The effects of dexmedetomidine on trauma-induced secondary injury in rat brain

Ahmet Sen*, Basar Erdivanlı†, Levent Tümkaya‡, Hüseyin Avni Uydu§, Tolga Mercantepe*, Şule Batıcık and Abdullah Ozdemir

*Department of Anesthesiology and Reanimation, Trabzon Faculty of Medicine, University of Health Sciences, Trabzon, Turkey;
†Department of Anestesiology and Reanimation, Faculty of Medicine, Recep Tayyip Erdogan University; ‡Histology and Embryology, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey; §Histology and Embryology and Biochemistry, Faculty of Medicine, Recep Tayyip Erdogan University, Rize, Turkey

ABSTRACT

Background: The objective of this study was to investigate the effect of dexmedetomidine (Dex), a sedative drug with little or no depressant effect on respiratory centers, on secondary injury in rat brain tissue by means of the Na+/K+ ATPase enzyme, which maintains the cell membrane ion gradient; malondialdehyde, an indicator of membrane lipid peroxidation; glutathione, an indicator of antioxidant capacity; and histopathological analyses.

Methods: Eighteen rats were randomized into three groups: the trauma group received anesthesia, followed by head trauma with a Mild Traumatic Brain Injury Apparatus; the Trauma+Dex group received an additional treatment of 100 µg/kg intraperitoneal dexmedetomidine daily for three days; the Control group received anesthesia only.

Results: The highest MDA levels compared to the Control group were found in the Trauma group. Mean levels in the Trauma+Dex group were lower, albeit still significantly high compared to the Control group. Glutathione levels were similar in all groups. Na/K-ATPase levels were significantly lower in the Trauma group compared to both the Control group and the Trauma+Dex group. Histopathologic findings of tissue degeneration including edema, vascular congestion and neuronal injury, and cleaved caspase-3 levels were lower in the Trauma+Dex group compared with the Trauma group.

Conclusions: Dexmedetomidine administered during the early stage of traumatic brain injury may inhibit caspase-3 cleavage. However, the mechanism does not seem to be related to the improvement of MDA or GSH levels.

1. Introduction

As traumatic brain injury (TBI) has the highest morbidity and mortality rates among all traumatic injuries in the world, it is generally referred to as a silent epidemic. In the U.S.A., 1.4 million cases of TBI and 50,000 deaths are recorded per annum [1–3]. Although one has no control over the initial injury, the secondary injury is an important and controllable determinant of the outcome [4].

The primary injury is due to an external mechanical force causing extensive neurodegeneration [5–7]. This initial injury may be limited, or widespread to include the neurons and vascular endothelium, leading to edema and hemorrhage [8]. In any case, the ensuing inflammatory response triggered by injury to the endothelial cells or neuronal ischemia may progress to secondary injury [9]. Increased intracranial pressure due to cellular damage or patient-related factors like coughing, agitation or hypertension further aggravates ischemia. This vicious cycle may ultimately lead to death [10]. Therefore, it is vital to follow these patients in the ICU to promptly recognize the signs of secondary injury and provide the appropriate treatment [10].

Sedation and analgesia are commonly used to prevent coughing or agitated movements, which may increase intracranial pressure [11,12]. On the other hand, the current drug therapy involving the use of barbiturates, benzodiazepines, opioids or propofol may cause respiratory depression or loss of protective airway reflexes and necessitate endotracheal intubation. Dexmedetomidine is a potent agonist of α2 adrenoceptors [13]. Presynaptic activation of α2 receptors inhibits norepinephrine release and pain, while postsynaptic activation in the central nervous system decreases sympathetic activity, heart rate and blood pressure [14].

Secondary traumatic brain injury (TBI) results in the activation of inflammation, excitotoxicity, increased vascular permeability, and oxidative damage that causes irregularities in ion concentrations and mitochondrial functions [15]. Under normal
circumstances, antioxidants establish homeostasis by cleaving reactive oxygen species. However, the excessive production of ROS after trauma renders antioxidants insufficient. Glutathione, which is one of the most important markers of the antioxidant system, depletes in conditions of excessive ROS production [16]. Following excessive ROS production, components of the cell and the membrane become targets for free radicals [17]. One of these targets is the Na+/K + ATPase enzyme, which is a membrane protein that maintains the integrity of ion transfer in the cell membrane and the cell membrane potential [18]. The alpha and beta subunits of this enzyme are targets for oxygen radicals due to their thiol content [19]. The inhibition of the activity of this enzyme as a result of TBI leads to membrane phosphorylation and lipid peroxidation [20]. An important marker of membrane lipid peroxidation is increased malondialdehyde [10].

