Effect of non-steroidal anti-inflammatory drugs on behavioral actions of diazepam in mice

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ABSTRACT

الأهداف: دراسة التدخل الدوائي لتأثير السلوكي لديازيبام مع مضادات الالتهاب الغير الستيرودية.

الطريقة: تم إعطاء 15 و 24 مجموعة على التوالي من الفئران نوع ألبينو (n=8) مضادات الالتهاب غير الستيرودية غير الانتقائية للأنزيم سيكلواوكسجينيز (الأسبرين 100mg/kg، ديكلوفيناك 10mg/kg، و الاندوميتاسين 10mg/kg)، و مضادات الالتهاب الانتقائية لسيكلواوكسجينيز1- المتمثلة في جرعة صغيرة من الأسبرين 10mg/kg و سيلوكوكسيب الانتقائي لسيكلواوكسجينيز-2 10mg/kg قبل إعطاء الديازيبام المنوم (20mg/kg). وفي المجموعات السادسة والرابعة الأخرى (n=10) وباستخدام الحلبة المفتوحة واختبار الشد العضلي، تم إعطاء5mg/kg و 10mg/kg من الديازيبام كمهدىء و لإرخاء العضلات، ثم إعطاء 2mg/kg من الديازيبام لأربعة مجموعات (n=6) لعلاج الأرق بعد إعطاء الأسبرين 10mg/kg. أجريت الدراسة في جامعة الفاتح للعلوم الطبية – طرابلس – ليبيا خلال الفترة من فبراير حتى مايو 2009م.

النتائج: قللت مضادات الالتهاب غير الستيرودية غير الانتقائية للأنزيم سيكلواوكسجينيز و مضادات الالتهاب الانتقائية لسيكلواوكسجينيز-1 من فترة النوم الناتج عن طريق ديازيبام في فئران التجارب بنسبة تتراوح من 60-75%. بينما استخدام مضادات الالتهاب الانتقائية لسيكلواوكسجينيز-2 لم يفلح في التأثير على النوم الناتج من الديازيبام في فئران التجارب $p>0.05$. لكن، آثار استخدام الديازيبام في علاج الأرق و القلق و استرخاء العضلات لم يتأثر بالأسبرين.

خاتمة: يستنتج أن مضادات الالتهاب غير الستيرودية غير الانتقائية و الانتقائية لسيكلواوكسجينيز1- قللت من فترة النوم الناتجة من الديازيبام و هو نتيجة تأثير آلية الدواء.

Objectives: To investigate the behavioral pharmacological interactions of diazepam with non steroidal anti-inflammatory drugs.

Methods: Non selective cyclooxygenase enzyme inhibitors (100 mg/kg acetylsalicylic acid, 10 mg/kg indomethacin, and 10 mg/kg diclofenac), a selective cyclooxygnase-1 inhibitor (10 mg/kg acetylsalicylic acid), and a selective cyclooxygnase-2 inhibitor (10 mg/kg celecoxib) of non steroidal anti-inflammatory drugs were individually pretreated to 15 and 24 groups of Albino mice for dose and time dependent models (n=8, each treatment) before sleeping induced by diazepam (20 mg/kg, intraperitoneally). In 6 groups using an open field and 4 groups using traction test models (n = 10), 5 and 10 mg/kg of diazepam, intraperitoneally were given to induce sedation and muscle relaxation, and 2 mg/kg to induce anxiolytic action after treatment with acetylsalicylic acid (10 mg/kg) to 4 groups (n = 6). This study was carried out at the Al-Fateh Medical Science University, Tripoli, Libya between February and May 2009.

Results: In dose and time dependent models non selective cyclooxygenase and selective cyclo-oxygnase-1 inhibitors significantly reduced the duration of sleep induced by diazepam in mice by 60-75%, while the selective cyclooxygnase-2 inhibitor did not ($p>0.05$). However, anxiolytic, muscle relaxant, and sedative effects of diazepam were unchanged by acetylsalicylic acid.

Conclusion: Non-steroidal anti-inflammatory drugs, most likely cyclooxygenase selective-1 inhibitors reduced the duration of sleep induced by diazepam, and this interaction could be of a pharmacodynamic type.

