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PRENATAL STRESS+MORPHINE AND POSTNATAL RE-EXPOSURE TO STRESS ALTER PENTYLENTETRAZOL-INDUCED EPILEPTIC MANIFESTATIONS IN RATS

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We studied effects of restraint-induced stress and morphine co-administration within the prenatal period and of re-exposure to stress at the end of infancy on the body mass and pentylenetetrazol-induced epileptic manifestations in rats. Pregnant rats were divided into six groups (control, restraint-stressed, saline, morphine, stress+saline, and stress+morphine). In the stressed groups, pregnant rats were subjected to restraint stressing twice per day for three consecutive days (starting on pregnancy day 15). Rats in saline and morphine groups received saline and morphine subcutaneously at the same days. In the morphine/saline+stressed groups, rats were exposed to stress and received morphine/saline simultaneously. Control rats were left intact. The pups were weighed at postnatal days (PD) 1, 15, and 22. On P22, half of the pups were re-exposed to stress; then, pentylenetetrazol (PTZ)-induced seizures were recorded. The offspring body mass was significantly smaller in stressed, morphine, and stressed+morphine groups compared to the control. The time to onset of the first tonico-clonic seizure was shorter, while the duration and number of tonico-clonic attacks were greater significantly in the stressed+morphine group compared to other groups. Re-exposure to stress decreased the number of clonic seizures. The number of leg-opening and tail rigidity episodes were smaller in female offspring compared to male ones. Co-administration of restraint stress and morphine within the prenatal period reduces the offspring body mass and increases the seizure vulnerability more severely compared to the respective individual effects In addition, prenatal stress exerts stronger effects on the neural development and epileptic behaviors of the offspring than postnatal stress.

Keywords: restraint stress, prenatal stress, re-stressing, morphine, seizure, pentylenetetrazol (PTZ), rat.

INTRODUCTION

Prenatal environmental factors exert severe and profound influences on the offspring postnatal development, including preterm birth, fetal growth retardation, delays in the motor development, and behavioral abnormalities [1]. Prenatal stress can affect the susceptibility to seizures in rats during postnatal development, which can persist until adulthood [2]. The appearance of such changes depends on timing of the maternal stress, its intensity and duration, and sex of the offspring [3]. On the other hand, animal studies indicated that administration of morphine during gestation causes morphological and behavioral aberrations in rat offspring, including changes in the seizure susceptibility and long-term alterations in the adult brain and behavior [4–7]. Many abused drugs, including opiates, can cross the placenta and affect the development of the CNS [8]. Complex effects of morphine on seizure manifestations have been reported in terms of the type and experimental conditions. According to previous reports, the effect of morphine on seizure activity in several seizure models appears to be biphasic, potentiating seizures at doses of or above 2 mg/kg and inhibiting seizures at lower doses [9–11]. Morphine excites dopaminergic neurons in the ventral tegmental area of the brain through inhibition of GABAergic inhibitory interneurons [12]. Pentylenetetrazol (PTZ) acts as a $GABA_A$ receptor antagonist [13]. Therefore, both morphine and PTZ function as GABA receptor inhibitors [14].

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Intraperitoneal (i.p.) administrations of PTZ are used to investigate the development of acute and chronic epileptic seizures in animals [15, 16].

Although there are ample studies of the effect of prenatal stress [17–22] and morphine [18, 23–27] on the susceptibility of offspring to seizures, limited attention was paid to concomitant effects of stress and morphine in this context. Meanwhile, there are no literature data indicating the impact of re-exposure of prenatally stressed rats to the same and/or other stresses on the seizure susceptibility. Therefore, our study aimed to investigate effects of co-administration of restraint-induced stress and morphine within the prenatal period and re-exposure to stress at the end of offspring infancy on the body mass and PTZ-induced epileptic manifestation in male and female offspring of rats.

