Scholars LITERATURE

La Prensa Medica Argentina

Research Article

DOI: https://doi.org/10.47275/0032-745X-214
Volume 106 Issue 4

Effect of Diclofenac, Meloxicam and their Interaction with Diazepam in Lithium-Pilocarpine Induced Status Epilepticus in Mice

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Abstract

Background: Status Epilepticus (SE) is a neurological emergency that necessities rapid control to prevent its bad sequelae. Benzodiazepines, like diazepam and lorazepam, are widely used drugs to terminate seizure activity but SE becomes more refractory to these agents. SE causes cyclooxygenase-2 (COX-2) induction and pharmacological targeting of specific pro-inflammatory pathways after SE may show antiepileptogenic effects.

This work was designed to study the effect of two COX inhibitors: diclofenac and meloxicam, as well as their interaction with diazepam on lithium-pilocarpine model of SE in mice.

Methods: Fifty-six mice were randomly divided into 7 equal groups; control, lithium-pilocarpine, diclofenac, meloxicam, diazepam, diclofenac-diazepam and meloxicam-diazepam groups. All groups, except control, were injected with pilocarpine, 24 hrs after lithium chloride, to induce SE. Latency period to first seizures, percentage of convulsed and protected mice as well as death rate were recorded. At the end, TNF-α, IL-1β and PGE2 levels were assessed in brain homogenate.

Results: Diazepam and diclofenac-diazepam, meloxicam-diazepam groups were protected from convulsion and death while diclofenac or meloxicam alone increased the latency to seizures with partial protection from convulsions and death. SE increased levels of TNF-α, IL-1β and PGE2 in brain tissue while, diclofenac, meloxicam and diazepam decreased their levels. The later effect was enhanced by diclofenac-diazepam and meloxicam-diazepam combinations.

Conclusion: Diclofenac and meloxicam have anticonvulsant effect that was enhanced by combination with diazepam and effect could be attributed partially to their anti-inflammatory action.

Keywords: Diclofenac; Meloxicam; Status Epilepticus; Diazepam; COX inhibitors

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Citation: Elgarhi RI, Shehata MM, Abdelsameea AA, et al. (2020) Effect of Diclofenac, Meloxicam and their Interaction with Diazepam in Lithium-Pilocarpine Induced Status Epilepticus in Mice. Prensa Med Argent, Volume 106:4. 214. DOI: https://doi.org/10.47275/0032-745X-214.

Received: February 11, 2020; **Accepted:** February 25, 2020; **Published:** March 02, 2020

Introduction

Epilepsy is a chronic disorder of the brain that affects people worldwide. It is usually defined as a tendency to recurrent seizures [1]. Benzodiazepines bind to GABAA receptors between α and γ subunits. This extracellular binding opens the chloride channel and permits chloride influx, making generation of an action potential more unlikely due to hyperpolarization of neurons [2].

The role of inflammatory molecules in seizures generation was first noted when anti-inflammatory drugs as steroids were shown to control seizures in pediatric refractory epilepsies, like infantile spasm [3]. The inflammatory mediators (i.e., cytokines and prostaglandins) were detected in epilepsy brain specimens not only promoting local inflammation but also function directly on their cognate receptors to modulate neuronal excitability [4].

Non-steroidal anti-inflammatory drugs (NSAIDs) inhibit prostaglandin synthesis by inhibition of cyclooxygenase (COX) [5]. Diclofenac is a unique member of the NSAIDs that inhibits COX and lipoxygenase as well as phospholipase A2 pathways. It also may block voltage-dependent sodium channels, acid-sensing ion channels while opens potassium channels thus decreases neuronal excitability [6]. Meloxicam exhibits anti-inflammatory, analgesic and antipyretic activities via reduction of prostaglandin production through inhibition of COX. In vitro and in vivo studies have confirmed that meloxicam is a preferential COX-2 inhibitor [7].