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Over several years, interactions between drugs were recognized as having potential to influence patient outcomes through alteration in drug disposition and actions.[1] Thus, a drug interaction is a pharmacological or clinical response to the administration of a drug with another substance that changes the patient's response to the drug. Drug interactions can be the result of pharmacokinetic, pharmacodynamic, or a combination of both mechanisms.[2] Pharmacokinetic interaction takes place when a drug changes the absorption, distribution, metabolism, and/or excretion of another drug.[3] A pharmacodynamic interaction occurs when more than one drug has a mechanism of action that results in the same physiological outcome. Pharmacodynamic interactions are classified into synergistic, antagonistic and additive.[2,3] In some clinical cases such as low back pain, non-steroidal anti-inflammatory drugs (NSAIDs) are prescribed with benzodiazepines (BZs). Diazepam (DZP), a protype of BZs, binds to BZs gamma-aminobutyric acid (GABA) receptors complex type A that contains α subunits, α_1, α_2, α_3, or α_5 $GABA_A$ receptors. It has been reported that α_1 $GABA_A$ receptors mediate the sedative and anticonvulsant actions of DZP. However, anxiolytic actions are mediated through α_2/α_3 and muscle-relaxant actions are through α_2 $GABA_A$ receptors. Anterograde amnesic actions are through α_1/α_5.[4] Pharmacologically, NSAIDs produce their actions by inhibiting the production of prostaglandins (PGs), inflammatory residues that are synthesized from arachidonic acid under the control of cyclooxygenase (COX) enzyme. The cyclooxygenase system is present in 2 forms, COX-1 and COX-2.[5] The COX-1 is expressed constitutively in most tissues of the body where COX-2 is increased during an inflammatory response.[6,7] Selective inhibition of COX enzyme activity by NSAIDs can be classified as the non selective COX group, as acetylsalicylic acid (ASA), diclofenac and indomethacin,[6] the COX-1 selective group as low dose ASA[8] and the COX-2 selective group as celecoxib.[9] In our laboratory, preliminary experimental studies have indicated that ASA reduced the duration of sleep induced by DZP. Thus, the present work aimed to study which type of COX inhibitor affects the interaction between DZP and NSAIDs and if this interaction would occur with other hypnotics, other BZs derivatives and other actions of DZP.

Methods. *Chemicals.* Diazepam was obtained from C.P. Pharmaceuticals Ltd, Wrexham, UK, midazolam and clonazepam from Roche (Basel, Switzerland), ASA and pentobarbitone from Sanofi–Synthelabo (Paris, France). Diclofenac from Gulf Pharmaceutical Industries (Ras Alkhaimaah, UAE), indomethacin from Ajanta Pharmaceuticals Ltd (Mombai, India) and celecoxib from Pfizer, (New York, USA).

Animals. Male Swiss Albino mice weighing 25-30 g and Wistar rats weighing of 150-250 g were obtained from the local animal house (Al-Fateh Medical Science University, Tripoli, Libya). All drugs were administrated intraperitoneally (i.p.) with an injection volume of 10 ml/kg.[10,11] This study was carried out at the Department of Pharmacology, Faculty of Pharmacy, Al-Fateh Medical Science University, Tripoli, Libya from February to May, 2009. Ethical approval for the experiment and animal use was obtained from the ethics committee of Al-Fateh Medical University.

Dose-dependent study. Mice were divided into 15 groups (n = 8, total 120). Water (vehicle) was given to the first group to serve as a control. The NSAIDs were administrated to the remaining groups as following: ASA in doses of 50, 100 and 200 mg/kg,[12] diclofenac in doses of 5, 10 and 20 mg/kg,[13] indomethacin in doses of 5, 10 and 20 mg/kg.[14] Celecoxib in doses of 5, 10 and 20 mg/kg[15] as a COX-2 selective inhibitor of NSAIDs and ASA in doses of 5, 10 and 20 mg/kg[14] as a COX-1 selective inhibitor of NSAIDs. Then, 20 mg/kg of DZP was administrated 30 minutes after treatment with the vehicle or NSAIDs.[14]

Time-dependent study. Mice were divided into 24 groups (n = 8, total 192). Diazepam (20 mg/kg) was given to the first group as a control. The NSAIDs as mentioned above in selected doses were administrated, simultaneously with DZP (20 mg/kg) to the second group. The same treatments were administrated to the remaining groups at 30, 60 and 120 minute intervals, except ASA (non selective) at 0.5, 1, 2, 24, 48, 72 and 96 hours. The other study shows the effect of ASA on other BZ derivatives induced sleep in mice. In this study mice were divided into 2 groups (n = 10, total 20), the vehicle was administrated to the first group and ASA (10 mg/kg) to the second group. Clonazepam (20 mg/kg)[16] was then administrated 30 minutes after treatment by the vehicle and ASA. The same procedures were carried out for midazolam (90 mg/kg).[16]

To study this interaction in other species, ASA (100 mg/kg)[11] and DZP (40 mg/kg) in the same manner were administrated to 2 groups of rats (n = 6, total 12). In another 7 groups of mice (n = 8, total 56), pentobarbitone sodium (50 mg/kg), (selected upon pilot study) was used for comparison to investigate the interaction with NSAIDs in dose and time dependent models.