METHODS

Male and female Wistar rats were obtained from the Animal Facility at Urmia University of Medical Sciences, Urmia, Iran. The animals were 8 weeks old on delivery. They were housed in groups of four per cage and kept under standard conditions (12/12 h light/dark cycle, 22 ± 2°C, and food and water *ad libitum*). When rats were 12 weeks old, all females were mated with sexually experienced males of the same genotype. Each female was paired with one male at 8 a.m. and checked for plugs at 3 p.m. The pregnant rats were immediately moved to new cages (three per cage) for the entire gestation period. They were divided into six groups (n = 6 in each), control, stressed, saline, morphine, stressed+saline, and stressed+morphine. The stressed groups were exposed to restraining on gestation days (GD) 15, 16, and 17 [20–22]. The morphine groups were treated with 10, 12, and 15 mg/kg morphine i.p. at GD15, GD16, and GD17, respectively [18]. The doses for prenatal morphine exposure were chosen according to previously reported studies [28–30]. The saline groups received 0.5 ml saline i.p. on the same days. In the morphine/saline-stress groups, rats were exposed to stressing and received morphine/saline simultaneously. The control rats were transported to the experimental room on GD15, GD16, and GD17 and handled similarly to the stressed rats. This gestational age ("late-gestational period") was chosen because of its importance in developing of the opioid system [31], hypophyseal-pituitary-adrenal (HPA) axis, and nervous system [32]. Prenatal stress, particularly during the third week of pregnancy, plays an important role in increasing the seizure vulnerability in rat offspring [18, 21].

Restraint Stress Procedure. For stressed rats, stressing involved transporting from the home cage to the experimental room and placing the pregnant female in a restraint chamber (a transparent plastic cylindrical chamber, 6 cm in diameter and 16 cm in length) under normal room conditions. The animals were restrained for 120 min twice per day (between 08:00-10:00 and 15:00-17:00) for three consecutive days. This protocol has previously been shown to cause alterations in regulation of the HPA axis in the offspring [1, 33].

Pups. After parturition, the pups of each group were weighted, mixed, and equally divided in the dams in the case their birth date was the same. Each dam along with her pups was maintained in the individual cage [21]. On PD15 and PD22, the pups were weighted again. On PD22, half of the pups in each group (two pups per dam, one male and one female) were re-exposed to stressing for one hour, and PTZ (60 mg/kg, i.p.) was injected to all of the pups (n = 24 of both sexes, 12 stressed and 12 non-stressed). After the injection, behavior of each rat was observed and documented for 60 min by direct observation and a digital camera. In re-stressed pups, PTZ was injected 60 min after the stressing procedure.

Behavioral Assessment. The seizure rating was assessed using an early proposed point scale [34]: 0, normal; 1, immobilization, sniffing; 2, head nodding, facial and forelimb clonus; 3, continuous myoclonic jerk, forelimb clonus, tail rigidity; 4, generalized limbic seizures with violent convulsions, and 5, continuous generalized seizures (tonic or tonico-clonic convulsions). In rats, the occurrence of seizures evoked by convulsant agents increases during the second and third postnatal weeks [35–37] and decreases between PD 30 to 35, just prior to puberty [38].

Statistical Analyses. The data related to the offspring body mass were normally distributed, and, thus, analyzed using parametric techniques. For the comparison of the parameters between experimental groups at each age (P1, P15, or P22), one-way analysis of variance (ANOVA) was performed and followed by the Tukey's *post-hoc* test, when indicated. The data related to some epileptic behaviors that were not normally distributed were analyzed using the Mann–Whitney U-test and/or Kruskal–Wallis one-way ANOVA. Also, *post-hoc* analyses were done using the Tukey's test. Data related to some epileptic behaviors (normally distributed) were analyzed using

two-way ANOVA. Results are expressed below as means ± s.e.m. Intergroup differences were considered statistically significant at $P < 0.05$.

RESULTS

Effects of Prenatal Morphine Administration and Stress Exposure on the Postnatal Body Mass. Exposure to both prenatal stress and prenatal morphine led to a low birth mass, and the effect lasted until P15. There was no significant difference between females and males with respect to the body mass; therefore, both data (male and female) were mixed and analyzed together. The results of measurements of the postnatal body mass are shown in Fig. 1 (one-way ANOVA).

Latency of the First Tonico-Clonic Seizure. There were significant differences between the control and saline groups ($P = 0.002$), control and stressed+saline groups ($P = 0.024$), saline and stressed+morphine groups ($P = 0.011$), and stressed+saline and stressed+morphine groups ($P = 0.012$). The results of the data analysis by the Kruskal-Wallis test for the latency of the first tonico-clonic seizure are shown in Fig. 2.

