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Involvement of nitric oxide pathway in the anti-inflammatory effect of modafinil on indomethacin-, stress-, and ethanol -induced gastric mucosal injury in rat

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ABSTRACT

Gastric ulcer is a prevalent disease with various etiologies, including non-steroidal anti-inflammatory drugs (NSAIDs), stress conditions, and alcohol, resulting in an inflammatory condition in the gastric mucosa. The aim of this study was to explore the protective effects of modafinil on gastric erosions induced by indomethacin, water-immersion stress, and alcohol in rats and to evaluate the role of nitric oxide (NO) pathway. Animals were allocated to the three experimental models of gastric ulcer – indomethacin (30 mg/kg PO), water-immersion stress, and ethanol (5 ml/kg PO). Induction of gastric ulcer in all models caused an increase in J-score (macroscopic assessment), biochemical markers, including tumor necrosis factor-alpha (TNF-α), interleukin-1beta (IL-1β), and myeloperoxidase (MPO), and microscopic destructions. Administration of modafinil (50 and 100 mg/kg i. p) significantly improved J-score in the indomethacin ($P < 0.05$) and stress models ($P < 0.001$). Moreover, the level of TNF-α IL-1β, and MPO was deceased after modafinil administration ($P < 0.001$). However, modafinil did not have any effects on gastric injury induced by ethanol. In addition, co-administration of L-NAME (a non-specific NO synthase inhibitor) and aminoguanidine (an inducible NO synthase inhibitor) with modafinil significantly neutralized the gastroprotective effect of modafinil in the indomethacin and water-immersion stress groups ($P < 0.05$, and $P < 0.01$; respectively), while 7-nitroindazole (a neuronal NO synthase inhibitor) did not show such reversing effects. In conclusion, modafinil possesses gastroprotective effects on the gastric lesions induced by indomethacin and stress, which are probably mediated via the inflammation inhibition and NO pathway modulation.

1. Introduction

Gastric ulcer is a common disease among gastrointestinal disorders, which may be associated with a significant morbidity and mortality (Chen et al., 2015). Several risk factors have been identified for gastric ulcer, including non-steroidal anti-inflammatory drugs (NSAIDs), stress, and alcohol (Everhart et al., 1998; Zheng et al., 2014). Several mechanisms have also been implicated in the pathogenesis of gastric ulcer such as inhibition of prostaglandin and bicarbonate secretion in the gastric mucosa, decreased gastric blood flow and the consequent oxidative stress, and impairment of the protective layer of epithelial cells. Moreover, it has been reported that pro-inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α) and interleukin-1beta (IL-1β) play a major role in the pathogenesis of gastric ulcer (Eamlamnam et al., 2006; Raeesi and Eskandari-Roozbahani, 2019; Richardson, 1990). In general, an imbalance between mucosal defense mechanisms and gastro-toxic agents results in erosion and ulceration in the gastric tissue (Ribeiro et al., 2016).

Modafinil, 2-[(diphenylmethyl) sulfinyl acetamide, is a non-amphetamine stimulant drug used to treat wakefulness diseases – shift work sleep disorder (SWSD), narcolepsy, and obstructive sleep apnea/hypopnea syndrome (OSAHS). It has been found that modafinil exerts

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anti-inflammatory effects by reducing the level of TNF-α and IL-1β (Ballon and Feifel, 2006; Han et al., 2018; Minzenberg and Carter, 2008). In addition, modafinil improves inflammation and reduces IL-6, TNF-α, and IFN-γ via inhibiting the Akt/NF-κB pathway in the apoE-deficient mouse model (Han et al., 2018). A previous study found that modafinil had anti-inflammatory effects on acetic acid-induced colitis in rats through decreasing the level of TNF-α and IL-1β (Dejban et al., 2020). Nitric oxide (NO) is a very small biologically active molecule that is produced from L-arginine amino acid by three isoforms of nitric oxide synthase (NOS), including neuronal NOS (n NOS)—expressed in neuronal tissues—endothelia NOS (eNOS)—presented in endothelial tissues—and inducible NOS (iNOS)—induced by phagocytes and leukocytes during inflammation in various tissues. It has been reported that NO has a protective role against gastric erosion and ulcer. With this in mind, NO modulates the healing process of gastric ulcer through enhancing the blood flow and angiogenesis in the ulcerated area as well as increasing the mucosal secretion (Rojas-Martínez et al., 2013). Indeed, NO, as a vasodilator, maintains the blood flow of the gastric mucosa; therefore, reduced NO production may lead to gastric mucosal ischemia and increased susceptibility of the gastric mucosa to erosion and ulcer (Konturek et al., 1993). The NO pathway has been considered as one of the mechanisms by which modafinil exerts its effects. For example, modafinil exhibits an anticonvulsant effect in mice through the NO pathway (Bahramnjead et al., 2018). Some animal studies also found that the effects of modafinil were neutralized by the injection of NOS inhibitors, including N (G)-nitro-l-arginine methyl ester (L-NAME) and 7-nitroindazole, non-specific inhibitors and neuronal inhibitors of NOS, which is a possible proof for the involvement of the NO pathway in the mechanism of action of modafinil (Gupta et al., 2014). Therefore, the aim of this study was to assess the effect of modafinil on gastric erosion induced by indomethacin, water-immersion stress, and ethanol in rats and to evaluate the possible role of the NO pathway.

2. Material and methods

2.1. Animal

All research procedures were performed in accordance with the ‘‘Principles of Laboratory Animal Care’’ (NIH publication 82–23, revised in 1985 and further implemented in 1996) and were approved by the Local Ethical Committee of Tehran University of Medical Sciences, No. 98-3-101-45584. Adult male Wistar rats (n = 216) weighing 200–250 gr were provided from the Animal Center of Pharmacology Department, Tehran University of Medical Sciences and kept in a temperature-controlled room in 12-h light/12-h dark cycles with free access to standard rodent food and water. One day before starting the experiment, the animals were deprived of food; however, they had free access to water. In addition, to prevent coprophagy, the animals were housed in cages with a raised floor of a wide mesh.

2.2. Chemical

Modafinil, indomethacin, L-NAME (nonspecific nitric oxide synthase inhibitor), 7-nitroindazole (a neuronal NOS inhibitor), and aminoguanidine (an inducible NOS inhibitor) were purchased from Sigma (St. Louis, Missouri, United States). Modafinil and 7-nitroindazole were dissolved in normal saline + Tween 80 1%, indomethacin was suspended in 1% carboxymethyl cellulose solution, and L-NAME and aminoguanidine were dissolved in 0.9% saline solution. Absolute ethanol (95%) (Merck, Germany) was diluted 1:1 (vol/vol) in water.

