:1) of sulfonamide and NEDD were added and incubated for 30 minutes at 37°C. After the reaction and color formation, the light absorption resulting from the formation of the color substance was read at 540 nm via the ELISA reader, and the concentration of the samples was measured using the standard curve. Sodium nitrite was used as a standard in concentrations of 50, 25, 12.5, 6.25, and 3.125 µM/l.

2.5. Immunohistochemistry (IHC) of p-GS^{Ser640}

Prefrontal cortex IHC of 3 animals in each group was carried out to evaluate the levels of phosphorylation of serine 640 GS as a downstream target of GSK3β. Deparaffinization of samples was done by xylene, then washed with alcohol and rehydration by citrate buffer was done. After washing with the washing solution and then blocking, the primary p-GS^{Ser640} antibody was added to the sample. After the sample was washed the secondary antibody was added and incubated

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for one hour at 37°C. Afterwards, we rinse it and add the substrate solution (this step should be done in the dark and away from light); after 5 minutes, we washed it with distilled water. Then it was dehydrated, starting with 70 degrees' alcohol until we reached absolute alcohol. For clarification, we put the sample in Xylol for 5 minutes. We mounted the lamella on the lamella with Entellan™ glue.

2.6. Statistical analyses

Ultimately, the effectiveness of different drug doses was compared to the control group. Mean±SEM was used using the software to determine statistical differences with One-way analysis of variance (ANOVAs) followed by post hoc Tukey's tests and two-way ANOVA repeated measure, followed by Bonferroni post hoc test were performed for statistical analysis [24].

3. Results

3.1. Inducing analgesic effect of morphine in mice

As indicated in figure 1 repeated morphine injections over five days decreased analgesic effect in mice in both hot plate test and tail flick compared to day one. There was a significant difference between third day compared to the first day ($P < 0.01$), and the fifth day compared to the first day ($P < 0.001$).

3.2. Effects of naringenin on morphine tolerance

The results depicted in figure 2 (A-F) indicate that administering multiple doses of naringenin (25, 50, and 100 mg/kg) 45 minutes before morphine injection significantly reduces tolerance to the analgesic effect in both the hot plate and flick tail tests as compared to the control and morphine groups ($P < 0.001$ and $P < 0.001$, respectively). As seen in the figures below, naringenin alone has no analgesic effect in either test group compared to the control group.

3.3. Effects of naringenin on nitrite level

Results of the Griess test show that chronic treatment with morphine significantly ($P < 0.001$) increased the level of nitrite compared to the control group during five days (Figure 3). Naringenin injection 45 minutes before morphine injection significantly reversed the increase in nitrite level in brain tissue compared to the morphine group ($P < 0.01$ for naringenin 25 and $P < 0.001$ for naringenin 50 and 100 mg/kg). Multiple doses of naringenin alone did not produce a significant difference in nitrite levels in comparison to the control group (data not shown).

3.4. Effects of naringenin on p-GS^{Ser640} phosphorylation

The study found that continuous use of morphine resulted in a significant enhancement in phosphorylation of GS at serine 640, which is regulated by GSK-3β, in comparison to the control group ($P < 0.001$) (Figure 4). However, pretreatment with naringenin at doses of 25, 50, and 100 mg/kg before each morphine administration significantly prevented the increase of p-GSSer640 phosphorylation ($P < 0.001$). These findings indicate that the inhibitory impact of GSK-3β is responsible for decreasing p-GSSer640 levels after naringenin administration.