Number of Tonico-Clonic Seizures. There was significant differences between the control and stressed+saline group ($P = 0.040$), saline and stressed+morphine groups ($P = 0.017$), and stressed+saline and stressed+morphine groups ($P = 0.012$). The results of the respective analysis for the number of tonico-clonic seizures are shown in Fig. 3.

Duration of Tonico-Clonic Seizures. Data analyzed by two-way ANOVA on the duration of tonico-clonic seizures in the examined offspring showed that the effects of the group ($P = 0.063$), sex ($P = 0.061$) and re-stress ($P = 0.419$) were not significant (while in the former two cases the respective trends were quite clear). Interaction between the group and sex

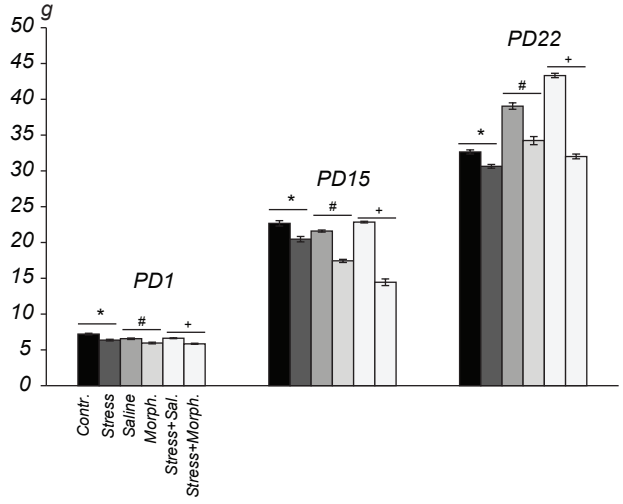


Fig. 1. Mean body mass, g, of the offspring of different experimental groups at postnatal day (PD) 1, 15, and 22. Asterisks, # signs, and crosess show significant differences ($P < 0.001$ or, in PD22, $P = 0.004$) between the groups shown.

Р и с. 1. Середня маса тіла (г) щурят-нащадків різних експериментальних груп, виміряна в постнатальні дні (PD) 1, 15 та 22.

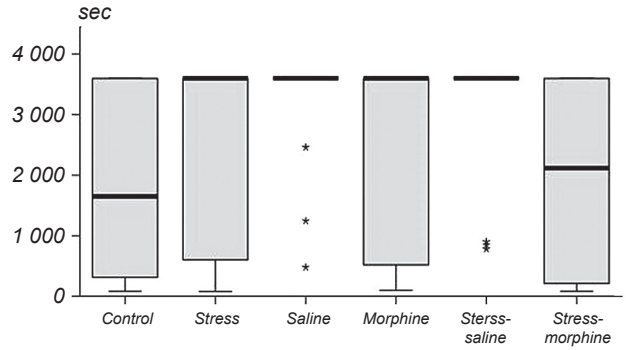
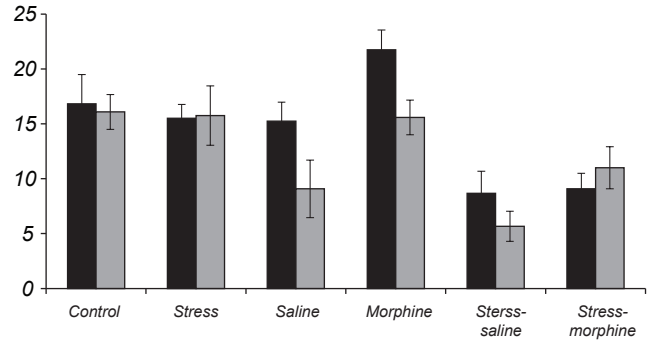


Fig. 2. Latencies of the first tonico-clonic seizure (sec) in the offspring. An SPSS-created graph; asterisks here indicate uneven data distribution. Significant differences were observed between the control and saline groups ($P = 0.002$), control and stressed+saline groups ($P = 0.024$), saline and stressed+morphine groups ($P = 0.011$), and stressed+saline and stressed+morphine group ($P = 0.012$).