In the recent years, several studies have shown that dexmedetomidine decreases oxidative stress and reduces lipid peroxidation and apoptosis by protecting the cell membrane and the receptors on the membrane from oxidative stress [21]. Studies have demonstrated that, in brain injury, apoptosis is elevated in the injured region and that dexmedetomidine reduces the apoptosis of neuronal cells by inhibiting the expression of proteins associated with apoptosis [21–23]. Caspase-3 mediates cell apoptosis and the level of caspase-3 is a good marker of the level of apoptosis [24]. Dexmedetomidine was shown to decrease apoptosis by inhibiting caspase-3 levels [21].

Traumatic brain injury is a highly variable clinical condition. Accordingly, several animal models have been used to investigate the pathophysiology of the secondary injury. We hypothesized that dexmedetomidine would alleviate the destruction of the lipid membrane due to inflammatory peroxidation, as well as the apoptosis of neuronal cells. We designed an experimental study to investigate the effects of dexmedetomidine on the abovementioned pathophysiological mechanisms in trauma-induced secondary injury of brain tissue in rats.

2. Materials and methods

Three-to-five-month-old female Sprague Dawley rats weighing 250–350 g were procured from the Animal Care and Research Unit of our university. All animals received humane care according to the criteria outlined in the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by the National Institutes of Health. This study was approved by University Animal Ethics Committee (ID: 2017/1).

2.1 Study design

Animals were cared for in University Animal Care and Research Unit. All animals were maintained and fed under aseptic conditions. Humidity and room temperature were kept at 55–60% and 22 ± 2°C, respectively. A 12 h light/12 h dark cycle was provided. Rats were allowed access to commercially available standard rat chow and tap water ad libitum throughout the experiment.

After sufficient time to adapt to the laboratory conditions, 18 experimental animals were randomized into three groups. The trauma group (n = 6) received head trauma following anesthesia. Trauma+Dex group (n = 6) received anesthesia and head trauma on the same day, and 100 µg/kg intraperitoneal dexmedetomidine daily for three days, starting from the next day. The dose was determined according to the literature [25]. The control group (n = 6) received anesthesia only.

Anesthesia was provided with 50 mg/kg intraperitoneal ketamine hydrochloride (Ketalar®, Parke-Davis,) and 10 mg/kg intraperitoneal xylazine HCl (Alfazyne®, Alfasan International BV Woerden, Holland).

Head trauma was performed with the Mild Traumatic Brain Injury Apparatus developed by Richelle Mychasiuk et al., by dropping a 150 mg weight onto the skulls of sedated rats through a 100-cm-long tube in free-fall motion [26]. Each rat was prepositioned on a 38 x 27 x 27 cm³ box-shaped platform which housed a 38 x 25 x 15 cm³ sponge. There was a 10 cm gap between the 15 cm tall sponge and the upper edge of the box. An aluminum foil was placed on the upper surface of the box where the rats were laid during trauma. The distal end of the tube and the head of the rat were positioned at a distance of 4 cm.

On the fourth day, all rats were sacrificed by decapitation; the brain was immediately removed and dissected into two parts for biochemical and histopathological analyses [27]. Biochemical, histopathological, and immunohistochemical analysis procedures are detailed below.

2.2 Tissue homogenization

The homogenization solution was prepared by mixing 20 mM sodium phosphate and 140 mM potassium chloride with the pH adjusted to 7.4 [28]. One mL of this solution was mixed with 100 mg of tissue and the mixture was centrifuged at 800 G for 10 mins at 4°C. The supernatant was used to measure the concentration of malondialdehyde produced due to the degradation of unstable lipid peroxides via Thiobarbituric Acid Reactive Substances (TBARS), as well as the intracellular glutathione concentration.
2.3 TBARS measurement

The TBARS level was measured according to the protocol of Ohkawa [29]. The mixture of 200 µL of tissue supernatant, 50 µL of 8.1% sodium dodecyl sulfate, 375 µL of 20% acetic acid at a pH of 3.5, and 375 µL of 0.8% thiobarbituric acid was vortexed. After incubation in boiling water bath for 1 h, it was cooled in ice water for 5 min and centrifuged at 750 G for 10 min. The resulting pink color was read on a spectrophotometer at 532 nm. The results were calculated as nmol/mg prt.