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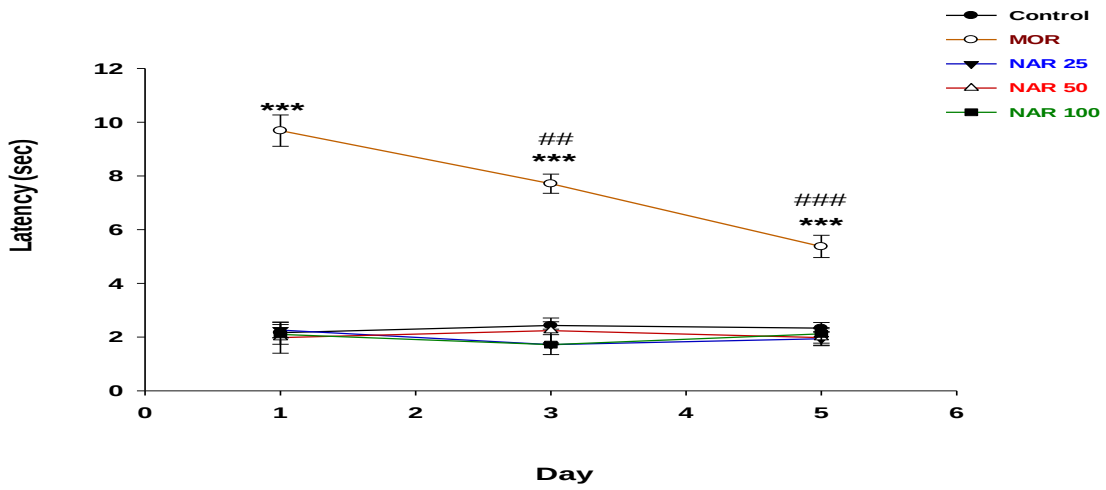
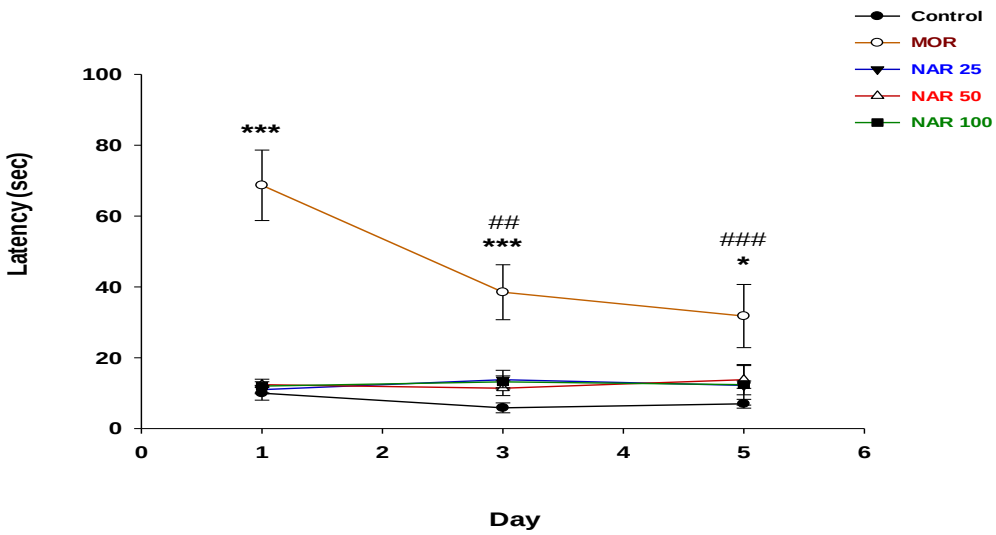
A





B

Fig. 1. After five-day administration of morphine development of analgesic tolerance was evaluate via (A: tail flick test B: hot plate test). ($^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared to the control group and $^{\#\#\#}P < 0.001$ compared to the first day of the morphine group).