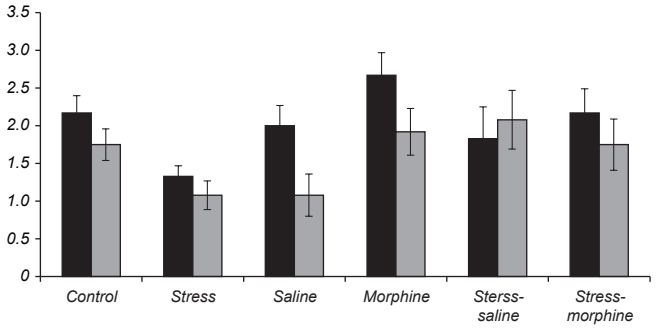
Р и с. 2. Латентні періоди (с) першої тоніко-клонічної судоми у щурят-нащадків.

Fig. 3. Number of tonico-clonic seizures in the offspring observed within a 60-min-long period. An SPSS-created graph; asterisks and circles indicate here uneven data distribution. Significant differences were observed between the control and stressed+saline groups ($P = 0.040$), saline and stressed+morphine groups ($P = 0.017$), and stressed+saline and stressed+morphine groups ($P = 0.012$).

Р и с. 3. Кількість тоніко-клонічних судом у щурят-нащадків, спостережувана протягом 60-хвилинного періоду.



F i g. 4. Mean numbers of the opening-leg episodes observed within a 60-min-long period in the offspring. Light and dark columns) Males and females; asterisk indicates significant difference ($P < 0.001$) between the groups shown.
Р и с. 4. Середня кількість епізодів рухів кінцівками, спостережувана протягом 60-хвилинного періоду.



F i g. 5. Mean numbers of the tail erections in the offspring. Designations are similar to those in Fig. 4. Significant differences were observed between the groups shown ($P < 0.05$ and $P < 0.04$, respectively).
Р и с. 5. Середня кількість епізодів випрямлення хвоста у щурят-нащадків.

($P = 0.078$) and that between sex and re-stress ($P = 0.226$) were also insignificant, but interactions between the group and re-stress and between the group, sex, and re-stress were quite significant ($P = 0.030$ and $P = 0.025$, respectively).

The Number of Clonic Seizures. The results of the data analysis by two-way ANOVA on the number of clonic seizures showed that the effects of the group ($P < 0.001$) and re-stress ($P = 0.020$) were highly significant, while the effect of sex was insignificant ($P = 0.435$). Interaction between the group and sex was also insignificant ($P = 0.254$), whereas interaction between the group and re-stress ($P = 0.024$) and that between sex and re-stress ($P = 0.011$) overcame the significance level. Interaction between the group, sex and re-stress was highly significant ($P < 0.001$).

Number of Opening-Leg Episodes. The so-called opening leg is a behavior event that commonly occurs in PTZ-induced seizures in rats. Data analyzed by two-way ANOVA on the number of opening-leg episodes in the offspring showed that the effects of the group ($P < 0.001$) and sex ($P = 0.011$) were significant. At the same time, effect of re-stress was insignificant ($P = 0.303$). Interactions between the group and sex ($P = 0.045$), group and re-stress ($P < 0.001$), and sex and re-stress ($P < 0.001$) were significant (as can be seen, highly in the two latter cases). Interaction between the group, sex, and re-stress was also highly significant ($P < 0.001$, Fig. 4).

Number of Tail Erections. Data analyzed by two-way ANOVA on the number of tail erection episodes in the offspring showed that the effects of the group ($P = 0.002$) and sex ($P = 0.007$) were significant. At the same time, the effect of re-stress did not reach the significance level ($P = 0.278$). Interaction between the group and sex ($P = 0.319$) was also insignificant. Interactions between the group and re-stress ($P < 0.001$) and between sex and re-stress ($P < 0.001$), were, however, highly significant. Interaction between the group, sex, and re-stress was also significant ($P = 0.023$, Fig. 5).

