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Therapeutic Effects of Trolox and Neocuproine on Experimental Mild Traumatic Brain Injury in Rats

Troloks ve Neokuproin'in Sıçanlarda Deneysel Hafif Travma Sonucu Beyin Hasarı Üzerindeki Terapötik Etkisi

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ABSTRACT

Aim: This study aimed to evaluate the therapeutic efficacy of Trolox and neocuproine treatment in an experimental rat model of mild traumatic brain injury (mTBI).
Material and Methods: Forty rats were grouped as Control, TBI, Trolox, and neocuproine. After the Marmarou Weight Drop Model was used to create TBI, 80 mg/kg/day Trolox (i.p.), and 100 μM/day Neocuproine (i.p.) treatments were applied in the 2nd hour. Open field, attention, and short-term memory novel object recognition tests were performed to measure locomotor activity. IL-1β, IL-10, TNF-α, and TGF-β levels in the hippocampus tissues of the rats were analyzed by the ELISA method.
Results: Open field and novel object recognition test results showed that the total path traveled was significantly decreased in the TBI group compared to the control group ($p<0.001$). A significant increase in locomotor functions was observed in the Trolox ($p=0.018$) and neocuproine ($p=0.002$) groups compared to TBI. Short-term memory test results decreased in TBI compared to control ($p<0.001$), while they increased in the Trolox and neocuproine groups compared to TBI (both $p<0.001$). Hippocampus IL-1β ($p=0.012$) and TNF-α ($p=0.011$) levels increased, while IL-10 ($p=0.031$) and TGF-β ($p=0.007$) levels decreased compared to control in the TBI group. While all proinflammatory cytokine levels decreased, antiinflammatory cytokine levels increased in the Trolox and neocuproine groups compared to TBI.
Conclusion: The present findings showed that Trolox and neocuproine treatments in TBI provided significant improvements in short-term memory, and locomotor activity levels by reducing proinflammatory cytokine production, and increasing antiinflammatory cytokine production.
Keywords: Neocuproine; Trolox; traumatic brain injury; locomotor activity; neuroinflammation.

ÖZ

Amaç: Bu çalışmanın amacı hafif travmatik beyin hasarı (hTBH) olan deneysel rat modelinde Troloks ve neokuprin tedavilerinin terapötik etkinliğinin değerlendirilmesidir.
Gereç ve Yöntemler: Kırk rat, kontrol, TBH, Troloks ve neokuprin olarak gruplandırıldı. TBH oluşturmak amacı ile Marmarou Weight Drop Model uygulandıktan sonraki 2. saat diliminde 80 mg/kg/gün Troloks (i.p.) ve 100 μM/gün neokuproin (i.p.) tedavileri yapıldı. Lokomotor aktiviteyi ölçmek için açık alan ve dikkat ve kısa süreli hafıza yeni nesne tanıma testleri gerçekleştirildi. Sıçanların hipokampüs dokularındaki IL-1β, IL-10, TNF-α ve TGF-β düzeyleri ELISA yöntemi ile analiz edildi.
Bulgular: Açık alan ve yeni nesne tanıma test sonuçları, alınan toplam yolun TBH grubunda kontrol grubuna kıyasla önemli ölçüde azaldığını gösterdi ($p<0,001$). Troloks ($p=0,018$) ve neokuproin ($p=0,002$) gruplarında ise TBH'a kıyasla lokomotor fonksiyonlarında anlamlı bir artış gözlendi. Kısa süreli bellek test sonuçları, TBH'da kontrole kıyasla azalırken ($p<0,001$), Troloks ve neokuproin gruplarında ise TBH'a kıyasla arttı (her iki $p<0,001$). Kontrole kıyasla TBH grubunun hipokampüs IL-1β ($p=0,012$) ve TNF-α ($p=0,011$) seviyeleri artarken, IL-10 ($p=0,031$) ve TGF-β ($p=0,007$) seviyeleri azaldı. Troloks ve neokuproin gruplarında TBH'ye kıyasla tüm proinflamatuar sitokin seviyeleri azalırken, antiinflamatuar sitokin seviyeleri arttı.
Sonuç: Elde edilen bulgular, TBH'da Troloks ve neokuproin tedavilerinin proinflamatuar sitokin üretimini azaltarak ve antiinflamatuar sitokin üretimini artırarak kısa süreli bellek ve lokomotor aktivite düzeyinde anlamlı iyileşmeler sağladıklarını göstermiştir.
Anahtar kelimeler: Neokuproin; Troloks; travmatik beyin hasarı; lokomotor aktivite; nöroinflamasyon.