2.3. Experimental procedure

The rats were randomly allocated to three experimental models of gastric injury induction as follows:

Model 1 (ulcer induction by indomethacin): Ulcers and erosions were induced by oral administration of indomethacin 30 mg/kg by oral gavage tube (Bhattacharya et al., 2007).

Model 2 (ulcer induction by water immersion restraint): Stress ulcers were induced by maintaining the rats in a restrainer and immersing them in cold water, up to the level of their necks, for 3.5 h (Warzecha et al., 2001).

Model 3 (ulcer induction by 95% ethanol): Ulcers and erosions were induced by the oral administration of 5 ml/kg ethanol by means of an oral gavage tube (1:1 v/v) (Hajrezaie et al., 2015).

Animals in each of the three experimental models were divided into different groups of sixteen rats, including untreated control group (gastric mucosal injury was not induced and animals only received saline without any treatment), gastric injury control group (gastric mucosal injury was induced and animals received saline), gastric injury + modafinil (20 mg/kg i. p) group (gastric mucosal injury was induced and animals were treated with modafinil 20 mg/kg), gastric injury + modafinil (50 mg/kg i. p) group (gastric mucosal injury was induced and animals were treated with modafinil 50 mg/kg), and gastric injury + modafinil (100 mg/kg i. p) group (gastric mucosal injury was induced and animals were treated with modafinil 100 mg/kg). To evaluate the role of the NO pathway, non-effective doses of L-NAME (10 mg/kg i. p), 7-nitroindazole (40 mg/kg i. p), and aminoguanidine (50 mg/kg i. p) were administrated alone and in combination with an effective dose of modafinil (100 mg/kg i. p) in the indomethacin and water-immersion stress models. None of the doses of modafinil had protective effects on gastric erosions induced by ethanol; therefore, NOS inhibitors were not applied in this model. In the three models, all injections were performed 30 min before ulcer induction. Ultimately, the animals were killed by cervical dislocation 4 h after inducing gastric mucosal injury with indomethacin, 3.5 h after the beginning of the water-immersion stress test, and 1 h after ethanol-induced gastric erosion. Gastric tissues were extracted, opened along the greater curvature, and washed with ice-cold saline. After macroscopic evaluation, half of the samples (n = 8) in each group were fixed in the 10% formalin solution for histopathologic assessment and rest of the samples (n = 8) in each group were kept at −80 °C for the measurement of myeloperoxidase (MPO) activity. In addition, blood samples were taken from animals in each study group to measure the levels of TNF-α and IL1-β by ELISA (enzyme-linked immunosorbent assay) method.

2.4. Macroscopic evaluation

The J-scoring method was applied to assess the macroscopic characteristics of the specimens in terms of ulcer severity. The score of each group was calculated by evaluating the diameter of erosions (mm) by an observer blind to the study. A diameter of 0–1 mm, 1–2 mm, and more than 2 mm was scored 1, 2, and 3, respectively. The summation of sample scores was described as the ulcer index in each group (Dehpour et al., 1999).

2.5. Microscopic assessment

For histopathologic study, the samples were fixed in 10% formalin solution and embedded in paraffin wax to prepare blocks. Each block was cut into the sections of four μm and stained by hematoxylin and eozine (H & E). Histopathologic assessments were carried out by a histopathologist blind to the study.

2.6. Measurement of gastric TNF-α and IL-1β

To evaluate inflammatory cytokines, the serum levels of TNF-α and IL-1β were measured using an enzyme-linked immunosorbent assay (ELISA) kit according to the manufacturer’s instructions, rat tumor necrosis factor-alpha ELISA kit (RAB0479, Sigma Aldrich, United States) and rat IL-1β ELISA kit (RAB0277, Sigma Aldrich, United States). In brief, blood samples were taken using a cardiac puncture and

centrifuged at 700 g for 10 min for serum separation. The samples were stored at −80 °C until analysis. TNF-α and IL-1β levels are presented as pg/ml.

2.7. Measurement of gastric MPO activity

The gastric tissue of each animal was used to evaluate the MPO level. First, the tissue was homogenized and the homogenate was centrifuged at 25,200 g for 20 min. Then, the supernatant was separated for the measurement of MPO activity using an ELIZA kit (Sigma Aldrich, United States). The MPO level is presented as U/gr tissue.

2.8. Statistical analysis

Data were analyzed using Graphpad Prism (version 9) and Statistical Package for Social Sciences (SPSS) version 22. Normal distribution of data was evaluated using the Shapiro-Wilk test. Macroscopic scores (J-score) were analyzed by the Mann–Whitney test and Kruskal Wallis Test. In addition, data of TNF-α, IL-1, and MPO levels were analyzed by One-way and two-way ANOVA, and Tukey post hoc test was conducted to detect the differences between groups. P values < 0.05 were considered statistically significant.

3. Results

3.1. Effect of different doses of modafinil on J-score and macroscopic damages induced by indomethacin

As illustrated in Fig. 1A, a remarkable increase was observed in the J-score in gastric injury control group compared to the untreated control group ($P < 0.001$); on the contrary, after treatment with modafinil 50 and 100 mg/kg, the J-score significantly decreased in comparison with the gastric injury control group ($P < 0.05$ and $P < 0.001$, respectively), and remarkable improvement was observed in the gastric erosion and ulceration. Administration of modafinil 20 mg/kg did not have any decreasing effects on the J-score.

3.2. Effect of L-NAME, 7-nitroindazole, and aminoguanidine administration on J-score and macroscopic damages induced by indomethacin

As shown in Fig. 1B, C, and 1D, intraperitoneal administration of non-effective dose of NOS inhibitors alone, i.e. L-NAME (10 mg/kg i. p), 7-nitroindazole (40 mg/kg i. p), and aminoguanidine (50 mg/kg i. p), did not cause any marked changes in J-score compared to the gastric injury control group and the results were similar. Amazingly, co-administration of modafinil (100 mg/kg i. p) with L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) reversed the ameliorating effect of modafinil on macroscopic erosions, and the J-scores significantly increased in comparison with the modafinil (100 mg/kg i. p) group ($P < 0.05$ and $P < 0.01$, respectively). By contrast, after co-administration of modafinil with 7-nitroindazole (40 mg/kg i. p), the above changes were not observed, and the J-score did not change markedly compared to the modafinil (100 mg/kg i. p) group.