DISCUSSION

In our study, pregnant rats were subjected to restraint stress for three consecutive days started GD15. Rats of the saline and morphine groups received saline and morphine injections at the same days. In the morphine/saline+stressed groups, rats were subjected to combined influences (exposed to stress and received morphine/saline) simultaneously. As was mentioned above, control rats were left fully intact. The pups obtained from experimental females were weighed at PD1, PD15, and PD22. On P22, half of the pups were re-exposed to stress; then, PTZ-induced epileptic behavioral phenomena were assessed.

Prenatal stress increases the concentration of plasma glucocorticoids and, correspondingly, increases the activity of the HPA axis. This, in turn, results in a long-term increase in adrenal secretion, abnormally increased plasticity of neurons, increased vulnerability with respect to seizures, and body mass loss [39, 40]. It is thought that such changes in the evolutionary

path are based on exposure to glucocorticoids within the prenatal period induced by long-term intense stress during pregnancy [40]. Our findings in general confirm these data and statements.

It was shown that opioid receptors play an important role in the physiological regulation of water and food intake. Morphine effects on food intake are mostly inconsistent because they are related to both stimulating and inhibitory effects from this aspect [41]. It was reported that injections of morphine in cumulative doses did not result in significant body mass changes in infant rats [14]. In another study, it was mentioned that prenatal morphine introduction increased the offspring mass measured on days 6, 15 and 25 after birth, and such increase, compared to the control group, was significant [42]. Our results showed that morphine introduction decreases, in general, the body mass of offspring. Thus, our results were inconsistent with the above reports. In support of our findings, it can be mentioned that drops in the body mass of offspring due to prenatal morphine were demonstrated by another research group [43]. Morphine introductions can reduce the birth body mass of the offspring via inhibiting water and food intake in pregnant rats. In our study, we did not assess the amount of food and water.

As was shown, exposure to stressful situations can lead to profound changes in the electrical properties of neurons; this, in turn, can increase the sensitivity of neurons to the induction of epileptic (epileptiform) activity [44]. It was also demonstrated that stress-related effects on the severity of neonatal seizures are age-dependent. According to one of the reports, prenatal stress potentiates seizures in 15-day-old pups but is ineffective with respect to 25-day-old offspring [42]. Our study was conducted on 22-day-old offspring, and we found that epileptic behaviors did not show significant differences between the stressed and control groups. The explanation for this observation could be related to the effect that the effects of prenatal stress is more severe in younger offspring than in older ones, and the effect of prenatal stress on the seizure susceptibility of rat pups decreases with age [23]. In previous studies, it was reported that exposure to opioids within the prenatal period may affect the development of the CNS and change the number of opioid receptors [45]. In our study, morphine administration decreased the number and duration of PTZ-induced tonico-clonic attacks; this is consistent with the results of previous studies. Morphine exerts a dose-dependent biphasic effect on seizures [46]; this agent potentiates seizures at high doses and shows an antiepileptic effect at lower doses [11]. With respect to the effects of co-administration of stress and morphine within the prenatal period on epileptic behaviors, in a study conducted in 2014, pregnant rats were exposed to forced-swimming stress and morphine on GD 17, 18 and 19, then, epileptic behaviors of the pups studied by PTZ injection were studied on PD15 and PD25. It was found that co-administration of morphine attenuated the effect of stress on epileptic behaviors. In our study, co-administration of restraint-induced stress and morphine within the prenatal period caused an earlier onset of the first tonico-clonic seizure and increased the number and duration of tonico-clonic attacks in the offspring more severely than individual effects of the above factors. The discrepancy between the results of these two studies may be due to differences in the type of stress, time of exposure to the latter, and some difference in timing of PTZ introduction to the offspring. It can be concluded that co-administration of morphine not only does not offset the effects of prenatal stress, but stabilizes the effects of this stress on offspring during lifetime. In fact, morphine intensifies the effects of stress on epileptic behaviors and seizure symptoms.

As was mentioned earlier, one half of prenatally stressed pups were re-exposed to stress at the end of their infancy. We found that prenatal stress exerts stronger effects on the neural development and epileptic behaviors of the offspring than postnatal stress does. Re-exposure to stress at the end of infancy decreased the number of clonic seizures and did not show any other significant effects on PTZ-induced seizures. Currently, it is impossible to explain this peculiarity in the absence of adequate studies in this field.