INTRODUCTION

Traumatic brain injury (TBI) is a substantial clinical disorder that affects survival, sequelae, mortality, and morbidity and occurs as a result of many traumatic incidents, or multiple underlying neuropathological causes (1).

Traumatic brain damage can lead to encephalopathy, cognitive deficits, behavioral abnormalities, epileptic seizures, and perhaps a neurodegenerative condition resembling Alzheimer's disease (2).

There are two forms of TBI. The first type results in central nervous system damage to the brain caused by trauma such as fractures or other traumas. Secondary TBI develops sometime after the initial brain injury and may result from pathological causes such as mitochondrial dysfunction, inflammation, neurotransmitter activity, calcium, and gene activity on a cellular basis (3,4).

α-tocopherol is a powerful antioxidant that protects the membrane from oxidative agents, and Trolox is a water-soluble analog of α-tocopherol vitamin E (5). Neocuproine is the chemical form that acts as a copper chelator (6,7). Trolox has been evaluated as a control group in experimental studies due to its antioxidant analog properties of vitamin E. In one study, it was reported that incubation of glial cells with Trolox prevented neuronal cell death through interleukin-1beta (IL-1β) expression (8). In a study on isoprostane found in the rat cerebral cortex, it was shown that treatment with antioxidant Trolox prevented isoprostane release under basal conditions (9).

A few of the studies on neocuproine in the literature are on smooth muscle activity, which is on the effect on relaxation in the rat corpus cavernosum (10). Another rat study using neocuproine showed its protective role against cardiac damage in isolated perfused rat hearts (11). In a cancer study, it was stated that neocuproine could be considered an antitumor candidate (12). Another study using the copper chelate neocuproine discussed its effects on DNA synthesis (13).

TBI continues to be a major public health concern worldwide, with the need for effective research models to better understand its pathophysiology and develop therapeutic interventions. The use of Trolox and neocuproine in TBI studies has not yet been included in the literature. It is questionable whether Trolox and neocuproine can be considered as an alternative treatment option in terms of anti-neuroinflammatory effects in eliminating abnormal locomotor activity and cognitive behaviors. The working hypothesis is that separately administering Trolox and neocuproine to TBI rats will reduce neurodegenerative processes through anti-inflammatory effects. To demonstrate the therapeutic efficacy of Trolox and neocuproine in TBI, cognitive function and locomotor activity were assessed using the open field (OF), and novel object recognition (NOR) tests. Proinflammatory, and antiinflammatory markers such as IL-1β, interleukin-10 (IL-10), tumor necrosis factor-alpha (TNF-α), and tumor growth factor beta (TGF-β) levels were investigated in the cerebral hippocampus preparations.

MATERIAL AND METHODS

Experimental Groups and Protocol

This study used 3-month-old Wistar Albino male rats weighing 200-250 g. The Bolu University Animal Care and Ethics Committee authorized, and oversaw the implementation of all procedures involving the use of animals in experiments (Approval no: 2024/22, Date: 07.08.2024). Forty rats were divided into four groups each with n=10, as Control, TBI, Trolox, and neocuproine. The TBI model was created using the Marmarou method. The control group was administered Trolox, and neocuproine solvent DMSO intraperitoneally (i.p.). The Trolox and neocuproine groups were administered 80 mg/kg/day Trolox (i.p.), and 100 μM/day neocuproine (i.p.) immediately after TBI. The method used to determine the therapeutic effective dose was taken into account in the relevant studies using Trolox and neocuproine, and also a single dose was preferred in this preliminary study to prove whether Trolox and neocuproine, which is the hypothesis of the study, could be a treatment option in TBI (14-17).