3.3. Effect of modafinil on J-score and macroscopic damages induced by water-immersion stress

As depicted in Fig. 2A, data analysis showed a significant increase in the J-score in the gastric injury control group in comparison with the untreated control group ($P < 0.001$) after induction of gastric injury. A marked decrease was observed in the J-score following treatment with modafinil (50 mg/kg and 100 mg/kg) in comparison with the gastric injury control group ($P < 0.05$, $P < 0.001$, respectively). However, administration of modafinil 20 mg/kg did not have any decreasing effects on the J-score.

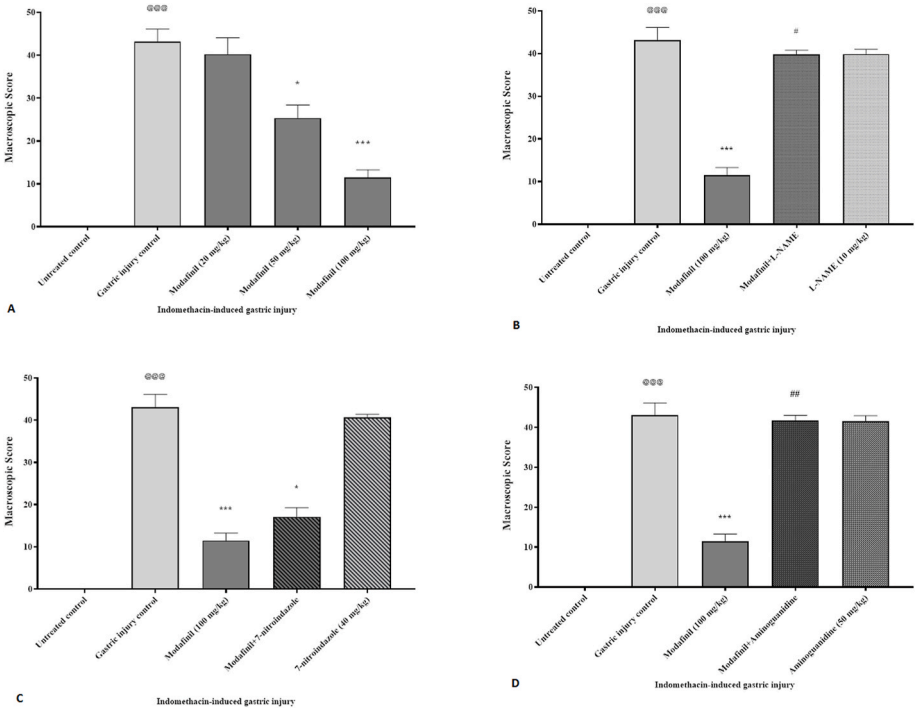

Fig. 1. Effects of oral administration of indomethacin on J-score. Data were analyzed by Mann–Whitney test and the Kruskal Wallis Test. Bars represent mean ± S.E.M (8 rats per group). $^{@@@}p < 0.001$ significantly different from untreated control group $p < 0.05$ and $^{***}p < 0.001$ significantly different from gastric injury control group. $^{\#}p < 0.001$ and $^{\#\#}p < 0.01$ significantly different from modafinil 100 mg/kg group.

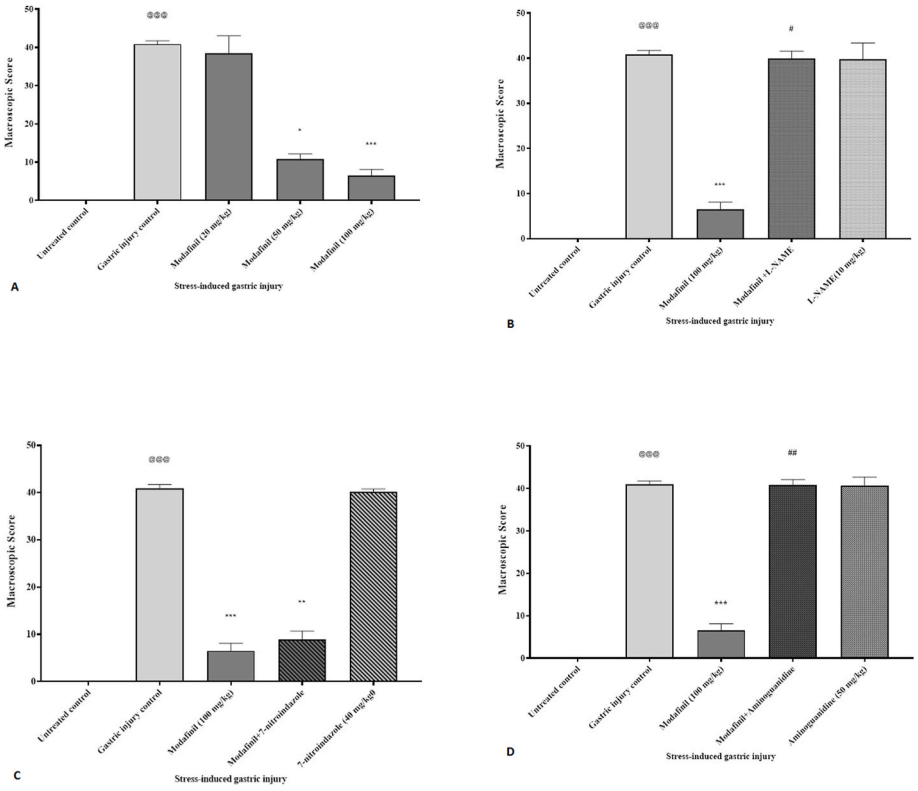

Fig. 2. Effects of water-immersion stress on J-score. Data were analyzed by Mann–Whitney test and the Kruskal Wallis Test. Bars represent mean ± S.E.M (8 rats per group).$^{@@@}$ $p < 0.001$ significantly different from untreated control group * $p < 0.05$, **$p < 0.01$, and ***$P < 0.001$ significantly different from gastric injury control group$^{\#}$ $p < 0.001$ and $^{\#\#}p < 0.01$ significantly different from modafinil 100 mg/kg group.