Effect of ASA on DZP induced sedation in an open field model of locomotor activity. Each mouse was tested for 5 minutes in an open field apparatus (44 × 44 and 30 cm side wall) in which locomotor movements were monitored automatically with infrared beams.[17] Mice were observed for ambulatory, non ambulatory and number of movements by placing them gently and individually in the center of the open field.[18] The

experiment was carried out in a calm environment illuminated with low light (10 watt) between 9:00 am and 1:00 pm,[17] in constant environmental conditions (temperature, humidity and 12/12 light/dark cycle). Six groups of mice (n= 10, total 60) were used: group A (control), group B (10 mg/kg ASA), group C (5 mg/kg DZP), group D (10 mg/kg ASA + 5 mg/kg DZP) group E (10 mg/kg DZP) and group F (10 mg/kg ASA + 10 mg/kg DZP). The time interval between treatments was 30 minutes.[14,19] The test was carried out 30 minutes after the last treatment in all the groups.[20]

Effect of ASA on DZP induced anxiolytic action using an elevated plus maze model of anxiety. The elevated plus maze model of anxiety of mouse consisted of 2 opposed open arms (30 × 5 cm), and 2 opposed closed arms (30 × 5 cm) with a 15 cm side wall and connecting central platform (5 × 5 cm). Each mouse was placed in the central platform and allowed to explore the maze for 5 minutes.[21] The following were recorded: number of entries into arms and time permanence on arms. The ratios "number of entries into open arms (NEOA)/total number of arm entries and "time permanence on open arms (TPOA)/total time permanence on all arms" were calculated and percentage of entries (PEOA) and time of permanence on open arms (PTOA) were calculated.[22] Mice were divided into 4 groups (n = 6, total 24): group A (control), group B (2 mg/kg DZP), group C (10 mg/kg ASA) and group D (10 mg/kg ASA + 2 mg/ kg DZP). The time interval between the treatments was 30 minutes. The test was carried out 30 minutes after the treatment in all the groups.[23,24]

Effect of ASA on DZP induced muscle relaxant action using the traction test. The forepaws of each mouse were placed on a small twisted wire rigidly supported above a bench top. Normal mice grasped the wire with forepaws and when allowed to hang freely placed at least one hind foot on the wire within 5 seconds. Inability to put up at least one hind foot constituted failure of the traction test.[25] The test was carried out in 4 groups (n = 10, total 40). Group A (control), group B (10 mg/kg ASA), group C (10 mg/kg DZP) and group D (10 mg/kg ASA + 10 mg/kg DZP). The time interval between the treatments was 30 minutes and the test was carried out 30 minutes after the last treatment.[25]

All data were tested for normal distribution by Kolmogorov-Smirnov test. Analysis of Variance (ANOVA) test was performed followed by post hoc LSD test for multiple comparisons. The student 't' test was used when necessary. Data were expressed as mean ± standard error and $p<0.05$ was considered statistically significant difference by the Statistical Package for Social Sciences (SPSS Inc, Version 11, Chicago, IL, USA).

Results. Table 1 shows the effect of administration of different doses of NSAIDs (ASA, diclofenac, and

Table 1 - Effect of different doses of non-steroidal anti-inflammatory drugs on diazepam induced sleep in Albino mice.

Treatment (mg/kg)	Duration of sleep in minutes	Percentage change	*P*-value
Control	230.50 ± 6.21	---	---
ASA @			
50	81.75 ± 12.41	65	0.0001
100	80.88 ± 8.55	65	0.0001
200	60.50 ± 6.88	74	0.000
Diclofenac			
5	69.15 ± 13.12	70	0.000
10	67.25 ± 5.92	71	0.000
20	60.25 ± 11.94	74	0.000
Indomethacin			
5	82.28 ± 10.32	64	0.0001
10	80.25 ± 7.75	65	0.0001
20	72.33 ± 17.92	69	0.000
ASA #			
5	83.43 ± 3.96	64	0.0001
10	96.88 ± 6.92	58	0.0001
20	84.00 ± 11.89	64	0.0001
Celecoxib			---
5	229.29 ± 21.41	1	---
10	207.12 ± 22.01	10	

ASA@ as non-selective COX inhibitor and ASA# as COX-1 selective inhibitor, ASA - acetylsalicylic acid. Data expressed mean ± SEM, *p*-value is comparison with the control (ANOVA followed by LSD test).