The present work aimed to study the potential anticonvulsant effect of meloxicam and diclofenac as well as their interactions with diazepam in lithium-pilocarpine model of status epilepticus (SE). The rationales behind our study were based on many previous studies of NSAIDs such as celecoxib, aspirin, roficoxib and diclofenac in different models of epilepsy.

Materials and Methods

Animals

Male Swiss albino mice 8 weeks old, weighing 15-35 g, purchased from the faculty of veterinary medicine, Zagazig University, Egypt and kept in colony cages with free access to food and tap water, under standardized housing conditions (temperature of 22±1℃). After 7 days of adaptation to laboratory conditions, the animals were randomly assigned to seven experimental groups. All experimental protocols were approved by the Ethics Committee of Zagazig University (IRB approval number 3578).

Drugs

Meloxicam: powder (Adwia, Egypt). Diazepam: powder (Roche, Switzerland). Diclofenac sodium: powder (Novartis, Switzerland). Lithium chloride powder (sigma, Egypt). Pilocarpine hydrochloride: powder (sigma, Egypt). Scopolamine: powder (Boehringer Ingelheim, Tokyo).

Lithium-pilocarpine model of SE

Lithium chloride 127.17 mg/kg;i.p, was injected 24 hours before pilocarpine. Scopolamine 1 mg/kg was injected i.p 5 min prior to pilocarpine injection (350 mg/kg;i.p). The later will induce SE. About 15 min after pilocarpine administration, animals exhibited intense salivation, immobility, facial automatisms, and head tremors. After 15-60 min, animals showed increased head tremors with vigorous mastication, forelimb clonus, rearing, and falling with convulsive tonus of the hind limbs. Once initiated, these behaviors occurred every 2-5 min and developed into SE [8]. The Latency period to first seizures, percentage of convulsion and percentage of protection from convulsions as well as death rate were recorded. Lastly, mice were euthanized by decapitation and brains were quickly removed in liquid nitrogen then brain tissue was perfused with PBS (phosphate buffered saline) solution, pH 7.4, Containing

0.16 mg/ml heparin to remove any red blood cells and clots. The tissue was homogenized in 5-10 ml cold buffer (i.e., 50 mM potassium phosphate, pH 7.5. 1 mM EDTA) per gram tissue. The homogenate was centrifuged at 100,000 x g for 15 min at 4℃. The supernatant was removed for assessment of TNF-α, IL-1β and PGE2.

Experimental design

56 mice were randomly assigned into 7 equal groups; Saline control group: mice were injected normal saline (10 ml/kg mice, i.p). Lithium chloride group; Mice were injected with lithium chloride 127.17 mg/kg; i.p [9], then pilocarpine hydrochloride 350 mg/kg; i.p [9], after 24 hrs. to induce SE. Diazepam group: Mice were injected with lithium chloride then after 24 hrs. a protective dose of diazepam (5 mg/kg); i.p was injected as a control treated group [10], diazepam dose was based on previous experiments, followed by pilocarpine hydrochloride 30 min later. Meloxicam group: Mice were injected with lithium chloride then after 24 hrs. meloxicam 10 mg/kg; i.p was injected [11], (this dose was confirmed by dose response curve with different doses of meloxicam (5, 10, 15 mg/kg)), followed by pilocarpine hydrochloride 30 min later. Diclofenac group: Mice were injected with lithium chloride, after 24 hrs. diclofenac 10 mg/kg; i.p was injected [12], (this dose was confirmed by dose response curve with different doses of diclofenac (5, 10, 15 mg/kg)), then after 30 min pilocarpine hydrochloride was injected. Meloxicam-diazepam group: Mice were injected with lithium chloride then after 24 hrs. meloxicam 10 mg/kg; i.p was injected followed by sub-protective dose of diazepam (3 mg/kg); i.p 15 min later and lastly pilocarpine hydrochloride was injected after 30 min. Diclofenac-diazepam group: Mice were injected with lithium chloride, then after 24 hrs. diclofenac 10 mg/kg; i.p was injected followed by sub-protective dose of diazepam (3 mg/kg); i.p after 15 min then after 30 min pilocarpine hydrochloride was injected.