3.4. Effect of L-NAME, 7-nitroindazole, and aminoguanidine administration on J-score and macroscopic damages induced by water-immersion stress

Fig. 2B, C, and 2D demonstrate the effect of intraperitoneal administration of NOS inhibitors alone and in combination with the effective doses of modafinil. NOS inhibitors did not have any effects on the J-score compared to gastric injury control group; in contrast, the co-administration of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) with modafinil (100 mg/kg i. p) totally reversed the protective effect of modafinil ($P < 0.05$ and $P < 0.01$). No similar effect was observed after co-administration of 7-nitroindazole and modafinil.

3.5. Effect of modafinil on macroscopic damages induced by ethanol gavage

According to Fig. 3, ethanol led to a significant increase in the J-score in the gastric injury control group compared to the untreated control group ($P < 0.001$). However, no significant difference was found in the J-score after treatment with the modafinil doses compared to the gastric injury control group. Indeed, modafinil did not possess any protective effects on gastric mucosal injury induced by ethanol.

3.6. Effect of different doses of modafinil on TNF-α in indomethacin model of gastric mucosal injury

The serum level of TNF-α in the three models of ulcer induction is reported in Table 1. Administration of indomethacin (30 mg/kg PO) resulted in a significant increase in the level of TNF-α compared to the untreated control group ($P < 0.001$). Nonetheless, pre-treatment with modafinil (50 and 100 mg/kg i. p) reduced TNF-α markedly ($P < 0.05$ and $P < 0.001$ respectively) compared to the gastric injury control group. However, pre-treatment with modafinil (20 mg/kg i. p) did not show any significant decrease in the level TNF-α. ‘

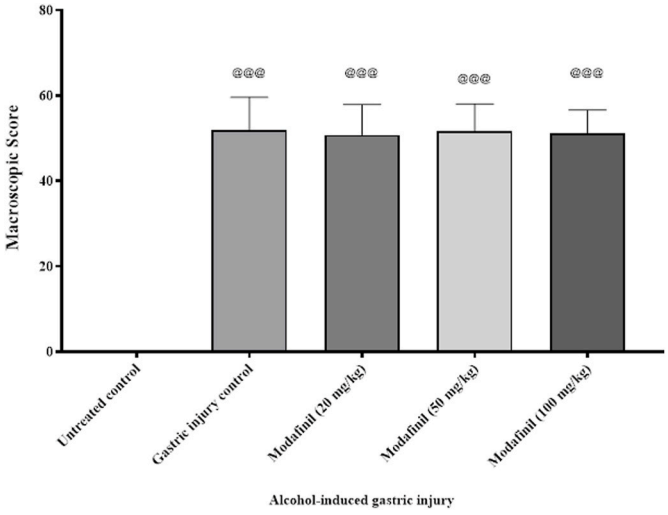

Fig. 3. Effects of the oral administration of ethanol on J-score. Data were analyzed by Mann–Whitney test and the Kruskal Wallis Test. Bars represent mean ± S.E.M (8 rats per group).$^{@@@}$ $p < 0.001$ significantly different from untreated control group.

3.7. Effect of L-NAME, 7-nitroindazole, and aminoguanidine administration on TNF-α in

Indomethacin model of gastric mucosal injury.

According to Table 1, administration of the non-effective dose of NOS inhibitors alone did not affect the level of TNF-α in comparison

Table 1
Serum level of TNF-α (pg/ml) in the gastric mucosal injury induced by indomethacin, water-immersion stress, and ethanol in rat.

	Indomethacin	Stress	Ethanol
Untreated control	50 ± 0.123	49 ± 0.111	49.5 ± 0.097
gastric injury control group	85 ± 0.014^{aaa}	80 ± 0.322^{aaa}	95.5 ± 0.011^{aaa}
Mucosal injury + Modafinil 20 mg/kg	80 ± 0.111	75 ± 0.422	90.5 ± 0.467
Mucosal injury + Modafinil 50 mg/kg	63 ± 0.214^{b}	60 ± 0.018^{bb}	82 ± 0.121
Mucosal injury + Modafinil 100 mg/kg	52.5 ± 0.108^{bbb}	47.5 ± 0.11^{bbb}	81 ± 0.222
Mucosal injury + L-NAME 10 mg/kg	85 ± 0.204	78 ± 0.788	–
Mucosal injury + 7-Nitroindazole 40 mg/kg	80 ± 0.118	75 ± 0.122	–
Mucosal injury + Aminiguanidin 50 mg/kg	80 ± 0.012	78 ± 0.012	–
Mucosal injury + Modafinil 100 mg/kg + L-NAME 10 mg/kg	80 ± 0.514^{ccc}	75 ± 0.433^{ccc}	–
Mucosal injury + Modafinil 100 mg/kg + 7-Nitroindazole 40 mg/kg	48.5 ± 0.11	49 ± 0.66	–
Mucosal injury + Modafinil 100 mg/kg + Aminiguanidine 50 mg/kg	78 ± 0.216^{c}	72 ± 0.012^{cc}	–

Two-way ANOVA was applied to comparison between untreated control groupand gastric injury control group. One-way ANOVA was used to compare between gastric injury control group and other treated groups (8 rats per group).
aaa $P < 0.001$ compared to the untreated control group.
b $P < 0.05$.
bb $P < 0.01$, and.
bbb $P < 0.001$ compared to gastric injury control group.
c $P < 0.05$.
cc $P < 0.01$, and.
ccc $P < 0.001$ compared to the mucosal injury + modafinil 100 mg/kg group.

with the gastric injury control group. Interestingly, co-administration of the modafinil effective dose (100 mg/kg i. p) with non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) significantly neutralized ($P < 0.001$ and $P < 0.05$, respectively) the effect of modafinil (100 mg/kg i. p) on TNF-α in the indomethacin model, and TNF-α increased compared to the modafinil (100 mg/kg i. p) group. However, the level of TNF-α did not change after the co-administration of modafinil and 7-nitroindazole (40 mg/kg i. p) compared to the modafinil (100 mg/kg i. p) group.

3.8. Effect of different doses of modafinil on TNF-α in the water-immersion stress model of gastric mucosal injury

Water-immersion stress led to a marked increase in the level of TNF-α in the gastric tissue compared to the untreated control group ($P < 0.001$). However, treatment with modafinil (50, 100 mg/kg i. p) markedly lowered TNF-α ($P < 0.01$ and $P < 0.001$, respectively) compared to the gastric injury control group. Additionally, no significant changes were observed in the level of TNF-α after administration of modafinil 20 mg/kg.