Table 2 - Effect of different time intervals of non-steroidal anti-inflammatory drugs on diazepam induced sleep in Albino mice.

Time intervals	Duration of sleep in minutes	Percentage change	*P*-value
Control	230.50 ± 6.21	---	---
ASA @			
0:00 (hrs)	82.00 ± 7.55	64	0.0001
0:30	80.88 ± 8.55	65	0.0001
1:00	77.28 ± 9.99	66	0.0001
2:00	72.42 ± 5.81	67	0.000
24:00	72.29 ± 10.66	67	0.000
48:00	76.75 ± 4.87	67	0.000
72:00	71.00 ± 7.92	69	0.000
96:00	90.00 ± 9.59	61	0.0001
Diclofenac			
00 (min)	71.37 ± 10.22	69	0.000
30	67.25 ± 5.92	71	0.000
60	82.87 ± 8.69	64	0.0001
120	79.00 ± 12.78	66	0.0001
Indomethacin			
00 (min)	73.85 ± 10.91	68	0.000
30	80.25 ± 7.75	65	0.0001
60	76.33 ± 12.44	67	0.000
120	86.83 ± 8.32	62	0.0001
ASA #			
00 (min)	86.14 ± 18.18	63	0.0001
30	96.88 ± 6.91	58	0.0001
60	77.87 ± 7.35	66	0.0001
Celecoxib			
00 (min)	212.29 ± 16.63	8	---
30	229.29 ± 21.41	1	---
60	200.02 ± 25.21	13	---
120	221.56 ± 16.08	14	---

ASA@ as non-selective COX inhibitor and ASA# as COX-1 selective inhibitor, ASA - acetylsalicylic acid. Data expressed mean ± SEM, *p*-value is comparison with the control (ANOVA followed by LSD test).

indomethacin) as non-selective COX inhibitors and COX-1 selective inhibitor (low dose ASA) on sleeping time induced by DZP in mice. A highly significant reduction in the duration of sleep induced by DZP was observed. The percentage of reduction in the sleeping time as compared with the control group ranged from 58-74% (p=0.000 and p=0.0001). However, there was no dose dependent effect observed in the doses studied. The administration of the selective COX-2 inhibitor celecoxib in 5 and 10 mg/kg did not produce any significant changes compared with the control group (**Table 1**).

Table 2 shows the effect of non-selective NSAIDs (100 mg/kg of ASA, 10 mg/kg of diclofenac and 10 mg/kg of indomethacin) and COX-1 selective inhibitor ASA (10 mg/kg) on DZP induced sleep in a time dependent model (30-120 minutes). A highly significant reduction in the duration of sleep induced by DZP was found in comparison with the control group (58-71%, p=0.000 and p=0.0001). However, there was no time dependent effect observed in different time intervals studied. The administration of the selective COX-2 inhibitor celecoxib in a time dependent model did not produce any significant changes in sleeping time. However, the administration of ASA in 10 mg/kg as a selective COX-1 inhibitor was not found to be statistically significantly change the duration of sleeping time of either midazolam or clonazepam in mice (data not shown). In another experiment, using another class of hypnotic drugs, pretreatment of mice with ASA showed no significant changes in the sleeping time induced by pentobarbitone sodium (data not shown). However, using other species, administration of 100 mg/kg of ASA to rats significantly reduced the duration of sleep induced by DZP (40 mg/kg) to 52% (p=0.000) as shown in **Figure 1**.

Table 3 - Effect of acetylsalicylic acid on diazepam induced sedative effect in mice.

Treatments	Ambulatory movements	Non-ambulatory movements	Number of movements
Control	353.16 ± 90.90	326.80 ± 36.33	93.85 ± 9.29
ASA	358.80 ± 81.07	220.80 ± 24.55	80.11 ± 14.74
DZP (5 mg)	294.16 ± 106.97	161.44 ±73.55 (p=0.041)	59.30 ± 12.82 (p=0.04)
ASA+DZP (5 mg)	263.17 ± 119.03	151.50 ± 43.40 (p=0.035)	46.85 ± 11.53 (p=0.03)
DZP (10 mg)	28.88 ± 8.37 (p=0.000)	47.99 ± 12.74 (p=0.000)	12.37 ± 3.54 (p=0.000)
ASA+DZP (10 mg)	26.99 ± 6.27 (p=0.000)	43.16 ± 10.50 (p=0.000)	12.66 ± 3.00 (p=0.000)

ASA - acetylsalicylic acid, DZP - diazepam. Data expressed mean ± SEM, p-value is comparison with the control (ANOVA followed by LSD test).