Secondary injury plays a role in the pathophysiology of TBI and secondary injuries begin to appear in the first hours of TBI. After the 4th hour, the blood-brain barrier is damaged and neuroinflammation and apoptotic processes begin, leading to loss of motor and cognitive function. Therefore, treatments should usually be initiated at an early stage to prevent secondary damage. Neuroinflammation and apoptotic processes play a role in the pathophysiological damage mechanism at 24, 48, and 72 hours of TBI. In our study, in order to investigate the efficacy of Trolox and neocuproine treatments on neuroinflammation in TBI-induced motor and cognitive dysfunctions, drug treatment was started at the 2nd hour of TBI and terminated at the 48th hour (4,18,19).

The NOR test evaluated learning, and memory activities, whereas the OF test evaluated locomotor activity. The subjects were sacrificed after the behavioral experiments, the brains were removed, and IL-1β, IL-10, TNF-α, and TGF-β levels in the hippocampus tissues were assessed using the ELISA method (Figure 1).

Marmarou Weight Drop Model

In this study, the most preferred among weight drop models, Marmarou's (20) weight drop method was used (Figure 2). Animals were anesthetized with 5 % isoflurane. The skull was placed between lambda, and bregma without surgical operation using a polyacrylamide adhesive, with a stainless steel disk (10 mm diameter, 3 mm depth). A moderate chronic traumatic encephalopathy (CTE) model was created by dropping a 450 g brass weight directly onto the steel disk from a height of 1 meter on the animals placed on the sponge.

Behavioral Experiments

Open Field (OF) Test

The locomotor activity is measured with the OF test. OF experiments will be conducted in a square-shaped, 80x80 cm, black mat-based setup with a wall height of 40 cm. The area was divided into 16 equal small circles of 20 cm^2. At the beginning of the experiment, rats were placed one by one in the center of this area, and their movements were examined for 3 minutes. The number of squares entered by each rat, the total distance covered, and the average speed were measured (21,22).

Novel Object Recognition (NOR) Test

The NOR test is particularly effective in measuring attention, and short-term memory studies, and has three stages: habituation, training, and retention. In the habituation stage, rats were placed in the middle of an

80×80 cm setup, 40 cm high, and allowed to explore the area for five minutes without any items. In the training stage, rats were placed in the area from the center and were given 5 minutes to examine two objects placed in the environment. During the experiments, the apparatus was cleaned with 70% ethanol to prevent the rats from moving according to their sense of smell. In the retention stage, one of the objects was replaced with a new object, and the rats' behaviors were recorded for 5 minutes. During this process, it is expected that the rats will spend more time examining the new object (23). In the NOR test, the discrimination index (%), and the time spent on the new object (sec) values were analyzed.

Discrimination Index = ((time spent on new object - time spent on old object) / total time) * 100.

Biochemical Assessment

Tissue Homogenization Process

Tissues were roughly homogenized with liquid nitrogen in a ceramic mortar. Then, brain tissue was transferred into an Eppendorf tube, its weight was weighed, and 10 times the volume of phosphate buffer was added. After homogenization for 60 seconds with a tissue homogenizer, it was centrifuged at 12000 rpm at + 4 °C for 10 minutes.

Tumor Necrosis Factor-Alpha (TNF-α) and Tumor Growth Factor Beta (TGF-β)

The measurement was carried out in compliance with the guidelines provided by the solid phase sandwich enzyme immunoassay (ELISA) kit, which is sold commercially. Specific monoclonal antibodies against rat TNF-α were placed onto 96-well plates together with standard solutions, and samples were made in serial dilutions at decreasing concentrations. TNF-α, and TGF-β molecules in the samples bound to antibodies in this antigen-antibody binding technique. It was incubated for ninety minutes at 37 °C in an incubator. Following the incubation period, 100 µl of concentrated biotinylated detection Ab was applied to each well after being diluted 1/100 with a biotinylated detection dilution solution. Following an hour of incubation at 37 °C, 750 milliliters of distilled water were added to the concentrated wash buffer, and three washes using the wash buffer solution were carried out to eliminate any unattached molecules. After that, 100 µl of streptavidin-bound peroxidase (horseradish peroxidase, HRP) conjugate was added and incubated at 37 °C for 30 minutes. After incubation, 5 washes were performed and substrate solution was added. The TNF-α and TGF-β concentrations in the samples were directly correlated with a color shift that was seen, and the reaction was stopped by adding 50 µl of stop solution to each well. Each well's absorbance value was calculated by reading at 450 nm using a microplate reader.