3.9. Effects of L-NAME, 7-nitroindazole, and aminoguanidine administration on TNF-α in water-immersion stress model of gastric mucosal injury

Administration of the non-effective dose of NOS inhibitors alone did not affect the level of TNF-α compare to the gastric injury control group. Interestingly, co-administration of the effective dose of modafinil (100 mg/kg i. p) with non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) significantly neutralized ($P < 0.001$ and $P < 0.01$, respectively) the effect of modafinil (100 mg/kg i. p) on TNF-α in the indomethacin model, and the level of TNF-α increased compared to the modafinil (100 mg/kg i. p) group. However, after the co-administration of modafinil with 7-nitroindazole (40 mg/kg i. p), the level of TNF-α did not alter compared to the modafinil (100 mg/kg i. p) group.

3.10. Effect of different doses of modafinil administration on IL-1β in indomethacin model of gastric mucosal injury

The serum level of IL-1β in the three models of ulcer induction is reported in Table 2. Administration of indomethacin (30 mg/kg PO) caused a significant increase in the level of IL-1β in the gastric injury control group compared to the untreated control group ($P < 0.001$). Pretreatment with modafinil (50 and 100 mg/kg i. p) markedly reduced the level of IL-1β ($P < 0.05$ and $P < 0.01$, respectively) compared to the gastric injury control group. However, no significant changes were observed in the level of IL-1β after the administration of modafinil 20 mg/kg.

3.11. Effect of L-NAME, 7-nitroindazole, and aminoguanidine administration on IL-1β in indomethacin model of gastric mucosal injury

Non-effective doses of NOS inhibitors alone did not affect the level of IL-1β compared to gastric injury control group. Co-administration of the effective dose of modafinil (100 mg/kg i. p) with the non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) markedly reversed ($P < 0.05$ and $P < 0.01$, respectively) the effect of modafinil (100 mg/kg i. p) on IL-1β in the indomethacin model, and the level of IL-1β increased compared to the modafinil (100 mg/kg i. p) group. However, the level of IL-1β did not change after co-administartion of modafinil with 7-nitroindazole (40 mg/kg i. p) compaed to the modafinil (100 mg/kg i. p) group.

Table 2
Serum level of IL-1β (pg/ml) in the gastric mucosal injury induced by indomethacin, water-immersion stress, and ethanol in rat.

	Indomethacin	Stress	Ethanol
Untreated control	30 ± 0.105	32 ± 0.310	31 ± 0.151
gastric injury control group	70 ± 0.177^{aaa}	65 ± 0.344^{aaa}	80 ± 0.122^{aaa}
Mucosal injury + Modafinil 20 mg/kg	65 ± 0.07	62.5 ± 0.124	77.5 ± 0.202
Mucosal injury + Modafinil 50 mg/kg	47.5 ± 0.088^{b}	45 ± 0.431^{b}	72.5 ± 0.008
Mucosal injury + Modafinil 100 mg/kg	35 ± 0.024^{bb}	37 ± 0.106^{bb}	75 ± 0.104
Mucosal injury + L-NAME 10 mg/kg	70 ± 0.023	65 ± 0.202	–
Mucosal injury + 7-Nitroindazole 40 mg/kg	68 ± 0.01	60 ± 0.161	–
Mucosal injury + Aminiguanidin 50 mg/kg	70 ± 0.047	63 ± 0.021	–
Mucosal injury + Modafinil 100 mg/kg + L-NAME 10 mg/kg	62.5 ± 0.022^{c}	61.5 ± 0.046^{ccc}	–
Mucosal injury + Modafinil 100 mg/kg + 7-Nitroindazole 40 mg/kg	37 ± 0.671	38.5 ± 0.121	–
Mucosal injury + Modafinil 100 mg/kg + Aminiguanidine 50 mg/kg	67 ± 0.012^{cc}	63 ± 0.103^{c}	–

Two-way ANOVA was applied to comparison between untreated control group and gastric injury control group. One-way ANOVA was used to compare between ulcer control group and other treated groups (8 rats per group).
aaa $P < 0.001$ compared to the untreated control group.
b $P < 0.05$ and.
bb $p < 0.01$compared to the gastric injury control group.
c $P < 0.05$.
cc $P < 0.01$, and.
ccc $P < 0.001$ compared to the mucosal injury + modafinil 100 mg/kg group.

3.12. Effect of modafinil administration on IL-1β in the water-immersion stress model of gastric mucosal injury

Water-immersion stress led to a marked increase in the level of IL-1β in the gastric tissue compared to the untreated control group ($P < 0.001$). However, treatment with modafinil (50, 100 mg/kg i. p) significantly lowered the IL-1β level compared to the gastric injury control group ($P < 0.05$ and $P < 0.01$, respectively). Additionally, no significant changes were observed in the level of IL-1β after the administration of modafinil 20 mg/kg.

3.13. Effects of L-NAME, 7-nitroindazole, and aminoguanidine administration on IL-1β in water-immersion stress models of gastric mucosal injury

Non-effective doses of NOS inhibitors alone did not affect the level of IL-1β compared to the gastric injury control group. Co-administration of the effective dose of modafinil (100 mg/kg i. p) with non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg, i. p) significantly reversed ($P < 0.001$ and $P < 0.05$, respectively) the effect of modafinil (100 mg/kg, i. p) on IL-1β in the indomethacin model, and the level of IL-1β increased compared to the modafinil (100 mg/kg i. p) group. However, the level of IL-1β did not alter after co-administartion of modafinil with 7-nitroindazole (40 mg/kg i. p) compared to modafinil (100 mg/kg i. p).

3.14. Effect of different doses of modafinil on MPO level in indomethacin model of gastric mucosal injury

According to Table 3, administration of indomethacin resulted in a marked increase in the MPO level in the gastric injury control group compared to the untreated control group ($P < 0.001$). However, pre-treatment with modafinil (50 and 100 mg/kg i. p) decreased the level of MPO compare to the gastric injury control group ($P < 0.01$ and $P < 0.001$, respectively). Administration of modafinil (20 mg/kg i. p) had no significant effects on the MPO level compared to the gastric injury control group.

Table 3
Gastric tissue level of MPO (U/gr) in gastric mucosal injury induced by indomethacin, water-immersion stress, and ethanol in rat.