The animals were pretreated with scopolamine (1 mg/kg) 5 min [13] before pilocarpine injection to prevent the peripheral effect of pilocarpine.

Biochemical assays

Estimation of IL-1β: This assay employs the quantitative sandwich enzyme immunoassay technique according to Prostmann T, et al. (1992) [14]. The enzyme reaction yields a blue product that turns yellow when the stop solution is added. The intensity of the color is measured using a microplate reader set to 450 nm. IL-1β ELISA kits were brought from Cusabio technology LLC

Estimation of TNFα: This assay employs the quantitative sandwich enzyme immunoassay technique according to Prostmann T, et al. (1992) [14].The enzyme reaction yields a blue product that turns yellow when the stop solution is added and measured, using a microplate reader set to 450 nm. TNFα ELISA kits were brought from Cusabio technology LLC

Estimation of prostaglandin E2: This experiment use double-sandwich ELISA technique and the ELISA Kit provided is typical according to Quintero-Ronderos P, et al. (2013) [15]. The pre-coated antibody is mouse PGE2 monoclonal antibody and the detecting antibody is polyclonal antibody with biotin labeled. Samples and biotin labeling antibody are added into ELISA plate wells and washed out with PBS. Then Avidin-peroxidase conjugates are added to ELISA wells in order; Use TMB substrate for coloring after reactant thoroughly washed out by PBS.

TMB turns into blue in peroxidase catalytic and finally turns into yellow and was read at 450 nm. Prostaglandin E2 ELISA kits were brought from Cusabio technology LLC

Statistical analysis: The results obtained were statistically analyzed using the SPSS 15.0 software package for Windows (SPSS Inc., Chicago, IL). Statistical analysis of data was performed with one-way ANOVA followed by the post hoc Tukey-Kramer test for multiple comparisons. The number of animals that did not reach SE and those that died during the experiment were calculated as percentages and compared with a nonparametric test (χ^2). In both situations, statistical significance was considered when $p<0.05$.

Results

Effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)-diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on latency period in lithium-pilocarpine induced status epilepticus (SE) in mice in the table (Table 1).

In lithium-pilocarpine control group, the latency period between pilocarpine injection and the occurrence of SE was 10.8±2.57 min. The latter was significantly ($p<0.05$) increased to 21.3±6.88 and 20.16±4.79 min in meloxicam and diclofenac groups respectively, in relation to lithium-pilocarpine control group. In diazepam group and combination groups of diazepam-diclofenac and diazepam-meloxicam none of the mice injected reached status epilepticus state, only 3 mice

in diazepam injected group showed jerks movements after 20 min of injection. There is no statistic difference between these groups.

Protective effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)- diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on lithium-pilocarpine induced status epilepticus (SE) in mice (Table 2).

All mice injected with pilocarpine (control group) reached status epilepticus state with 0% protection. In meloxicam and diclofenac groups, mice protected from SE were 12 and 25% respectively. In the diazepam, diclofenac-diazepam and meloxicam-diazepam combination groups, the protection was 100% as no mice showed SE.

Effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)-diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on case fatality percentage in lithium- pilocarpine induced status epilepticus (SE) in mice (Table 3).

In lithium-pilocarpine control group, 63% of mice died after they reached SE. In the group of mice injected with meloxicam prior to pilocarpine the case fatality rate was 38%. On the other hand, the group injected with diclofenac the case fatality rate was only 13%. There were no case fatalities in diazepam, meloxicam-diazepam and diclofenac-diazepam combination groups.

Effects of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)-diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on TNF-α, IL-1β and PGE2 levels in the brain in lithium-pilocarpine induced SE in mice (Table 4).