2.4 Total sulfhydryl content measurement

The sulfhydryl groups were measured with Ellman’s reagent. 1000 µL of 3 M Na2HPO4 and 250 µL of DTNB (4 mg DTNB prepared in 10 mL of 1% sodium citrate solution) were added to 250 µL of supernatant and vortexed. The absorbance was measured at 412 nm. The results were determined from the standard curve of reduced glutathione (1000 µM-62.5 µM) and given as nmol/ mg prt.

2.5 Na/K ATPase measurement

Na/K ATPase activity was measured by the method of Yoshimura [30]. 100 mg of brain tissue was homogenized after adding 1 mL of 10 mM Tris-HCl at a pH of 7.4. Homogenates were centrifuged at 4000 rpm for 10 minutes at 4°C. One tube was prepared by adding 250 µL of KCl (0.8 M), 250 µL of NaCl (4 M), 250 µgL of MgCl (0.4) and 200 µL of Tris-HCl (1 M) to 50 µL of the supernatant and incubated for 10 minutes. Another tube was prepared by adding 250 µL of ouabain (40 mM), 250 µL of NaCl (4 M), 250 µgL of MgCl (0.4) and 200 µL of Tris-HCl (1 M) to 50 µL of the supernatant and incubated at 37°C for 10 minutes. After 10 min, 60 µL of ATP (25 mM) was added to each tube and left for 1 h at 37°C. The reaction was stopped by adding 500 TCL of 10% TCA to each tube. Both tubes were centrifuged at 3000 G for 10 min at 4°C. Inorganic phosphate was measured by the Fiske and Subbarow method and the results were given as nmol/Pi/min/mg prt. A commercially available bicinchoninic acid kit was used for protein measurement.

2.6 Histopathological analysis procedure

The brain tissue samples were fixed in 10% neutral formaldehyde. After the fixation, specimens were dehydrated in an ascending series of alcohol, cleared in xylene and embedded in paraffin by routine laboratory methods. Following Hematoxylin&Eosin staining, evaluation with a light microscope (Leica DM6200, Germany; with Olympus DP20 camera attached) was performed by two histologists blinded to the study groups.

2.7 Immunohistochemical analysis

The following steps were performed for cleaved caspase-3 staining: the sections were deparaffinized and treated with 20 µg/mL proteinase-K solution in Phosphate Buffered Saline (PBS), rinsed in distilled water, immersed in 3% hydrogen peroxide. After several washes with PBS (pH 6.0), the sections were immersed in an equilibration buffer. The sections were incubated with anti-Cleaved Caspase-3 (1:200, ab2302, Rabbit polyclonal to active Caspase-3, Abcam, UK). After several washes, all sections were incubated with anti-digoxigenin-peroxidase. Cleaved caspase activity was revealed with 0.06% 3,3-diaminobenzidine tetrahydrochloride in PBS, and the sections were counterstained with Harris hematoxylin.

2.8 Semi-quantitative analysis

Brain tissue sections were stained with Hematoxylin&Eosin. For each animal, three slides were randomly selected from eight slides containing their brain tissues. On each slide, the presence of ischemic neurons, edema and vasoconstriction were evaluated. All sections (3–4 µm thick) prepared from brain tissue were stained with Caspase-3. The evaluation was performed with a light microscope (Leica DM6200, Germany, x40 magnification). One preparation per animal was randomly selected, and eight random areas were evaluated. On each slide, the presence of Cleaved caspase-3-positive cells was examined. Two histologists blinded to the study groups evaluated and graded the slides in four categories: neuronal ischemia, brain edema, vascular congestion, and positive cleaved caspase-3 staining. The findings were graded as none (<5%), mild (6–25%), moderate (26–50%) or severe (>50%).