	Indomethacin	Stress	Ethanol
Untreated control	2.1 ± 0.111	2 ± 0.354	2 ± 0.231
gastric injury control group	4 ± 0.154^{aaa}	3.5 ± 0.764^{aa}	7 ± 0.056^{aaa}
Mucosal injury + Modafinil 20 mg/kg	3.8 ± 0.067	3.2 ± 0.303	6.5 ± 0.043
Mucosal injury + Modafinil 50 mg/kg	3 ± 0.76^{bb}	2.5 ± 0.154^{b}	6 ± 0.565
Mucosal injury + Modafinil 100 mg/kg	2.5 ± 0.012^{bbb}	2.3 ± 0.032^{bb}	6.2 ± 0.01
Mucosal injury + L-NAME 10 mg/kg	3.3 ± 0.305	3.5 ± 0.111	–
Mucosal injury + 7-Nitroindazole 40 mg/kg	3.9 ± 0.115	3.3 ± 0.408	–
Mucosal injury + Aminiguanidin 50 mg/kg	3.7 ± 0.123	3.4 ± 0.014	–
Mucosal injury + Modafinil 100 mg/kg and L-NAME 10 mg/kg	3.9 ± 0.406^{c}	3.3 ± 0.651^{cc}	–
Mucosal injury + Modafinil 100 mg/kg and 7-Nitroindazole 40 mg/kg	2.6 ± 0.054	2.4 ± 0.112	–
Mucosal injury + Modafinil 100 mg/kg and Aminiguanidine 50 mg/kg	4 ± 0.012^{ccc}	3.5 ± 0.096^{cc}	–

Two-way ANOVA was applied to comparison between untreated control group and gastric injury control group. One-way ANOVA was used to compare between gastric injury control group and other treated groups (8 rats per group).
aa $P < 0.001$ and.
aa $P < 0.01$compared to the untreated control group.
b $P < 0.05$.
bb $p < 0.01$, and.
bbb $p < 0.001$ compared to the gastric injury control group.
c $p < 0.05$.
cc $p < 0.01$, and.
ccc $p < 0.001$ compared to the mucosal injury + modafinil 100 mg/kg group.

3.15. Effect of L-NAME, 7-nitroindazole, and aminoguanidine administration on MPO level in indomethacin model of gastric mucosal injury

Treatment with NOS inhibitors alone did not affect the level of MPO in the indomethacin model compared to the gastric injury control group. Co-administration of the effective dose of modafinil (100 mg/kg i. p) with non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) neutralized ($P < 0.05$ and $P < 0.001$, respectively) the decreasing effect of modafinil (100 mg/kg i. p) on the level of MPO, and the MPO level increased significantly compared to the modafinil (100 mg/kg i. p) group. However, the level of MPO did not change after co-administration of modafinil (100 mg/kg i. p) with the non-effective dose of 7-nitroindazole (40 mg/kg i. p) compared to modafinil (100 mg/kg i. p).

3.16. Effect of modafinil administration on MPO level in water-immersion stress model of gastric mucosal injury

In the stress model of gastric injury, although ulcer induction significantly increased the MPO activity in the gastric injury control group compared to the untreated control group ($P < 0.01$), the MPO level decreased significantly following the injection of modafinil (50 and 100 mg/kg i. p) compared to the gastric injury control group ($P < 0.05$ and $P < 0.01$, respectively). However, pre-treatment with modafinil (20 mg/kg i. p) did not decrease the level of MPO.

3.17. Effects of L-NAME, 7-nitroindazole, and aminoguanidine administration on MPO level in water-immersion stress model of gastric mucosal injury

Treatment with NOS inhibitors alone did not affect the level of MPO in the water-immersion stress model compared to the gastric injury control group. Co-administration of the effective dose of modafinil (100 mg/kg i. p) with non-effective doses of L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) neutralized the decreasing effect of modafinil (100 mg/kg i. p) on the MPO level ($P < 0.01$), and the MPO level increased significantly compared to the modafinil (100 mg/kg i. p) group. However, the level of MPO did not change after co-administartion of modafinil (100, mg/kg i. p) and non-effective dose of 7-nitroindazole (40 mg/kg i. p) compared to the modafinil (100 mg/kg i. p) group.

3.18. Effect of modafinil on histological damage induced by indomethacin, stress, and ethanol

Fig. 4 demonstrates the histopathologic appearance of experimental groups. Administration of indomethacin, water-immersion stress test, and oral gavage of ethanol led to a severe gastritis with mucosal erosion and hemorrhage compared to the untreated control group. Administration of modafinil (50 and 100 mg/kg i. p) in indomethacin and water-immersion stress models significantly improved microscopic lesions. The histologic appearance of the gastric tissue of the animals, which were ulcerated by ethanol gavage, did not show any decrease in the mucosal damages after treatment with modafinil.