In saline control group, level of TNF-α in brain tissue was 4±0.21 pg/g. the latter was significantly ($p<0.05$) increased to 40.06±4.77 pg/g in lithium-pilocarpine group in relation to saline control group. In diazepam group, the level of TNF-α in brain tissue was significantly ($p<0.05$) decreased from 40.06±4.77 pg/g in lithium-pilocarpine group to 22.51±1.09 pg/g. In diclofenac and meloxicam groups, TNF-α levels in brain tissue were significantly ($p<0.05$) decreased from 40.06±4.77 in lithium-pilocarpine group to 17.92±1.12 and 27.16±1.43 pg/kg respectively. The latter was significantly ($p<0.05$) increased in relation

Table 1: Effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)-diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on latency period in lithium- pilocarpine induced status epilepticus (SE) in mice.

Groups	Lithium- Pilocarpine	Diazepam	Diclofenac	Meloxicam	Diazepam+ diclofenac	Diazepam+ meloxicam
Latency period	10.8±2.57^{A}	0.00^{B}	20.16±4.79^{C}	21.3±6.88^{C}	0.00^{B}	0.00^{B}

- Values represent mean of latency period ± SD.
- Values without common superscript capital letters are significantly different ($P<0.05$).
- Number of animals in each group was 8 mice.
- SD = standard deviation.

Table 2: Protective effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), combination of meloxicam (10 mg/kg) with diazepam (3 mg/kg) and combination of diclofenac (10 mg/kg) with diazepam (3 mg/kg)on mice injected with lithium-pilocarpine.

Groups	Lithium- Pilocarpine	Diazepam	Diclofenac	Meloxicam	Diazepam+ Diclofenac	Diazepam+ Meloxicam
Percent protection from SE	0%^{A}	100%^{B}	25%^{C}	12%^{C}	100%^{B}	100%^{B}

- Values without common superscript capital letters are significantly different ($P<0.05$).
- Result for percentage protection was expressed as percentages of the number of animals from each experimental group.

Table 3: Effect of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)- diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on case fatality percentage in lithium-pilocarpine induced status epilepticus (SE) in mice.

Groups	Control (saline)	Lithium- Pilocarpine	Diazepam	Diclofenac	Meloxicam	Diazepam+ Diclofenac	Diazepam+ Meloxicam
Case fatality rate (percentage)	0%^{A}	63%^{B}	0%^{A}	13%^{C}	38%^{BC}	0%^{A}	0%^{A}

- Values without common superscript capital letters are significantly different ($P<0.05$).
- Result for percentage of dead animals was expressed as percentages of the number of animals from each experimental group.

Table 4: Effects of diazepam (5 mg/kg), meloxicam (10 mg/kg), diclofenac (10 mg/kg), meloxicam (10 mg/kg)- diazepam (3 mg/kg) and diclofenac (10 mg/kg)-diazepam (3 mg/kg) combinations on TNF-α, IL-1β and PGE2 levels in the brain in lithium-pilocarpine induced SE in mice.

Groups	Control (saline)	Lithium- Pilocarpine	Diazepam	Diclofenac	Meloxicam	Diclofenac + Diazepam	Meloxicam + Diazepam
TNF-α pg/g	4±0.21	40.06±4.77^{A}	22.51±1.09^{B}	17.92±1.12^{C}	27.16±1.43^{D}	6.15±0.55^{E}	9.11±0.58^{F}
IL-1β pg/g	6.19±0.25	59.22±3.82^{A}	25.23±1.48^{B}	19.11±1.21^{C}	30.04±2.07^{D}	7.09±0.84^{E}	11.54±0.77^{F}
PGE2 pg/g	4.32±0.12	33.13±2.48^{A}	19.01±1.02^{B}	14.44±0.84^{C}	20.09±1.79^{D}	6.36±0.63^{E}	9.27±0.83^{F}

Where: Data represent mean ± SD

^{A}Significant in relation to saline group.

^{B}Significant in relation to saline and lithium-pilocarpine groups.

^{C}Significant in relation to saline, lithium-pilocarpine and diazepam groups.

^{D}Significant in relation to saline, lithium-pilocarpine, diazepam and diclofenac groups.

^{E}Significant in relation to lithium-pilocarpine, diazepam, diclofenac and meloxicam groups.