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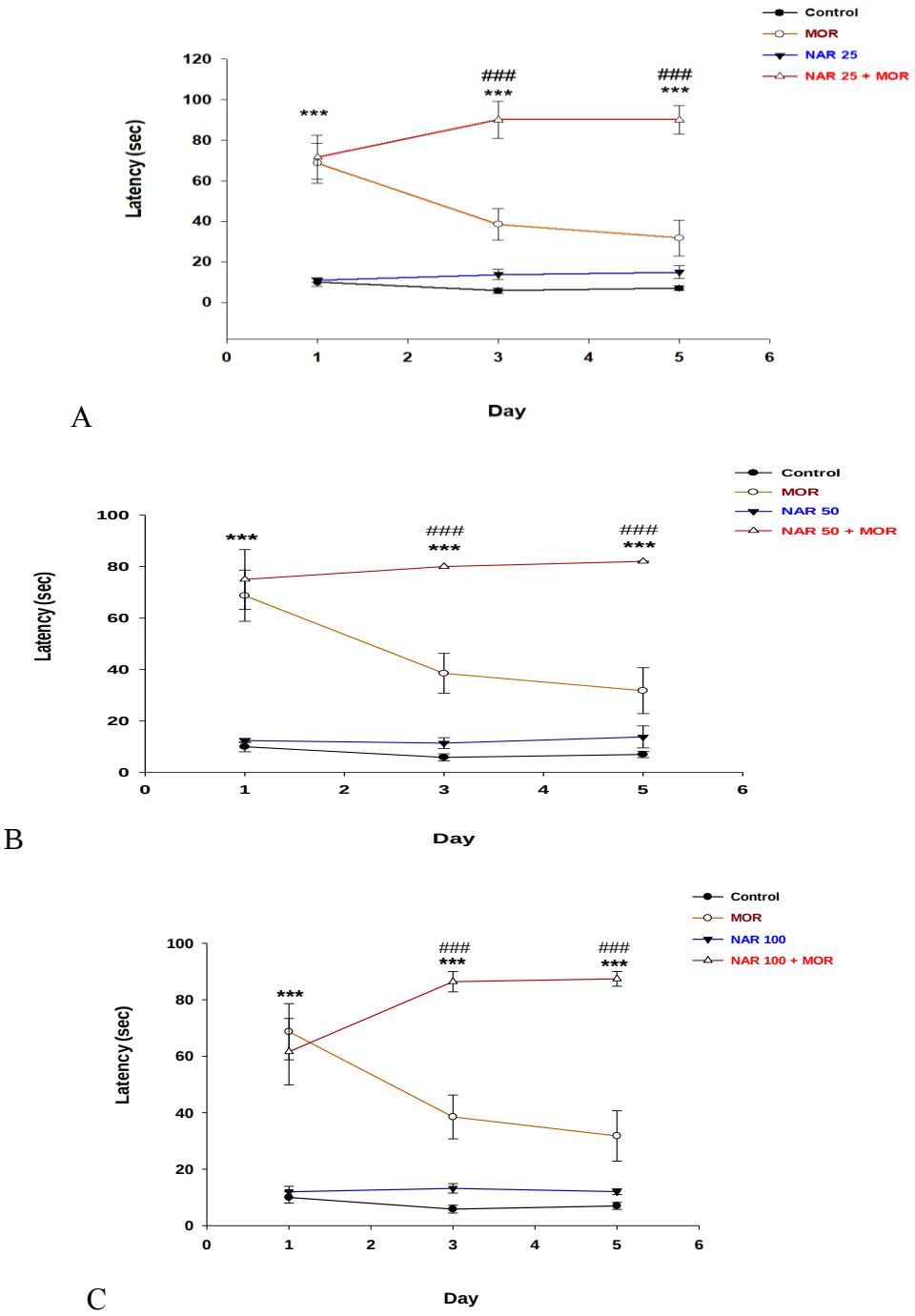

Fig. 2. The role of different doses of naringenin (25, 50, and 100 mg/kg) in reducing tolerance to the analgesic impact of morphine during five days of treatment. (A-C) the hot plate test and (D-F) the tail flick test. (***P < 0.001 compared to the control group and ###P < 0.001 to the morphine group).

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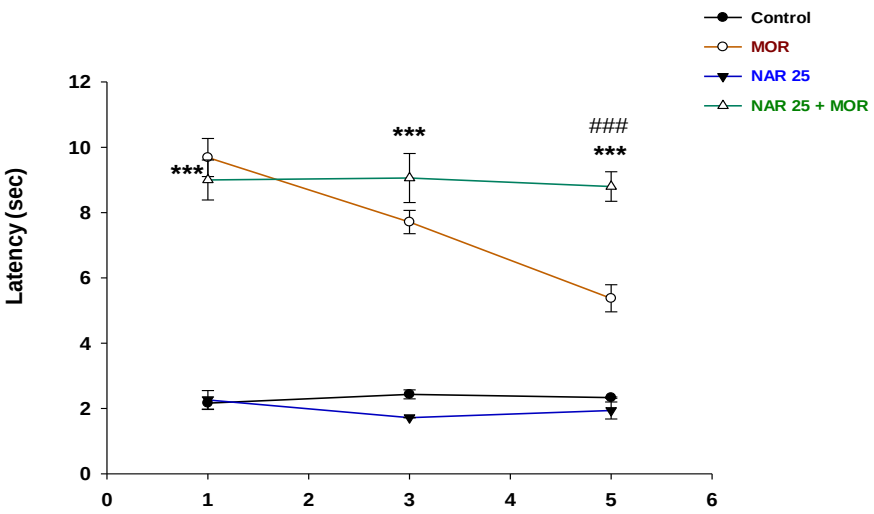