3.19. Effect of L-NAME, 7-nitroindazole, and aminoguanidine on histological damage induced by indomethacin and stress

The effect of the intraperitoneal administration of NOS inhibitors

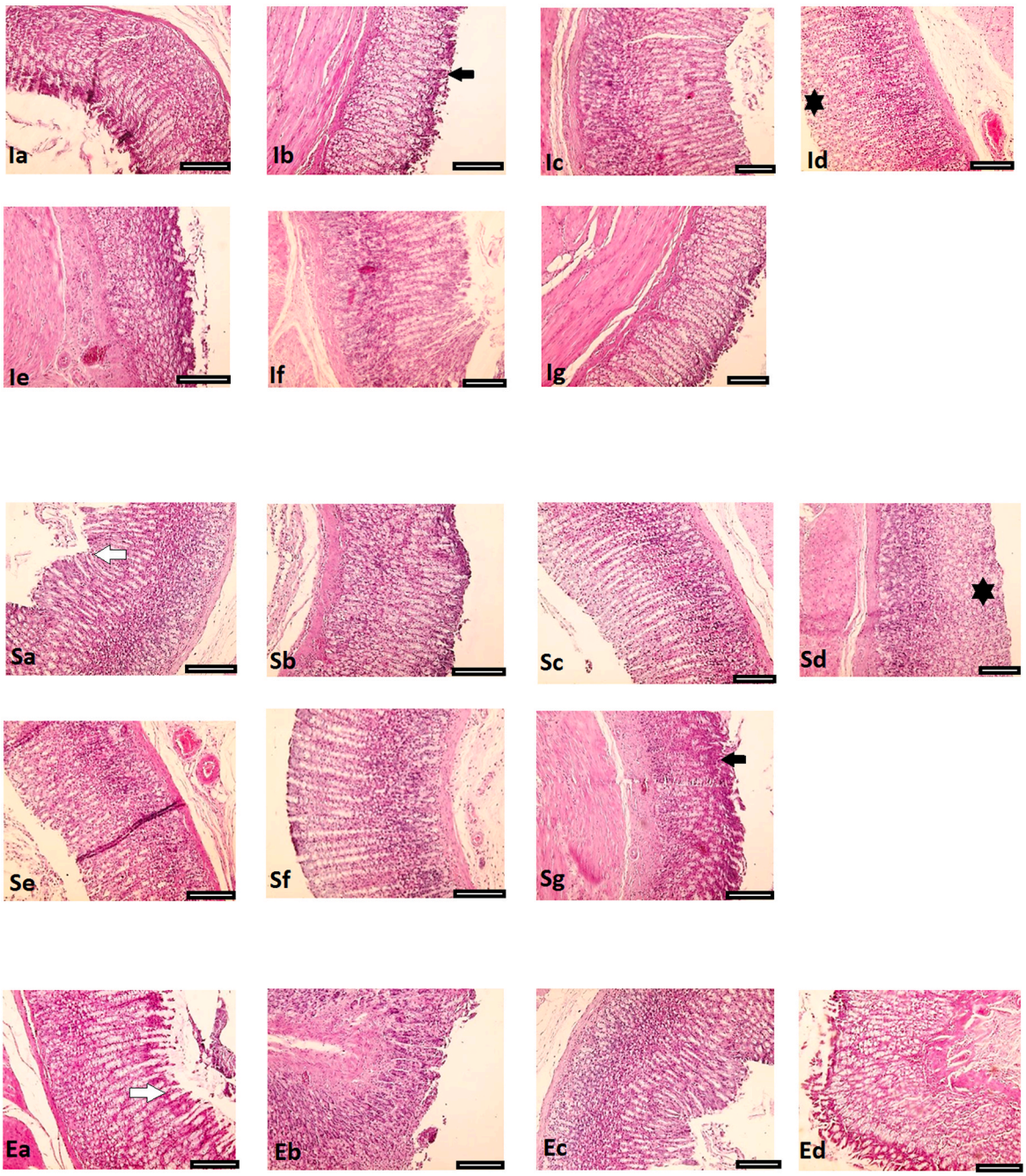

Fig. 4. Histopathologic feature of gastric mucosa of rat (8 rat per group). I: indomethacin, S: (Water-immersion) stress, E: ethanol. a: gastric injury control group, b: peptic ulcer + modafinil 20 mg/kg, c: peptic ulcer + modafinil 50 mg/kg, d: peptic ulcer + modafinil 100 m/kg, e: peptic ulcer + modafinil 100 mg/kg and L-NAME 10 mg/kg, g: peptic ulcer + modafinil 100 mg/kg and 7-nitroindazole 40 mg/kg, f: peptic ulcer + modafinil 100 mg/kg and aminoguanidine 50 mg/kg. Black flash: destroyed gastric epithelium, White flash: destroyed gastric gland, Black star: normal gastric epithelium and gastric pit. Scale bar: 100 μm.

alone and in combination with the effective dose of modafinil was assessed in the indomethacin and water-immersion stress models of gastric mucosal injury. It was observed that pre-treatment with L-NAME (10 mg/kg i. p), 7-nitroindazole (40 mg/kg i. p), and aminoguanidine (50 mg/kg i. p) alone did not improve the histologic damage compared to the gastric injury control group. However, a combination of the effective dose of modafinil (100 mg/.kg i. p) with L-NAME (10 mg/kg i. p) and aminoguanidine (50 mg/kg i. p) reversed the improving effects of modafinil (100 mg/kg i. p) on the histologic lesions in indomethacin and water-immersion stress models, and the samples in these groups developed severe gastric erosions. However, co-administration of the effective dose of modafinil and 7-nitroindazole (40 mg/kg i. p) did not affect the histologic damage, and the samples in these groups were similar to the modafinil (100 mg/kg i. p) group.

4. Discussion

The present experiment demonstrated that pretreatment with modafinil decreased macroscopic damage increased the levels of biochemical markers, and improved microscopic lesions, which appeared in the gastric tissue after oral administration of indomethacin, water-immersion stress, and ethanol gavage. As for macroscopic lesions, gastric ulcer induction in the three models increased the J-score, while administration of modafinil significantly decreased the macroscopic

lesions and J-score in indomethacin and stress models. However, no significant improvement was observed in the ethanol-induced lesions.

Regarding biochemical markers, TNF-α, IL1-β, and MPO increased after the three models of gastric ulcer induction; by contrast, intraperitoneal administration of modafinil caused a marked reduction in the level of the above markers in indomethacin and water-immersion stress models. Histological assessment also showed severe ulceration, erosions, and mucosal hemorrhage after inducing gastric erosions in the three models. By contrast, modafinil significantly improved histological lesions in indomethacin and water-immersion stress models. Interestingly, co-administration of L-NAME and aminoguanidine totally neutralized the protective effect of modafinil on macroscopic lesions and J-score, level of biochemical markers (TNF-α, IL-1β, and MPO), and histologic erosions induced by indomethacin and water-immersion stress, indicating the probable role of the NO pathway in the mechanism of action of modafinil.

It was previously reported that NSAIDs and stress conditions resulted in the development of macroscopic erosions in the gastric mucosa of rats (Zheng et al., 2014). Gastric lesions are developed due to a reduction in the gastric blood flow and an inhibition of the production of the of protective prostaglandins (PGs) – PG I_2 and PG E_2 – which are produced by cyclooxygenase 1 and 2 (COX 1 and COX 2) (Arakawa et al., 1981; De Souza et al., 2002; Wallace, 1997); subsequently, PGs inhibition causes an increase in the level of TNF-α and IL-1β (Hasgul et al., 2014). In addition, following the suppression of prostaglandins, a large number of neutrophils are recruited to the gastric mucosa (Elliott and Wallace, 1998; Nishida et al., 1998); consequently, the accumulated neutrophils in the gastric tissue start generating reactive oxygen species (ROS) that cause lipid and protein peroxidation, resulting in severe histological damage (Inas et al., 2011). Moreover, the MPO activity—an indicator of the neutrophil activation—increases following neutrophil accumulation (Kataoka et al., 2000). It has been reported that NSAIDs and stress conditions also lead to microscopic lesions, including inflammatory cells infiltration and hemorrhagic mucosal erosions in the rat gastric tissue (Ohba et al., 2006). In compliance with previous studies, oral administration of indomethacin—as a NSAID— and water-immersion stress resulted in macroscopic mucosal damage and increased the J-score of the gastric tissue and the levels of TNF-α, IL-1β, and MPO, indicating histopathologic damage.