2.9 Statistical analysis

Statistical analysis was performed with SPSS ver. 12 (SPSS Statistical Program, IBM Corporation, U.S.A.). The normality of data was tested with the Kolmogorov-Smirnov test and QQ-plots. TBARS and total sulfhydryl values were presented as mean±standard deviation. Groups were compared with a one-way analysis of variance, followed by the Tamhane test. Na/K-ATPase activity, histological injury scores and caspase-3 positivity scores were presented as median (25%-75% interquartile range). Groups were compared with Kruskal-Wallis Test. In case of a significant
difference, two-group comparisons were performed with one-way analysis of variance, followed by Tamhane’s test. A p-value <0.05 was considered statistically significant.

3. Results

3.1 Biochemical analysis

Results of biochemical tests are given in Figure 1. Briefly, TBARS levels were higher in the Trauma and Trauma+Dex groups compared with the Control group (p = 0.002 for both comparisons). Glutathione levels were similar among groups (p = 0.99). Na/K-ATPase levels were significantly lower in the Trauma group (p = 0.002 vs Control group, 0.026 vs Trauma+Dex group). The values of the Trauma+Dex group ranged between values similar to both the Control and the Trauma groups (p = 0.065 and 0.002, respectively).

3.2 Histopathological analysis

We observed that the brain tissue was normal in the sections of the control group (Figure 2a). In contrast, brain tissue sections of the Trauma group had atypical neurons, edematous areas, and vascular congestion (Figure 2b). We observed that typical neurons were common in the Dex treatment group (Figure 3c).

3.3 Immunohistochemical analysis

The progression of neuronal apoptosis as marked by cleaved Caspase-3 staining is shown in Figure 3. Results are given in Table 1. Briefly, the Trauma group had the highest number of apoptotic neurons, glial cells and oligodendrocytes. The Trauma+Dex group was similar to the Control group except for the number of apoptotic glial cells.

3.4 Semi-quantitative analysis

Histological injury scores are given in Table 2. Representative light microscopic images for each group are shown in Figure 2. We observed that the histopathological injury score (HPDS: 6.5(5.5–7)) of the Trauma group was higher compared to the control group (HPDS: 1(1–2). On the other hand, we found that the histopathological injury score (HPDS: 3(2–3.5) of the Dex treatment group was lower compared to the Trauma group (HPDS: 6.5(5.5–7))

4. Discussion

This study showed that dexmedetomidine administered in the early post-traumatic period may alleviate some aspects of secondary brain injury in a rat model. In addition, this study found that MDA levels, as measured by TBARS, increased in both groups of rats receiving

Figure 1. Results of biochemical tests. Each group contained six rats.
trauma. Lipid peroxidation is a consequence of the disruption of the cell membrane. MDA levels are higher in trauma patients [31,32]. An experimental study demonstrated that lipid peroxidation and MDA increased approximately one hour after spinal cortex injury [33]. However, the increase in MDA levels in rats receiving dexmedetomidine was limited compared to the Trauma group. This may be explained by the inhibitory effect of dexmedetomidine on the inflammatory cell response or oxidative stress [34].

The neuronal membranes in the central nervous system are rich in unsaturated fatty acids and the antioxidant system is weak [35]. Therefore, it is highly susceptible to oxidant activity. Antioxidants are thought to accelerate neurological recovery after trauma by limiting or preventing secondary injury [36]. On the other hand, Ignowski et al. observed no difference in brain tissue GSH levels 72 hours after trauma [37]. Similarly, in this study, we did not observe any difference in GSH levels between the groups. This suggests that dexmedetomidine is not involved in the thiol metabolism. However, there are other pathways involved in the inhibition of the oxidative metabolism and these were not investigated in this study, which constitutes a major limitation [38]. As discussed above, this study was not designed to explore the comprehensive state of the oxidative metabolism due to ethical and financial reasons.
Table 1. Histological injury scores. Data are given as median (25%-75% interquartile range) and analyzed with the Kruskal-Wallis test.