^{F}Significant in relation to saline, lithium-pilocarpine, diazepam, diclofenac, meloxicam and diclofenac-diazepam groups.

SD: standard deviation.

to diclofenac group. In diclofenac-diazepam combination group, the level of TNF-α in brain tissue was significantly ($p<0.05$) decreased to 6.15±0.55 compared to 40.06±4.77 pg/g in lithium-pilocarpine group. The latter was insignificant in relation to the normal group. In meloxicam-diazepam combination group, TNF-α in brain tissue was significantly ($P<0.05$) decreased from 40.06±4.77 pg/g in lithium-pilocarpine control group to 9.11±0.58 pg/g. The later was significantly ($p<0.05$) increased in relation to diclofenac- diazepam combination group. In the combination groups, TNF-α levels were significantly ($p<0.05$) decreased in relation to diazepam, meloxicam or diclofenac groups.

IL-1β level in the saline group was 6.19±0.25 pg/g brain tissue and significantly ($p<0.05$) increased to 59.22±3.82 pg/g in lithium-pilocarpine group. In diazepam group, IL-1β level was significantly ($p<0.05$) decreased to 25.23±1.48 pg/g in relation to lithium-pilocarpine group. IL-1β levels in both diclofenac and meloxicam groups were significantly ($p<0.05$) decreased in relation to lithium-pilocarpine group to 19.11±1.21 and 30.04±2.07 pg/g respectively. The later was significantly ($p<0.05$) increased in relation to diclofenac group. The IL-1β level was significantly ($p<0.05$) decreased in relation to lithium-pilocarpine group to 7.09±0.84 and 11.54±0.77 pg/g in diclofenac-diazepam combination and meloxicam-diazepam combination groups respectively. The later was significantly ($p<0.05$) increased in relation to diclofenac-diazepam combination group. In the combination groups, IL-1β levels were significantly ($p<0.05$) decreased in relation to diazepam, meloxicam or diclofenac groups.

The PGE2 level in brain tissue of saline control group was 4.32±0.12 pg/g, this level was significantly ($p<0.05$) increased in lithium-pilocarpine group to reach 33.13±2.48 pg/g in relation to normal group. In diazepam, diclofenac and meloxicam groups PGE2 level in brain tissue were significantly ($p<0.05$) decreased to 19.01±1.02, 14.44±0.84 and 20.09±1.79 pg/g respectively in relation to lithium-pilocarpine group. The latter was significantly ($p<0.05$) increased in relation to diclofenac group, but insignificant ($p>0.05$) in relation to diazepam group. In combination groups, the PGE2 levels in brain tissue were significantly ($p<0.05$) decreased from 33.13±2.48 pg/g in lithium- pilocarpine group to 6.36±0.63 pg/g in diclofenac-diazepam and 9.27±0.83 pg/g in meloxicam-diazepam combination groups. The latter was significantly ($p<0.05$) increased in relation to diclofenac-diazepam combination group. In the combination groups, PGE2 levels were significantly ($p<0.05$) decreased in relation to diazepam, meloxicam or diclofenac groups.

Discussion

Pilocarpine model of SE is a preclinical paradigm for temporal lobe epilepsy [16]. Pilocarpine induces SE via activation of the M1 muscarinic receptors, since M1 receptor knockout mice do not develop seizures in response to pilocarpine [17]. In cultured hippocampal neurons, pilocarpine, activates muscarinic receptors inducing imbalance between excitatory and inhibitory transmission resulting in the generation of SE [18]. Indeed, it was documented that following initiation by M1 receptors activation, seizures are maintained by NMDA receptor activation [19].

In the present study, pilocarpine induced sustained recurrent epileptic movements. Pretreatment of mice with diazepam totally protected from SE while, diclofenac or meloxicam induced partially protection. These drugs increased the latency period for occurrence of SE and decreased the case fatality compared to pilocarpine only injected mice. Also, combination of diclofenac or meloxicam with diazepam prevented occurrence of SE. Benzodiazepines enhance the GABA-mediated synaptic inhibition. This effect is more prominent in hippocampus, amygdala and cerebral cortex which are particularly rich in GABA and benzodiazepine receptors. Diazepam potentiates the effects of endogenous GABA in affected brain areas which counteracts the action of pilocarpine.