The modulatory role of NO has been investigated in gastric ulcer. In this regard, NO donors, like glyceryl trinitrate, significantly accelerate the healing process of gastric ulcers in rats, while NOS inhibitors impair this process (Teslovich et al., 2010). Konturek et al. explored the role of nitric oxide in gastric ulcer healing and found that NOS inhibitors caused a delay in ulcer healing by decreasing the blood flow and capillaries at the ulcer margin. They concluded that NO played a protective role against gastric ulcer (Konturek et al., 1993). Similarly, Calatayud et al. found that transdermal nitroglycerin protected against indomethacin-induced gastric lesions through maintaining the mucosal blood flow and reducing leukocyte-endothelial cell rolling and adherence (Calatayud et al., 1999). Apart from the above-mentioned roles, NO may protect against indomethacin-induced ulcer through enhancing prostaglandin synthesis. As a matter of fact, NO donors increase cyclooxygenase (COX) activity, while NOS inhibitors suppress PGE2 production (Salvemini et al., 1993). In addition, it has been demonstrated that the NO pathway modulates gastric lesions caused by water-immersion stress. With this in mind, NO synthesis inhibitors, like L-NAME, intensify and NO stimulators, like L-arginine, decrease gastric ulceration induced by water-immersion stress in rats (Ogle and Qiu, 1993), which is due to the modulatory effect of NO on neutrophil infiltration in the gastric tissue (Ohta and Nishida, 2001). In addition, NO maintains the proper function of goblet cells that are responsible for mucus secretion. Therefore, NO donors enhance mucus release in the gastric tissue in rats (Brown et al., 1993) and thicken the mucus gel in the rat stomach (Brown et al., 1992).

Some studies recently found the anti-inflammatory effects of Modafinil. In 2018, J Han et al. studied the anti-inflammatory role of modafinil in the prevention of atherosclerosis in an apoE deficient mouse model. They found that treatment with modafinil decreased the development of atherosclerosis by stimulating the anti-inflammatory cytokines and suppressing the pro-inflammatory cytokines and macrophage accumulation. Additionally, this study showed that modafinil led to the inhibition of macrophage proliferation (Han et al., 2018). In another animal study, Raineri et al. revealed that modafinil had a protective effect on neuroinflammation induced by methamphetamine in mice (Raineri et al., 2012). In our previous study, we also determined the anti-inflammatory effect of modafinil in a rat model of colitis. Colitis was induced by intrarectal administration of acetic acid; then, the animals were treated with modafinil. It was observed that modafinil significantly reduced TNF-α and IL-1β (Dejban et al., 2020), indicating the anti-inflammatory properties of modafinil. Moreover, it was indicated that the NO pathway played a role in the modafinil anti-inflammatory function. In this regards, Gupta et al. reported that administration of NOS inhibitors (i.e., L-NAME and 7-nitroindazole) before modafinil injection reversed hyperalgesia induced by modafinil. Hence, they suggested the involvement of the NO pathway in the mechanism of action of modafinil in mice (Gupta et al., 2014). In another animal study that investigated the effect of modafinil on the threshold of seizure induced by pentylenetetrazole, the results showed that the anticonvulsant effect of modafinil was thoroughly inhibited by NOS inhibitors (Bahramnjead et al., 2018). Our previous study also showed that the anti-inflammatory effects of modafinil were thoroughly reversed following the administration L-NAME, 7-nitroindazole, and aminoguanidine in a rat model of colitis. Therefore, it was concluded that modafinil had anti-inflammatory properties presumably through the NO pathway (Dejban et al., 2020). In line with previous studies, our present experiment also showed that pre-treatment with modafinil led to a significant improvement in the macroscopic, biochemical, and microscopic disorders following the oral administration of indomethacin and water-immersion stress. Furthermore, co-administration of modafinil with L-NAME and aminoguanidine reversed the protective effects of modafinil, indicating the role of NO in the mechanism of action of modafinil.

As for alcohol-induced gastric erosions, mast cells are the main inflammatory cells that regulate the inflammation and release large amounts of histamine in the gastric mucosa (Cho et al., 1985). Histamine also mediates the recruitment of neutrophils through adhesion molecules expression and potentiation in the gastric tissue following ethanol administration. (Chow et al., 1998). Moreover, acute ethanol ingestion alters the gastric mucosal barrier and has a direct effect on gastric mucosal cells, causing intensive injury with loss of surface epithelium, leading to mucosal layer exfoliation, hyperemia, hemorrhage, and severe erosions (Erkasap et al., 2005; MacMath, 1990). The effect of mast cells, histamine release, and direct damage to the mucosal cells following ethanol ingestion is probably the reason that modafinil did not improve ethanol-induced ulcer in the present study.

Generally, the limited number of antiulcer models for drug development against gastric ulcer studies has hindered the progress of targeted therapy in this field. If animals have longer time exposure with ethanol, stress, or indomethacin, more clinical symptoms would appear, for instance, diarrhea, nausea, and vomiting; therefore, we can evaluate the effect of treatment on clinical symptoms as well; however, because of considering ethical issues, we had limitations to expose animal for more time with damaging factors like ethanol. Another limitation was the concurrence of our study with Coronavirus pandemic. We planned to measure the arachidonic acid metabolites in this study; however, because of laboratory closing we could not do that.

5. Conclusion

In conclusion, modafinil has a protective effect against gastric erosions and ulcers through decreasing the level of pro-inflammatory

cytokines (TNF-α and IL-1β) and gastric MPO activity. In addition, co-administration of L-NAME and aminoguanidine with modafinil neutralizes the anti-inflammatory effects of modafinil, indicating that modafinil exerts its role via modulating the NO pathway.

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CRediT authorship contribution statement

Pegah Dejban: Conceptualization, Methodology, Supervision. **Faezeh Eslami:** Investigation, Project administration, Writing - original draft, Methodology. **Nastaran Rahimi:** Investigation, Project administration. **Nasrin Takzare:** Formal analysis, Writing - review & editing. **Mohamadmostafa Jahansouz:** Validation, Resources. **Ahmad Reza Dehpour:** Writing - review & editing.

Declaration of competing interest

The authors report no conflict of interest.

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