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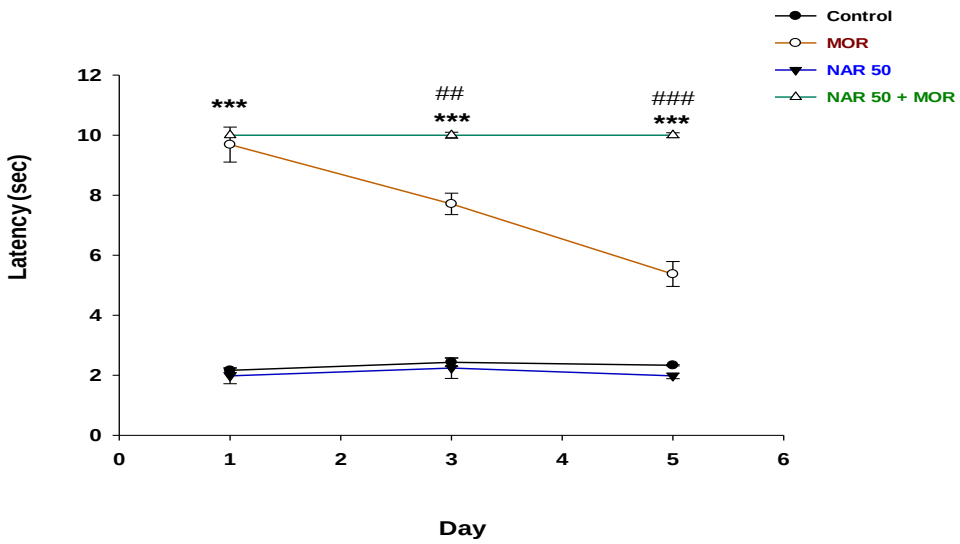

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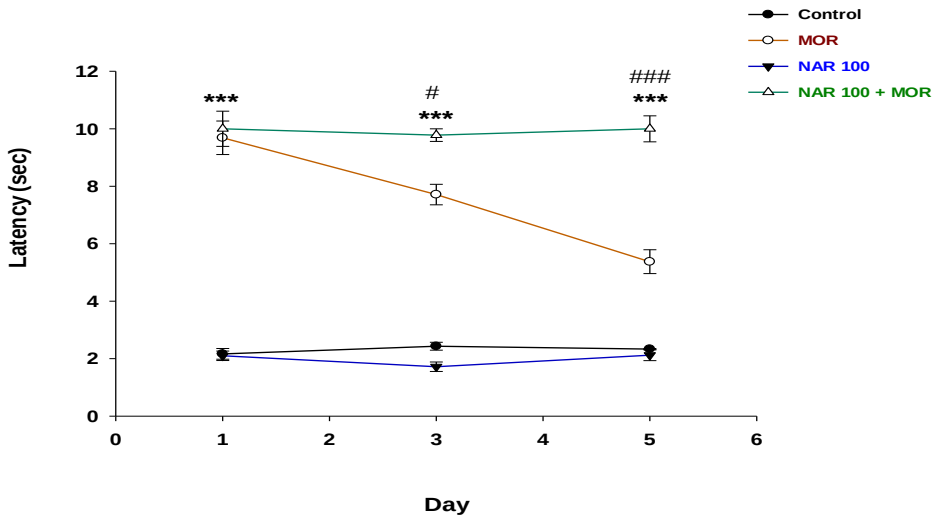

F

Fig. 2. The role of different doses of naringenin (25, 50, and 100 mg/kg) in reducing tolerance to the analgesic impact of morphine during five days of treatment. (A-C) the hot plate test and (D-F) the tail flick test. ($^{***}P < 0.001$ compared to the control group and $^{###}P < 0.001$ to the morphine group).

