Int J Clin Exp Med 2016;9(11):22807-22813
www.ijcem.com /ISSN:1940-5901/IJCEM0038087

Original Article

Cerebrovascular protective effect of combination of Tetradrine and Atorvastatin against cerebral ischemia-reperfusion injury in rats via inhibition of inflammatory mediators

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Received August 17, 2016; Accepted September 19, 2016; Epub November 15, 2016; Published November 30, 2016

Abstract: The present study was conducted to determine the protective effect of combination of terandrine (TET) and atorvastatin (ATR) against cerebral ischemia-reperfusion injury in rats. It was marked to note that, the individual treatment of TET and ATR causes significant decline in the level of TNF-α and IL-1β, with prominent inhibition reported by ATR-group. Moreover, in a combination, i.e. TET+ATR, the level of TNF-α and IL-1β found to be significantly reduced. The western blot analysis showed that effect of combination dose is more effective in lowering the expression of iNOS and COX-2 in ischemic cerebral tissues. Both, TET and ATR, causes improvement in the level of SOD and GSH and reduces MDA activity accompanied. It also causes significant reduction of NF-κB. The histopathological studies confirmed that, both the drugs in combination are effective in reversal of microstructural changes as compared by MCAO group. The combination of tetrandrine and atorvastatin are found to be more effective than alone for the protection of brain tissues against the cerebral ischemic injury via inhibition of NF-κB and other pro-inflammatory mediators together with antioxidant activity.

Keywords: Terandrine and atorvastatin, cerebral ischemia-reperfusion, TNF-α, IL-1β, antioxidant activity

Introduction

After the ischemic heart disease, illness affecting. It account for the death of approximately 6.2 million persons across the globe. A cerebrovascular stroke is generally considered as an ischemic stroke of brain or cerebral system, where the brain is become deprived of oxygen as a consequence of reduced blood supply. It leads to the damage of cerebral tissues and infarction of affected cerebral region. The underlying mechanism related with ischemic stroke is not confined to only one reason, yet it has been depend upon number of mechanism, such as, oxidative stress, inflammation, energy crisis and apoptosis which together contribute to the progression of ischemic stroke [1-3].

Thus, the therapies or agents which are able to target the aforementioned pathways are proved to be beneficial in the ischemic stroke. Recently, Tetrandrine a bis-benzylisoquinoline alkaloid obtained from the root of *Radix Stephania tetrandra* which is proved to exhibit protective role against cerebral ischemia and against vascular dementia [4-7]. On the other hand, Atorvastatin also proved to exert beneficial effect in cerebral ischemia via anti-oxidant and anti-inflammatory effect [8-10]. Due to multifactorial etiology of cerebral ischemia, no clinical trial has shown the beneficial effect of single agent in cerebral ischemia.

Prompted by the above, the present study was conducted to enumerate the protective effect of combination of tetrandrine and atorvastatin against cerebral ischemia-reperfusion injury in rats.

Material and methods

Animals

For the study, adult Sprague-Dawley male rats were selected and obtained from the animal

house. They were housed in polypropylene cages. Whereas, food and water were provided *ad libitum*. They were acclimatized in the laboratory condition for at least 6-7 days prior to start any experiment. The study has been duly permitted by the Institutional Animal Ethical Committee.

Focal cerebral ischemia-reperfusion model

The rats were made unconscious with the anaesthetic and subsequently underwent to MCAO as per the previous. Briefly, the origin of right MCA was obstructed by introducing an appropriate nylon monofilament into the carotid artery. The suture was inserted till the 18-20 mm from the external carotid artery to internal carotid artery. After 2 h, the filament has been removed after 2 h of occlusion and the resulting wound was sutured. The rats were then housed in normal laboratory condition. Apart from the treatment groups, control group underwent surgery without the MCAO. The internal temperature of the rats were maintained at 37°C during the surgery via heat pads during the surgery.

The rats were randomly categorized into five groups as follows: Group 1: Sham operated group; Group 2: MCAO group; Group 3: MCAO + TET (i.p, 20 mg/kg B.W.); Group 4: MCAO + ATR (i.p., 20 mg/kg B.W.); Group 5: MCAO + (TET+ATR) (i.p., 20 mg/kg B.W. each).