Interleukin (IL-1β and IL-10) Measurement

The measurement was carried out in compliance with the guidelines provided by the ELISA kit, which is sold commercially. Specific monoclonal antibodies against rat IL-1β and IL-10 were placed onto 96-well plates together with standard solutions and samples generated in serial dilutions at decreasing concentrations. The IL-1β and IL-10 molecules in the samples were attached to the antibody in this antigen-antibody binding technique. For ninety minutes, it was incubated at 37 °C in an incubator. Following the incubation period, 100 µl of concentrated biotinylated detection Ab was applied to each well after being diluted 1/100 with a biotinylated detection dilution solution. Following an hour of incubation at 37 °C, unattached molecules were eliminated by washing the concentrated wash buffer three times with the wash buffer solution made by adding 750 ml of distilled water to it. Subsequently, 100 µl of the HRP conjugate was added, and 30 minutes were spent incubating at 37 °C. This time, after incubation, a substrate solution was added and five washes were carried out. 50 µl of stop solution was given to each well to halt the reaction, and a color shift was seen that was directly correlated with the amounts of IL-1β and IL-10 in the samples. Using a microplate reader, the absorbance value of each well was measured by reading at 450 nm.

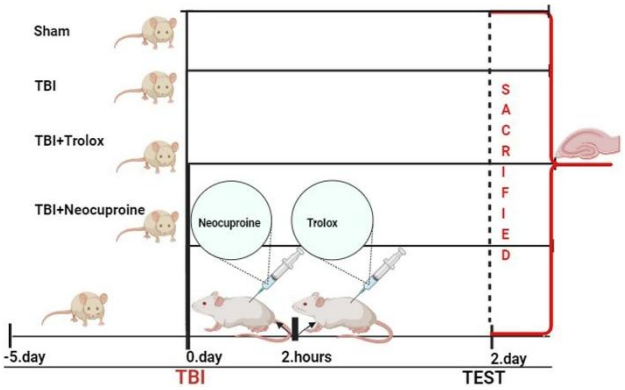

Figure 1. Experimental design (created by biorender.com)

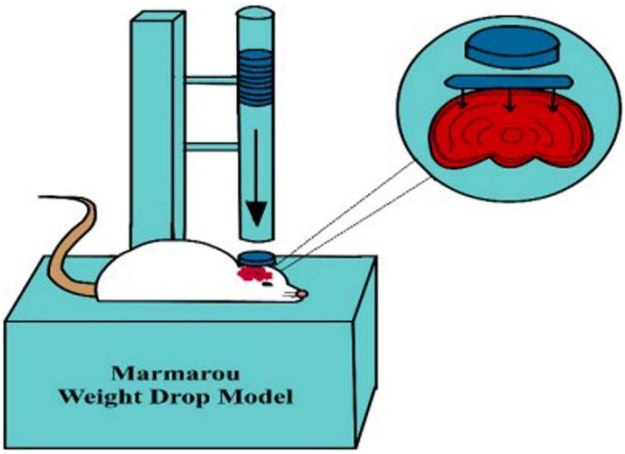

Figure 2. Marmarou weigth drop model (2)

Statistical Analysis

The IBM SPSS v.20.0 software was used to analyze the data. The Shapiro-Wilk test was used for normality assumption, and homogeneity of variances was evaluated with the Levene test. The One-way ANOVA or Welch test followed by post hoc Tukey test for pairwise comparisons between groups was used to analyze data that fit the normal distribution. The descriptive statistics of the data were presented as mean±standard deviation. A $p<0.05$ was deemed to be statistically significant.

RESULTS

The NOR test assessed learning, while the OF assessed locomotor activity (Table 1). The total distance traveled in the OF was significantly reduced in the TBI group compared to the control group ($p<0.001$). When comparing the TBI group to the control group, the total distance traveled in the

Table 1. Comparison of behavioral results and biochemical findings between experimental groups