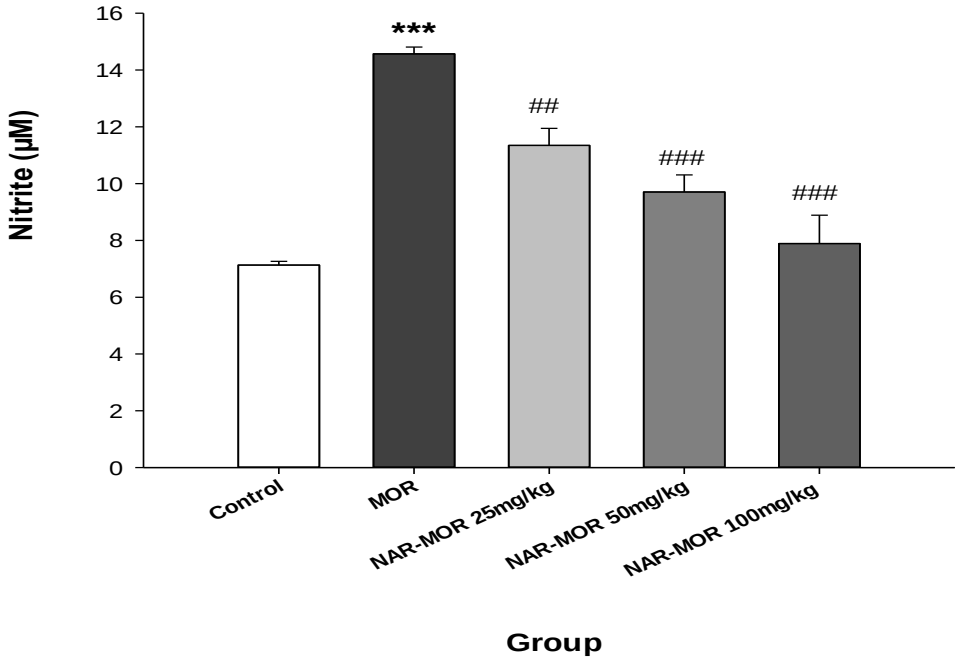

Fig. 3. Nitrite levels after chronic exposure to morphine and different doses of naringenin (25, 50, and 100 mg/kg) with morphine. Morphine significantly increased the nitrite level compared to the control group, which was reversed by oral administration of different doses of naringenin (25, 50, and 100 mg/kg). ($^{***}P < 0.001$ vs. the control group, $^{##}P < 0.01$, and $^{###}P < 0.001$ vs. the morphine group).

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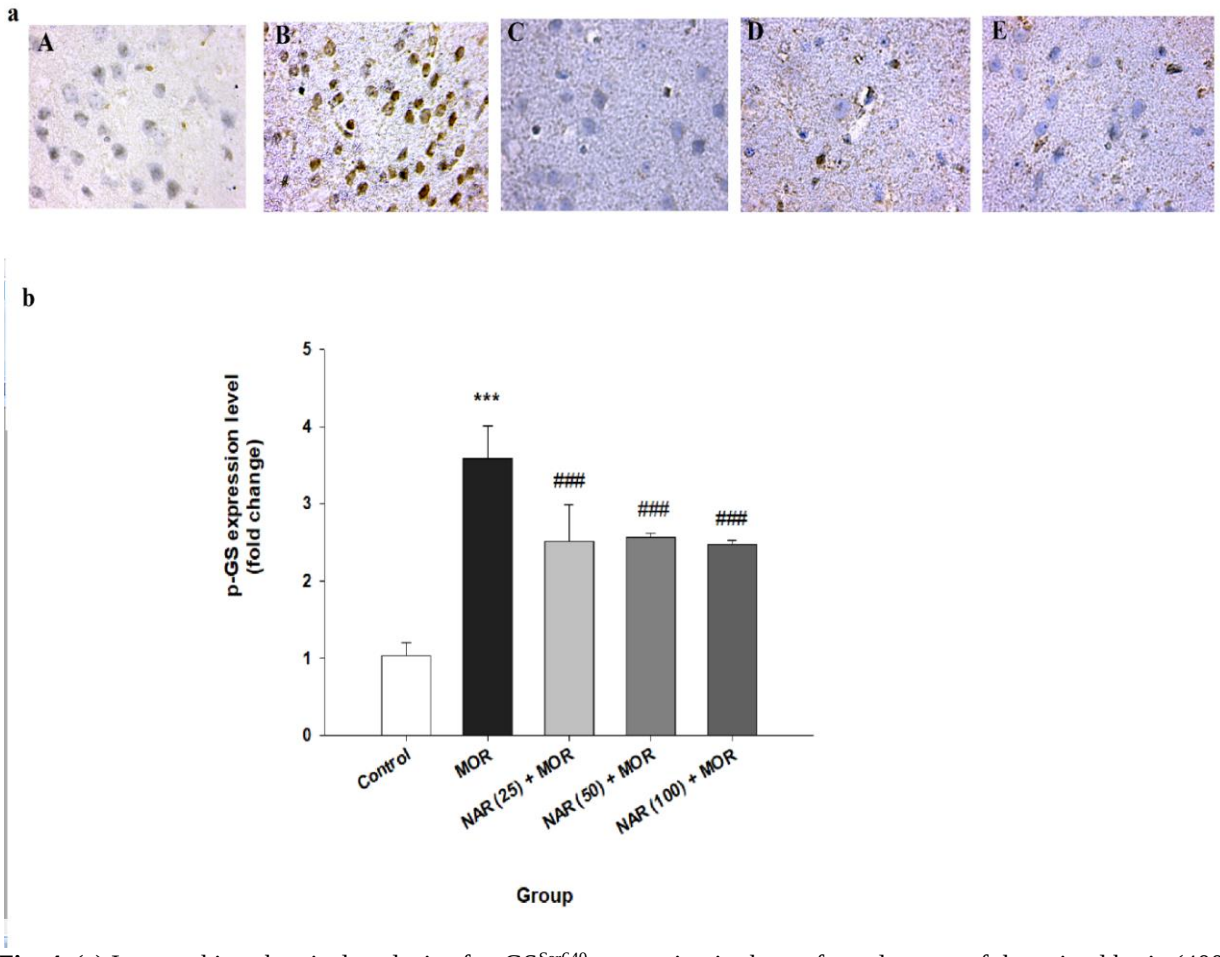