ELISA assay

Briefly, with the help of sonification, the collected ischemic cortical tissues were homogenized. The subsequent samples were then rapidlyfreezed and stored at -20°C. The level of tumor necrosis factor (TNF)-α and interleukin (IL)-1β was estimated using ELISA kit (Toray Fujibionics, Tokyo, Japan). Moreover, the activity of myeloperoxidase (MPO) was determined with the protocol supplied with the estimation kit (Nanjing Jiancheng Bioengineering Institute, China).

qRT-PCR

The total RNA (tRNA) was obtained from the cortical tissues of the ischemic brain. Later the 2 mg of the (tRNA) was then subjected to TaqMan one-step reverse transcription (Applied Biosystems, Foster City, CA), trailed by real-time PCR (Applied Biosystems). The primer used in the study was given as follows: inducible nitric oxide synthase (iNOS), 5'-ATCCCGAAACGCTACACTT-3' (forward primer) and 5'-TCTGGCGAAGAACAATCC-3' (reverse primer); cyclooxygenase-2 (COX-2), 5'-GAGAGATGTATCCTCCCACAGTCA-3' (forward primer) and 5'-GACCAGGCACCAGACCAAAG-3' (reverse primer); β-actin, 5'-ACT CGTCAT ACT CCT GCT-3' (forward primer) and 5'-GAAACT ACC TTC AAC TCC-3' (reverse primer) as the internal control.

The steps for the qRT-PCR was performed as per the established protocol.

Western blot analysis

Briefly, the total proteins was extracted from the ischemic cortical tissues for the estimation of protein expression of iNos and Cox-2. The nuclear extracts were used for the estimation NF-κB subunit p-65. For this, the extracted total protein was suspended in buffer sol for 15 min. The pellets together with extraction buffer were allowed to incubate on ice for 30 min with subsequent centrifugation at 10000 rpm for at 4°C, and the resultant supernatant was used for the estimation of NF-κB. The BCA Protein assay kit was used for the determination of protein concentration. The 40 μg of protein was the isolated using SDS-PAGE, followed by translocation on PVDF membrane (Millipore, Billerica, MA, USA). The 5% de-fat milk in TBST was used for blocking the membrane and then incubated with suitable primary antibodies for iNOS, COX-2, NF-κB p65, and GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 4°C overnight and further incubated with HRP secondary antibody.

Estimation of superoxide dismutase, malondialdehyde and glutathione concentration

The activity of superoxide dismutase (SOD), glutathione (GSH) and malondialdehyde (MDA) activity in the brain ischemic tissues were estimated following to the instructions provided with assay kits obtained from Nanjing Jiancheng Bioengineering Institute, China.

Histopathological analysis

Briefly, the brains of the rats were detached and immobilized in 4% PBS-buffered formaldehyde for 48 h. Thereafter, a 4 mm coronal sec-