	Control (n=10)	TBI (n=10)	Trolox (n=10)	Neocuproine (n=10)	p
TD (cm)	2112.00±72.99	1756.00±90.95	1907.00±146.59	1948.00±108.40	**<0.001**
DI (%)	13.11±1.17	8.01±1.14	11.03±1.20	11.43±1.33	**<0.001**
IL-1β (pg/100µg protein)	98.40±5.95	115.10±11.77	107.10±12.27	105.60±14.07	**0.023**
IL-10 (pg/100µg protein)	122.52±19.11	103.40±11.95	109.70±13.27	111.30±13.58	**0.047**
TNF-α (pg/100µg protein)	120.80±11.84	137.60±10.20	126.20±12.55	128.50±10.75	**0.018**
TGF-β (pg/100µg protein)	128.40±15.83	103.70±18.30	111.20±13.16	109.80±15.58	**0.009**

TBI: traumatic brain injury, TD: total distance, DI: discrimination index, IL-1β: interleukin 1 beta, IL-10: interleukin 10, TNF-α: tumor necrosis factor-alpha, TGF-β: tumor growth factor-beta, post hoc test results; TD: control vs TBI: p<0.001, control vs trolox: p=0.001, control vs neocuproine: p=0.009, TBI vs trolox: p=0.018, TBI vs neocuproine: p=0.002, trolox vs neocuproine: p=0.832; DI: control vs TBI: p<0.001, control vs trolox: p=0.003, control vs neocuproine: p=0.019, TBI vs trolox: p<0.001, TBI vs neocuproine: p<0.001, trolox vs neocuproine: p=0.875; IL-1β: control vs TBI: p=0.012, control vs trolox: p=0.337, control vs neocuproine: p=0.502, TBI vs trolox: p=0.411, TBI vs neocuproine: p=0.263, trolox vs neocuproine: p=0.991; IL-10: control vs TBI: p=0.031, control vs trolox: p=0.228, control vs neocuproine: p=0.337, TBI vs trolox: p=0.775, TBI vs neocuproine: p=0.631, trolox vs neocuproine: p=0.995; TNF-α: control vs TBI: p=0.011, control vs trolox: p=0.715, control vs neocuproine: p=0.440, TBI vs trolox: p=0.131, TBI vs neocuproine: p=0.295, trolox vs neocuproine: p=0.969; TGF-β: control vs TBI: p=0.007, control vs trolox: p=0.089, control vs neocuproine: p=0.058, TBI vs trolox: p=0.716, TBI vs neocuproine: p=0.824, trolox vs neocuproine: p=0.997

OF was considerably less (Figure 3A). Following therapy, the Trolox (p=0.018) and neocuproine (p=0.002) groups showed a substantial increase in locomotor activity in comparison to the TBI group. The NOR test was used to assess the groups' short-term memory tests (Figure 3B). The Trolox and neocuproine groups showed a substantial gain in learning compared to TBI group (both p<0.001), whereas the TBI group showed a significant decline in learning compared to the control group (p<0.001).

Effects of Neocuproine and Trolox on Locomotor Activity in TBI Groups

Biochemical Findings in Hippocampus Tissue

IL-1β, IL-10, TNF-α, and TGF-β levels in the hippocampus were shown in Table 1. Compared to the control group, IL-1β (p=0.012) and TNF-α (p=0.011) levels of the TBI group increased significantly, while the IL-10 (p=0.031) and TGF-β (p=0.007) levels decreased significantly. Both of the treatments reduced all proinflammatory cytokine levels in the Trolox and neocuproine groups while increasing anti-inflammatory cytokine levels (Figure 4).

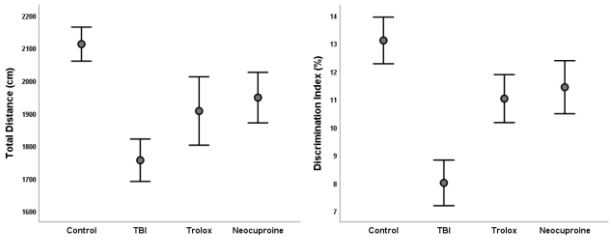

Figure 3. Behavioral results, **A)** total distance traveled (cm), and **B)** discrimination index of experimental groups