|                | Control   | Trauma     | Trauma+Dex |
|----------------|-----------|------------|------------|
| Atypical neurons | 1(1–1)    | 2(2–3)a    | 1.5(1–2)   |
| Edema          | 0(0–0)    | 2(2–2.5)b  | 1(0.5–1)c  |
| Vascular congestion | 0(0–0.5)| 2(2–2)b   | 1(0–1)d    |
| Histopathological Damage Score (HPDS) | 1(1–2) | 6.5(5.5–7)b | 3(2–3.5)e,f |

*p = 0.007 versus Control group,
*p = 0.000 versus Control group,
*p = 0.028 versus Trauma group,
*p = 0.005 versus Trauma group,
*p = 0.037 versus Control group,
*p = 0.005 versus Trauma group,
Kruskal Wallis/Tamhane T2 test

Table 2. Caspase-3 positivity scores. Data are given as median (25%-75% interquartile range) and analyzed with the Kruskal-Wallis test.

|                | Control   | Trauma     | Trauma+Dex |
|----------------|-----------|------------|------------|
| Neuronal Caspase-3 positivity | 0(0–0) | 1(1–1.5)a | 0(0–0.5)b  |
| Oligodendrocytic Caspase-3 positivity | 0(0–0.5)| 3(3–3)c   | 1.5(1–2)d  |

*p = 0.008 versus Control group,
*p = 0.024 versus Trauma group,
*p = 0.000 versus Control group,
*p = 0.000 versus Trauma group,

Na/K-ATPase activity was significantly reduced in the Trauma group, possibly because of the effects of the primary and secondary injuries. Na/K-ATPase activity partially improved in rats that received dexmedetomidine, which suggests that secondary injury was limited in this group. Oxidative damage is a significant component of the secondary injury [39]. Lower levels of MDA in rats receiving dexmedetomidine may suggest that the wide range of Na/K-ATPase activity in the Trauma+Dex group is a result of dexmedetomidine’s antioxidant effect. Since animal studies investigating intraperitoneal TQ reported consistent effects of the drug, a difference in distribution or metabolism is unlikely. The most likely explanation would be the low number of rats.

The effects of two molecules with supposed antioxidant properties, propofol and dexmedetomidine, were compared in rats with spinal cord injury-induced by Allen’s weight-drop method [40]. They found the lowest MDA levels in the dexmedetomidine group.

Aslan et al. showed similar results in a similar setting with rabbits [41]. They showed lower glutathione levels in the dexmedetomidine group. This is in agreement with the findings of the present study.

The components of the histological injury score obtained in this study suggest that the degree of ischemia is similar in the Trauma and Trauma+Dex groups. However, cleaved caspase-3 positivity scores suggest that cellular injury is less severe in the Trauma+Dex group compared to the Trauma group. It is notable that although there is significant neuronal damage in the Trauma+Dex group, neuronal cleaved caspase-3 positivity is similar to the Control group.

Three recent studies may shed light on possible pathways in the rat brain. Shen et al. showed that the activation of the PI3K/Akt/mTOR signaling pathway by dexmedetomidine may inhibit the apoptosis [42]. Zhang et al. showed that 15 µg/kg intravenous dexamethasone increased the expression of both Bcl-2 and heat-shock protein 70 [43]. Finally, Li et al. reported that 25 µg/kg intraperitoneal dexamethasone upregulated the nuclear factor erythroid 2-related factor, which may promote these changes in Bax- and Bcl-protein families [44]. These and similar studies performed in rats suggest that dexamethasone is capable of diverting the neuronal cells to an anabolic path rather than apoptosis. Our study showed similar findings; albeit with a comparatively high dose of 100 µg/kg. Additionally, it suggests a possible role of dexamethasone as an inhibitor of the inflammatory pathway.

Secondary injury due to post-traumatic neurodegenerative changes is mediated by many metabolites such as monoamines, free oxygen radicals, neuropeptides and extracellular calcium [45]. An anti-inflammatory role for dexamethasone was previously suggested by Can et al [46]. They showed that the administration of dexamethasone to rats with spinal cord injury reduced the production of inflammatory cytokines. Cosar et al. [47] administered dexamethasone to rabbits with subarachnoid hemorrhage and found that dexamethasone was neuroprotective based on the histopathological and biochemical findings in hippocampal tissues.
Kwiecien et al. reported that spinal cord injury initiates hemorrhage and ischemia, free radical release, severe inflammation, and cellular necrosis [48]. The present study found a typical histological appearance and no pathology in the Trauma+Dex group, in contrast with the prominently ischemic neurons, perineural vacuolization and severe brain edema in the Trauma group. This study did not determine inflammation in the Trauma group. We think this is because we dissected the rats on the fourth day. This study also showed that dexmedetomidine limited the degree of neuronal apoptosis.