Fig. 4. (a) Immunohistochemical analysis of p_GS^{Ser640} expression in the prefrontal cortex of the animal brain (400X). A, control; B, morphine; C, naringenin (25 mg/kg) + morphine; D, naringenin (50 mg/kg) + morphine; E, naringenin (100 mg/kg) + morphine. (b) Change of p_GS^{Ser640} expression in the prefrontal cortex. ($^{***}P < 0.001$ in comparison to the control group and $^{###}P < 0.001$ in comparison to the morphine group).

4. Discussion

Our study demonstrates the role of naringenin in preventing morphine-induced tolerance in mice by inhibiting GSK3β. Intraperitoneal injection of naringenin as a GSK3 inhibitor prior to administration of morphine meaningfully decreased the analgesic tolerance development. Results showed that naringenin has significant anti-tolerant impacts against chronic morphine-induced tolerance. These findings provide strong evidence that GSK3 is involved in modifying the chronic complications induced by morphine and that naringenin can inhibit morphine-induced tolerance.

Chronic exposure to morphine often results in the development of analgesic tolerance, requiring an increase in dosage to achieve an adequate effect. This can also increase the risk of withdrawal symptoms [25]. It has been reported that the chronic use of morphine triggers the activation of glial cells [26], upregulates inflammatory cytokines [27], and induces morphine tolerance through different mechanisms [26]. Opioids exert their action through G-protein-coupled receptors, including

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μ (19), delta [11], and kappa located in the brain and spinal cord (25). Opioid analgesia is linked strongly to the the μ opioid receptors activation located in the CNS [28].

GSK3β is a regulatory protein kinase [29]. There is a site on the GSK3β protein known as p-glycogen synthase (p-GS^{Ser640}), and phosphorylation of the p-GS^{Ser640} residue regulates GSK3β. It is usually observed that glycogen synthase activity is suppressed via an increase in phosphorylation [30]. The role of GSK3β in analgesia signaling, opioid tolerance, and dependence has been mentioned in numerous studies. GSK3β inhibitors were found to reduce chronic morphine tolerance and desensitization [29]. GSK3 is involved in various cellular processes and is linked to neurodegenerative diseases like Alzheimer's [31]. Recently, various types of GSK3 inhibitors have been proposed as possibe therapeutic agents for neurodegenerative conditions such as Alzheimer's disease [32]. For instance, Lithium, a non-selective GSK3 inhibitor, has been used to treat bipolar disorder [33].