It has been reported that the effects of opioids are sex-dependent [47, 48]. As was shown, prenatal morphine exposure induces age- and sex-dependent changes in the seizure susceptibility. Probably, the main factor determining the difference between male and female reactions to opioids is the action of sex hormones [49]. Recently, it was reported that prenatal stress increases the density of NMDA receptors in male pups but does not exert such effect in female ones. This can lead to an increased susceptibility to epilepsy in the male offspring. Results of our study demonstrated that there was significant difference between male and female offspring in the number of episodes of opening-leg and tail erection. The female offspring are, in general, more resistant to stress.

These results are consistent with those of other studies [21, 50].

It can be concluded that co-administration of morphine not only does not attenuate the effects of prenatal stress, but it potentiates the effects of this stress on the offspring during lifetime. Prenatal stress exerts more profound effects on the neural development and seizure susceptibility of the offspring than postnatal stress. Also, our data confirmed that the female offspring are more resistant to stress.

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All experimental protocols and procedures of this research were complied according to guidelines of the Helsinki Declaration (1975), as reflected in the guidelines of the Medical Ethics Committee, Ministry of Health, I. R. Iran, and also in the guidelines of the Regional Medical Ethics Committee in the West Azarbyjan province, I. R. Iran.

The authors of this study, Ve. Nakhjiri, E. Saboory, Sh. Roshan-Milani, Y. Rasmi, and H. Sayyadi, confirm that, in the course of performance of the experiments, they had no conflict of interest pertinent to commercial or financial relations and relations with organizations or persons somehow or other related to the study, as well as to relations within the research group.

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КОМБІНАЦІЯ ПРЕНАТАЛЬНОГО СТРЕСУ+ВВЕДЕННЯ МОРФІНУ ТА ПОСТНАТАЛЬНЕ ПОВТОРНЕ СТРЕСУВАННЯ ЗМІНЮЮТЬ ПРОЯВИ ІНДУКОВАНОЇ ПЕНТИЛЕНТЕТРАЗОЛОМ ЕПІЛЕПТИФОРМНОЇ АКТИВНОСТІ У ЩУРІВ

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Р е з ю м е

Ми досліджували на щурах впливи пренатально індукованого іммобілізаційного стресу, який комбінували з уведенням морфіну, а також повторного стресування щурів-нащадків наприкінці «періоду дитинства» на прояви індукованої пентилентетразолом (ПТЗ) епілептиформної активності у цих нащадків. Вагітні самиці щурів були розділені на шість груп (контроль, стресовані іммобілізацією, з уведенням фізіологічного розчину – ФР, з уведенням морфіну, стресовані+ФР та стресовані+морфін). Вагітні самиці стресованої групи були піддані сеансам іммобілізаційного стресування двічі за день протягом трьох послідовних днів (починаючи з 15-го дня вагітності). Щурициі груп ФР та «морфін» отримували відповідні підшкірні ін'єкції протягом тих самих трьох діб. У групах «морфін/ФР+стрес» щури піддавалися комбінації стресування та відповідних ін'єкцій; контрольні щури залишалися інтактними. Народжені щурята зважувались у постнатальні дні ПД, 1, 15 та 22. У ПД 22 половину щурят піддавали повторному стресуванню. Після цього в усіх нащадків реєстрували епілептиформну судомну активність, індуковану введенням ПТЗ. Маса тіла щурят стресованої, «морфінової» груп та групи «стрес+морфін» була вірогідно нижчою, ніж у щурят контрольної групи. Час до проявів першої тоніко-клонічної судоми у тварин групи «стрес+морфін» був вірогідно коротшим, ніж в інших групах. Тривалість та кількість тоніко-клонічних нападів у згаданій вище групі («стрес+морфін») були вірогідно більшими. Повторне постнатальне стресування призводило до зменшення кількості клонічних судом. Отже, комбінація пренатального іммобілізаційного стресу та введення морфіну викликає зменшення маси тіла та підвищення чутливості до судомної активності, і ці ефекти сильніші, ніж впливи вказаних факторів поодинці. Окрім того, пренатальний стрес спричинює сильніші впливи на розвиток нервової системи та прояви епілептиформної активності у нащадків, аніж постнатальний стрес.

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