Table 4 - Effect of acetylsalicylic acid on diazepam induced anxiolytic action in mice by using the elevated plus maze model of anxiety.

Treatments	TPOA	PPOA	NEOA	PEOA
	(minutes)			
Control	6.17 ± 2.37	2.27 ± 0.85	1.00 ± 0.26	7.51 ± 2.41
DZP	30.57 ± 12.38 (p=0.033)*	14.21 ± 6.07 (p=0.031)*	4.57 ± 1.84 (p=0.05)*	22.56 ± 7.77
ASA	3.17 ± 1.76 (p=0.018)†	1.32 ± 0.69 (p=0.021)†	0.67 ± 0.33 (p=0.03)†	4.23 ± 1.92 (p=0.028)†
ASA + DZP	17.14 ± 5.88	7.71 ± 2.74	3.14 ± 1.20	24.29 ± 7.03 (p=0.042‡ and 0.017†)

ASA - acetylsalicylic acid, DZP - diazepam. Data expressed mean ± SEM, *comparison with the control, †comparison with DZP, ‡comparison with ASA (ANOVA followed by LSD test).
TPOA - time permanence on open arms, PPOA - percentage permanence on open arms, NEOA - number of entries into open arms, PEOA - percentage of entry into open arms

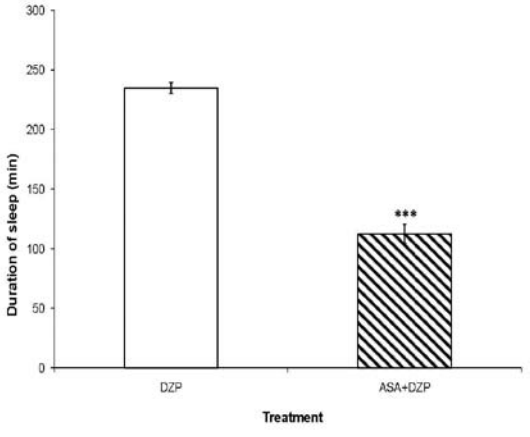

Figure 1 - Effect of acetylsalicylic acid on diazepam induced sleep in rats. ASA - acetylsalicylic acid. DZP - diazepam ***p=0.000 against control by Student t-test.

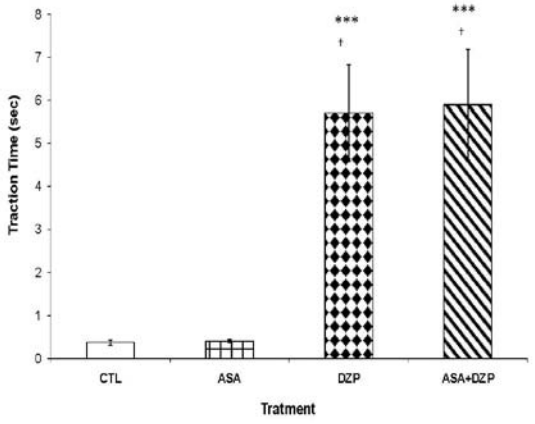

Figure 2 - Effect of acetylsalicylic acid (ASA) on diazepam (DZP) induced muscle relaxant action in mice by using traction test. ***p=0.0002 against control †p=0.0002 against ASA group (ANOVA followed by LSD test).

of mice. Thus, administration of DZP in 2 doses of 5 and 10 mg/kg significantly produced a sedative effect as represented in the ambulatory, non-ambulatory, and number of movements measured by photoelectrical cells. However, pretreatment with ASA did not produce any change in the sedative activity of DZP. In another test, as shown in **Table 4**, DZP alone in 2 mg/kg produced an anxiolytic action in mice as measured by the elevated plus maze model of anxiety represented by TPOA, percentage permanence on open arms [PPOA], NEOA and PEOA in comparison with the control group. However, pretreatment with 10 mg/kg ASA produced no significant changes in the parameters measured for the anxiolytic action of 2 mg/kg DZP. **Figure 2** shows the traction time induced by 10 mg/kg DZP alone or in combination with 10 mg/kg ASA in mice and a significant increase in the traction time by 10 fold-times was observed when using 10 mg/kg of DZP. However, pretreatment with ASA did not change the traction time induced by 10 mg/kg DZP in mice as shown in **Figure 2**.