Various studies have shown that naringenin, a flavonoid found in many sources, interferes with GSK3 pathways and downregulates GSK3 activity [34]. Naringenin has potent neuroprotective and antioxidant properties [35]. It has been demonstrated that citrus juices interfere with the opioids metabolism and maximize their impacts. Furthermore, oral administration of grapefruit juice increases morphine antinociception and prevents tolerance via enhancing the intestinal absorption of this agent [26]. Several studies indicate a correlation between flavonoids and morphine-tolerance. Alifarsangi et al. concluded that naringenin has a dose-dependent anti-tolerant impact toward chronic morphine usage and that the intensity of the impact is essentially dependent upon its concentration [26]. Also, Zhou et al. suggested that the naringenin contained in grapefruit juice may be useful for chronic pain and might help in the delay of opioid-related unwanted impacts [36].

NO is a gas synthesized from arginine via nitric oxide synthase (NOS). The NOS family comprises three isoforms: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS) [37]. NO has a complex role in pain perception. NO acts as a neuromodulator and neurotransmitter in the peripheral and central nervous systems, and is involved in nociceptive processing. In the spinal cord, NO facilitates pain transmission by enhancing the release of excitatory neurotransmitters like glutamate. In the brain, NO can inhibit pain transmission by activating descending pain inhibitory pathways [38]. NO has a dual role in pain perception - it can facilitate pain transmission peripherally and in the spinal cord, but also inhibit pain at supraspinal sites in the brain. The net effect of NO on pain depends on the specific site of action and the pain modality. Modulating NO signaling may represent a potential therapeutic target for pain management [39].

NO pathways play an essential role in the development of morphine-derived dependence and affect the development of opioid analgesic tolerance and physical dependence [40] [41]. NO/cyclic guanosine 3′,5′-monophosphate (cGMP) signaling pathway has been reported to participate in morphine tolerance. Morphine functions by binding to μ-opioid receptors located in the brain and causing an increase in the overexpression of both iNOS and nNOS. [37]. Repeated morphine treatment increases the secretion of NO. Therefore, inhibition of NOS and preventing NO overproduction eliminates morphine tolerance [42]. Previous study have indicated that there was a recognizable relationship between GSK3β and the levels of NO. In this study it has been shown that GSK3β

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increased the levels of NO production by iNOS via activation of NF-kB which was banned by GSK3β inhibitor [43].

Our data indicates that pretreatment with naringenin before each administration of morphine effectively reduced the effect of morphine on p-GS^{Ser640}. Moreover, when morphine was repeatedly administered for five days, a significant reduction in its analgesic effect was observed in the hot plate test and tail flick as compared to the first day of administration. The prefrontal cortex (PFC) phosphorylation level of GS was measured due to the proven role of PFC in pain processing. During acute and chronic pain many changes occurs in PFC such as alterations in gene expression, neuroinflammation, glial cell activity and neurotranmitters [44].

So, in this study we have analyzed the alteration in p-GS in PFC of animals. In the study, it was found that naringenin alone did not have any pain-relieving effects compared to the group receiving morphine. However, chronic administration of naringenin resulted in the loss of tolerance to morphine analgesic effects. All doses of naringenin reduced tolerance to the analgesic impact of morphine almost equally. The thermal hyperalgesia tests showed that naringenin was able to prevent the reduction of the pain threshold compared to the morphine group. Additionally, naringenin was found to significantly decrease the amount of nitric oxide caused by chronic injection of morphine, resulting in a decrease in morphine tolerance.

Also, long-term use of morphine significantly increased p-GS^{Ser640} levels in prefrontal cortex, confirming that morphine increases GSK3β activity. In contrast, pretreatment with naringenin before each morphine injection significantly reduces p-GS^{Ser640} and thus reduces the high activity of GSK3β.

Morphine analgesic dependence and tolerance are essential challenges in clinical settings, and finding novel ways seems necessary. Though, more research are required to discover the neural signaling and exact mechanisms in different brain areas involved in morphine dependence and tolerance.

5. Conclusion

It has been concluded that one of the effective ways to treat complications caused by morphine is to inhibit and modulate the activity of GSK3β by using inhibitors of this enzyme. Naringenin, a GSK3β inhibitor, has been found to improve the quality of long-term administration of morphine. Therefore, it can be inferred that naringenin can be used in combination with morphine for preventing tolerance to the morphine analgesic impacts. Yet, further research are needed to investigate the exact impact of naringenin on opioid-tolerance pathways.