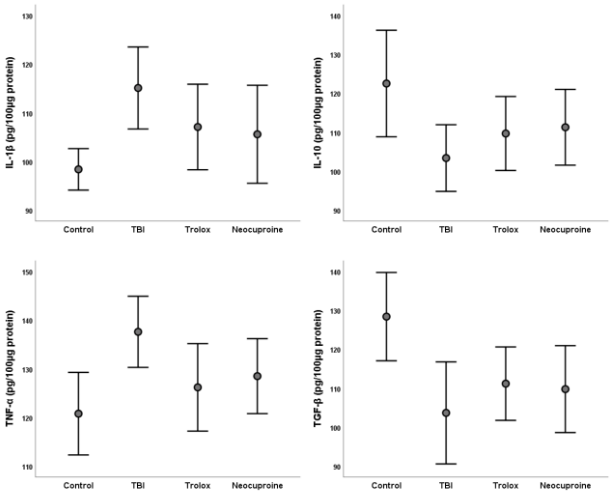

Figure 4. Results of neuroinflammation, **A)** IL-1β, **B)** IL-10, **C)** TNF-α, and **D)** TGF-β levels in the hippocampus

DISCUSSION

The problems observed in TBI cases in the clinical picture have not yet been completely resolved and treatment searches are ongoing (24). In primary brain damage and secondary brain damage caused by trauma, it is thought that secondary damage plays the leading role (18). Other mechanisms observed in the development of secondary damage and neuroinflammation cause motor function losses (25).

In the current study, it was determined that there was motor dysfunction in the experimental groups that developed TBI. It has not yet been shown that Trolox or neocuproine can be a therapeutic factor for motor function loss. Again, neither Trolox nor neocuproine has been included in a study on TBI in vivo study with its anti-inflammatory aspects. As a result of the findings presented in this study, it was observed that Trolox and neocuproine applications can have a therapeutic effect on both motor function loss of TBI and by affecting neuroinflammation levels. Motor function defects that developed with TBI in the experimental groups were recovered with Trolox and neocuproine treatment.

The Marmarou weight-drop model is a popular animal model used in TBI. The Marmarou model induces closed-head TBI without allowing for other complications caused by open-head injuries (26). In the experimental groups where the TBI model was performed, decreased locomotor activity was observed, and these results are consistent with the literature (27).

In addition, it is possible to say that while Trolox and neocuproine applied for treatment purposes for TBI regressed the loss of locomotor activity, similar gains were made for motor functions. A decrease in locomotor activity is a concurrent process with motor dysfunction (18). The therapeutic effectiveness of Trolox and neocuproine also draws attention to the similarity of the results to the control group. Another therapeutic effect that draws attention to the findings of this study is that according to the NOR test results of the short-term memory experiments of Trolox and neocuproine in rats, a significant decrease in learning

was observed in the TBI group compared to the control group, while a significant increase was observed in the Trolox and neocuproine groups compared to the TBI group.

In the clinic, it is mentioned that there may be both cognitive and physical function patterns in TBI patients (28). There is an underlined statement between the primary injury, and secondary injury processes that in patients with TBI, the secondary injury process, that is, the process in which TBI is observed at a more micro index, is associated with a poor prognosis (29). It is predicted that neuroinflammatory steps are involved in the progression of the process in the post-primary brain injury phase of clinical findings of TBI patients. In light of this information, the study aimed to determine the proinflammatory and anti-inflammatory cytokine levels in the hippocampus. The findings of the TBI in vivo rat study also suggest that diphenhydramine HCI application has a significant anti-inflammatory effect on TNF-α serum levels in the treatment groups, and reversals in brain damage findings (30).

Genes that are active in TBI may affect the severity of the injury, including pro, and anti-inflammatory cytokines, and neurotrophic genes are thought to affect repair and plasticity (31).

Considering the healing effects of Trolox and neocuproine, they can be used as preventive agents in cases where TBI may develop or occur again, with their antioxidant character as well as their therapeutic activity on TBI.

The limitations of the study are as follows: First, the dose intervals have not been tested in single applications to find the most effective dose for Trolox and neocuproine application in TBI. However, the current study is a preliminary study to evaluate whether Trolox and neocuproine have a place in TBI studies.

CONCLUSION

Trolox and neocuproine treatment resulted in significant improvements in short-term memory and locomotor activity levels in TBI. It is thought that this therapeutic effect is achieved by decreasing the production of proinflammatory cytokines and increasing the production of anti-inflammatory cytokines.

Ethics Committee Approval: The study was approved by the local ethics committee on animal experiments at Bolu Abant İzzet Baysal University (07.08.2024, 22)

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