Discussion. Experimentally, in this animal study, pretreatment of mice with non-selective and COX-1 selective inhibitors of NSAIDs in different doses and times (dose and time dependent models) significantly reduced the duration of sleeping induced by DZP in comparison with the control group. This effect was not found with the selective COX-2 inhibitor of NSAIDs (**Tables 1 & 2**). This finding concurs with a previous study,[14] which stated that administration of ASA, ibuprofen, indomethacin and piroxicam (non-selective NSAIDs) significantly reduced the duration of sleep in mice. In addition, using the valeryl salicylate (COX-1 selective NSAID) of the same procedure significantly reduced the duration of sleep while NS398 (COX-2 selective NSAID) was reported to be unchanged even when the dose was increased up to 20 mg/kg.[14]

The major PGs of the mammalian brain are D2 and E2. The first PG reported to induce sleep was PGD2 in rats and subsequently in humans.[14] Thus, PGD2 induces sleep by inhibiting histaminergic arousal neurons of the tuberomammillary nucleus (TMN) in the posterior hypothalamus by activating inhibitory neurons in the ventrolateral preoptic area,[26] while PGE2 causes wakefulness by activating arousal neurons in the TMN via AMPA type excitatory amino acid receptors.[14,26] The sites of action of PGD2 and PGE2 are located in the sleep and wake centers in or near the preoptic area and posterior hypothalamus.[27] Therefore, PGD2 acts as a sleep-inducer and PGE2 as a wakefulness-inducer and together regulates the generation of sleep and wakefulness in the brain.[14,26] These may be related to the present findings, by which PGE2 is the most likely product of COX-2,[9] inhibition of COX-2 activity decreases the production of PGE2 (wakefulness-inducer) and consequently PGD2 (sleep-inducer) is prominent. This may lead to an increase in the duration of sleep when COX-2 inhibitors are used and vice versa with COX-1 inhibitors. However, the negative effect of ASA on midazolam and clonazepam induced sleep may indicate that this interaction is specific to DZP. To investigate species variation regarding the effect of ASA on the duration of sleep induced by DZP, rats were pretreated with ASA. A significant reduction by around 50% in the duration of sleep was induced in this species (**Figure 1**). This may suggest that there is no species variation and may exclude metabolism and genetic effects. Further, it was reported that NSAIDs reduced ethanol induced sleep in mice, by using other species such as rats, the same effect was reported as in the mice when ASA and indomethacin were pre-administrated 15 and 30 minutes, before ethanol induced sleep.[28]

In this study, the lack of effect of ASA on pentobarbitone sodium induced sleep could be explained by the binding of barbiturates to the sites distinct from the complex of BZ binding sites at $GABA_A$ receptors. It is known that barbiturates are less selective in their action than BZs since they also depress the actions of excitatory neurotransmitters (glutamic acid) and exert nonsynaptic membrane actions in parallel with their effects on GABA neurotransmission.[29] Thus, the present finding is in line with the previous report of pretreatment of mice with NSAIDs and has no effect on pentobarbitone induced sleep.[14]

The main subunit of $GABA_A$ receptors is the α subunit, which occurs in many isoforms (α_1-α_5). It is known that α_1 $GABA_A$ receptors mediate the sedative and anticonvulsant actions of DZP. However, anxiolytic actions are mediated through α_2/α_3 and muscle-relaxant actions are through α_2 $GABA_A$ receptors.[4] Tobler et al[30] showed that DZP induced sleep is mediated by $GABA_A$ receptors other than α_1 (unknown isoform). This may agree with our present findings that the hypnotic effect of DZP occurs through isoforms other than those that induce other behavioral effects of DZP. This could also explain why interaction occurred with hypnotic effect, but not with other actions and supports the previous findings in which pretreatment with ASA has no effect on locomotor activity and muscle relaxant when ambulatory and rota-rod performance were measured.[14] Thus, it is concluded that NSAIDs, most likely, COX-1 inhibitors reduced the duration of sleep induced by DZP by a pharmacodynamic type interaction. Further studies should consider the anti-inflammatory and analgesic effect of ASA with repeated administration of DZP. As our study was carried out in experimental

animals with only a single treatment (acute effect), we recommend further study of repeated treatment (chronic effect) with diazepam, for example drug addiction in diazepam and how it can affect the anti-inflammatory activity of NSAIDs.

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