Author contributions

SS and MGM were involved in the conception and design of the study; SS acquired and analyzed data; GB, SB and MRM contributed into drafting the article; MRM and MGM contributed to designing of study; MHF designed the study and was involved in the analysis of data and critical revision.

Conflicts of interest

The authors declare that there is no conflict of interest.

Acknowledgment

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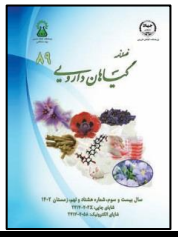

مقاله تحقیقاتی

بررسی اثر احتمالی نارینژنین بر تحمل ناشی از مورفین توسط مهار گلیکوژن سنتاز کیناز۳-بتا در موش سوری

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اطلاعات مقاله

گل‌واژگان:

نارینژنین

GSK

$p\text{-}GS^{Ser640}$

تحمل به مرفین

مواد افیونی

مدل حیوانی

چکیده

مقدمه: مواد افیونی برای درمان درد ضروری هستند، اما استفاده طولانی مدت از آنها منجر به تحمل دارویی می‌شود. نارینژنین، یک فلاونوئید طبیعی موجود در میوه، آنزیمی را که باعث تحمل مواد افیونی می‌شود، مهار می کند و آن را در درمان بیماری‌های عصبی موثر می‌سازد. **هدف:** در این مطالعه نقش نارینژنین در تحمل ناشی از مورفین و آنزیم گلیکوژن سنتاز کیناز-۳بتا مورد بررسی قرار گرفت. **روش بررسی:** برای القای تحمل مورفین، تزریق‌های مکرر مورفین به مدت پنج روز انجام شد و در روز پنجم، یک دوز مورفین به صورت داخل صفاقی تزریق شد. تست درد (هات پلیت و تیل فلیک) در روزهای اول، سوم و پنجم تزریق انجام شد. برای بررسی اثر نارینژنین، ۴۵ دقیقه پیش از هر تزریق مورفین، دوزهای ۲۵، ۵۰ و ۱۰۰ میلی‌گرم بر کیلوگرم به صورت خوراکی گاواژ شد. در روز آخر بافت‌های مغز از نظر فاکتورهای بیوشیمیایی و تغییرات فسفوریلاسیون آنزیم به روش ایمونوهیستوشیمی بررسی شدند. **نتایج:** نتایج نشان داد که مصرف همزمان نارینژنین به طور معنی‌داری تاخیر بی‌دردی را نسبت به گروه مورفین در روز سوم و پنجم افزایش می‌دهد ($P < ۰/۰۰۱$). نارینژنین در تمام غلظت‌ها باعث کاهش سطح نیتریت ناشی از مورفین شد. در روش ایمونوهیستوشیمی $p\text{-}GS^{Ser640}$ اثرات محافظتی بر تحمل مورفین نشان داد ($P < ۰/۰۰۱$) و فسفوریلاسیون $p\text{-}GS^{Ser640}$ توسط آنزیم گلیکوژن سنتاز کیناز-۳بتا را کاهش داد که با تجویز مزمن مورفین فعال می‌شود. **نتیجه‌گیری:** با توجه به نتایج مطالعه حاضر، تأثیر مفید نارینژنین بر آنزیم گلیکوژن سنتاز کیناز در تحمل ناشی از مورفین تأیید می‌شود، اما برای بررسی مکانیسم تأثیر آن به مطالعات بیشتری نیاز است.

مخففها: cGMP، گوانوزین مونوفسفات حلقوی؛ COX2، سیکلواکسیژناز ۲؛ eNOS، نیتریک اکساید سنتاز اندوتلیالی؛ GFAP، پروتئین اسیدی رشته‌ای گلیالی؛ GSK3، پروتئین کیناز فعال شده با میتوژن؛ iNOS، نیتریک اکسید سنتاز القایی؛ NO، نیتریک اکسید؛ NOS، نیتریک اکسید سنتاز؛ nNOS، نیتریک اکسید سنتاز عصبی؛ NF-κB، فاکتور هسته‌ای تقویت‌کننده زنجیره سبک کاپا از لنفوسیت‌های B فعال شده؛ $p\text{-}GS^{Ser640}$، p-گلیکوژن سنتاز

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