Hall et al. [49] investigated the cardioprotective effects of dexmedetomidine in rats with induced cerebral hypertension. They showed that dexmedetomidine inhibited the increase in plasma catecholamine levels and decreased myocardial MDA levels. Catecholamines are known to increase the production of free radicals and promote intracellular calcium accumulation. Both of these effects are associated with lipid peroxidation. Finally, Laudenbach et al. showed that dexmedetomidine reduced neuronal damage not only in the cortex but also in the white matter as indicated by reductions in the size of cystic lesions in five-day-old rats with hypoxic-ischemic brain injury induced by injections of NMDA-receptor agonists [50]. They reported that dexmedetomidine inhibited NMDA receptor activity and intracytoplasmic accumulation of calcium. Therefore, dexmedetomidine may directly or indirectly reduce the production of free radicals, as well as limit lipid peroxidation and cellular apoptosis.

This study has several limitations. Different animal models are used to investigate the pathophysiology of secondary injury after traumatic brain injury. Weight-drop models utilize the gravitational forces of free-falling [51]. They aim to achieve focal and extensive brain damage as a result of the external force delivered to the rat’s skull [52]. There are as many animal models of traumatic brain injury as there are mechanisms of pathogenesis, and this study used only one of these models. Secondly, this study only investigated the biochemical and histopathological end-results of trauma. In this study, we focused on the effects of secondary trauma-related brain damage on neurons and oligodendrocytes. Therefore, no conclusions can be made about the possible interactions between the axonal injury in the white matter of the secondary injury and the potential molecular mechanisms. In our study, TBARS, MDA and GSH levels in brain tissue were examined. Our study needs to be supported by studies examining other oxidant/antioxidant molecules that play a role in oxidative stress.

In addition, apoptosis should be supported by studies considering TUNEL, Ki-67, intracellular and mitochondrial calcium levels.

Another limitation is the absence of hemodynamic and respiratory monitoring. Dexmedetomidine is a sedative drug with unique properties such as vasodilation and bradycardia. The resulting decrease in cardiac output may increase cerebral ischemia. Sedative doses of dexmedetomidine decrease respiratory rate but do not affect the respiratory response to carbon dioxide [53]. The dose of dexmedetomidine in this study was based on a previous study, which showed that an intraperitoneal dose of 100 µg/kg reliably induced sedation in Sprague-Dawley rats [25]. However, this dose does not have a possible human application.

As dexmedetomidine was mostly studied in hypoxic-ischemic and hyperoxia-induced injury, this pilot study on traumatic brain injury investigated only a single dose of dexmedetomidine due to ethical reasons [54,55]. To limit the sample size, dose-response curves were not generated. In addition, there was no function test to evaluate the effect of Dex application in this study. Further laboratory studies that will incorporate a function test and investigate axon injury, the specific mechanisms, the dose-response relationship, and the impact of the time to treatment initiation are needed before clinical translational studies can be performed.

5. Conclusion

Dexmedetomidine administered during the early stage of traumatic brain injury may alleviate neuronal injury by inhibiting lipid peroxidation. The mechanism of action and possible alterations to the therapy, such as timing, dosing and duration, should be investigated further.

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Author contributions

AS and BE had full access to all of the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis. LT, TM and HAU were involved in the concept and design of the manuscript. All authors acquired, interpreted, and designed the manuscript. SB and AO drafted the manuscript. TM revised the manuscript for important intellectual and linguistic content. TM, LT, and HAU provided administrative, technical, and supervised data.
Data availability statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

Ethical approval

The study was approved by University Animal Ethical Committee (ID: 2017/1).

This research was approved by the Animal Research Ethics Committee of the University (2017–1). Procedures involving animals and their care were carried out in conformity with international laws and policies (Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 2011)).

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