In parallel with the present results Serrano GE, et al. (2011) concluded that ablation of COX-2 in forebrain neurons can be neuroprotective and diminish brain inflammation 4 days after SE [20]. In addition, Radu BM, et al. (2017) reported that in the pilocarpine model, NSAIDs are neuroprotective and diminish P-glycoprotein upregulation [21]. Multiple COX-2-selective and nonselective inhibitors have been evaluated for antiepileptic and neuroprotective effects in chemo convulsant or electrical models of acute seizures. These results were correlated with the in vivo effects of celecoxib, which blocked seizure-induced up-regulation of P-gp expression in brain capillaries of pilocarpine-induced epileptic rats [22].

Indeed, Jung KH, et al. (2006), explained that in the pilocarpine model, treatment with celecoxib had diminished the frequency and duration of seizures as well as attenuated COX-2 expression in hippocampal neurons and astrocytes. In addition, COX-2 specific inhibitor SC58236 reduced the production of PGE2 [23].

Moreover, mefenamic acid prevented seizures and protected rats from seizure-related brain damage induced by pilocarpine as well as reduced the behavioral correlates of SE and diminished electrical activity in rats induced with pilocarpine [24]. On the contrary, parecoxib was neuroprotective but not antiepileptogenic in the pilocarpine model of temporal lobe epilepsy in rats [25]. This could be related to the difference in drugs and animal species used**.** Parecoxib, decreased the levels of prostaglandin E2 and neuronal damage in the hippocampus and the piriform cortex in lithium-pilocarpine-induced SE model. This treatment also reduces the intensity of spontaneous seizures during the development of epileptogenesis.

Pharmacological targeting of specific pro-inflammatory pathways in status epilepticus shows antiepileptogenic effects. The pro-inflammatory molecules could modulate neuronal network hyperexcitability via both rapid, non-transcriptional effect on glutamate and alteration of BBB permiability [26].

The present results showed that pilocarpine induced SE elevated the levels of IL-1β, TNF-α and prostaglandin E. In parallel with the present findings, Vezzani A, et al. (2011) reported that IL-1R1 activation results in phosphorylation of ion channels increasing neuronal excitability like NMDA receptor that increases neuronal calcium influx and excitotoxicity [4]. Additional mechanisms of hyperexcitability to IL-1β and TNF-α include cytokine-mediated glutamate release from astrocytes and inhibition of glial glutamate reuptake**.** A reduction in GABA-mediated inhibition in inflamed brain tissue can be anticipated by the ability of IL-1β and TNF-α to reduce GABA-mediated chloride currents or GABAA receptor expression at neuronal membranes [27].

Moreover, Saad MA, et al. (2015), revealed that cytokines and other inflammatory mediators contribute to apoptotic neuronal death [28]. Apoptosis is mediated by TNF-α and death receptors leading to recruitment and activation of initiator caspases such as caspases 8 and 10. This leads to the activation of an effector caspase, typically caspase 3. The active caspase 3 is responsible for the cleavage of key intracellular structural and survival proteins and activate the enzyme responsible for the DNA fragmentation [29].

In line with the results of the present study, Trandafir CC, et al. (2015) reported that NS-398 (COX-2 inhibitor), when administered 30 min after the onset of SE decreased neuronal damage in the hippocampus [30]. Administration of diazepam with NS-398 potentiates the neuroprotective effect of the COX-2 inhibitor. These neuroprotective effects occurred with no detectable effect on electrographic SE.

In conclusion: Diclofenac and meloxicam had anticonvulsant effect in pilocarpine model of SE and decreased the associated proinflammatory mediators. These actions were enhanced by combination with diazepam and could be attributed to their anti-inflammatory action. Further clinical and experimental studies are needed to confirm these beneficial actions.

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