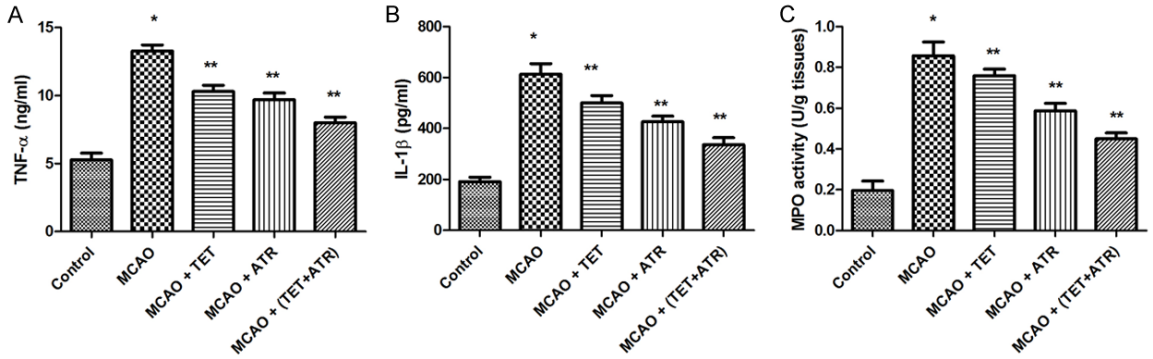

Figure 1. Effect of TET, ATR and TET+ATR in the cerebral ischemia/reperfusion injury of the rats, where (A) TNF-α, (B) IL-1β and (C) MPO activity. Results are conveyed as mean ± SD. *P < 0.05 vs the control; **P < 0.05 vs MCAO group.

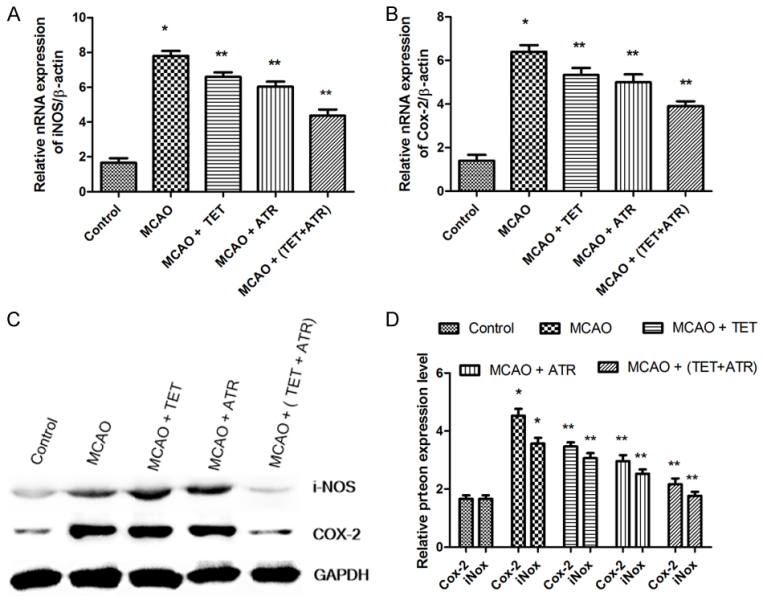

Figure 2. Effect of TET, ATR and TET+ATR on inflammatory mediators in the cerebral ischemia/reperfusion injury of the rats, where (A) iNOS and (B) COX-2 level with the help of qRT-PCR, (C) western blotanalysis for the expression of iNOS and COX-2. (D) The relative protein expression levels of iNOS and COX-2. Results are conveyed as mean ± SD. *P < 0.05 vs the control; and **P < 0.05 vs MCAO.

tion of the brain was cut 2.0 mm anterior and posterior to the bregma and the block was implanted in paraffin. The block was then cut into 5 μm coronal sections that were stained with hematoxylin-eosin (HE) using standard methods.

Statistical analysis

All results are reported as mean ± SD of three independent experiments. The one-way analysis of variance (ANOVA) followed by Bonferroni test for multiple groups or Student's t test between two groups was used for statistical analysis with P < 0.05 considered to be significant.

Results

Effect on inflammatory cytokines and MPO activity

The effect of individual and combined dose of TET and ATR were investigated on inflammatory mediators, such as, TNF-α and IL-1β using ELISA in the ischemic reperfusion injury of the rats. As shown in **Figure 1A** and **1B**, the level of TNF-α and IL-1β found to be significantly elevated in MCAO groups as compared with the control. It was marked to note that, the individual treatment of TET and ATR causes significant decline in the level of TNF-α and IL-1β, with prominent inhibition reported by ATR-group. Moreover, in a combination, i.e. TET+ATR, the level of TNF-α and IL-1β found to be significantly reduced might because of combination effect. A similar pattern of inhibition was observed in MPO activity **Figure 1C**.

Effect on the level of iNos and Cox-2 in cerebral ischemia/reperfusion of the rats

In the ischemic brain damage, the level of inflammatory mediators, such as, iNOS and COX-2 signifies the level of inflammation in the

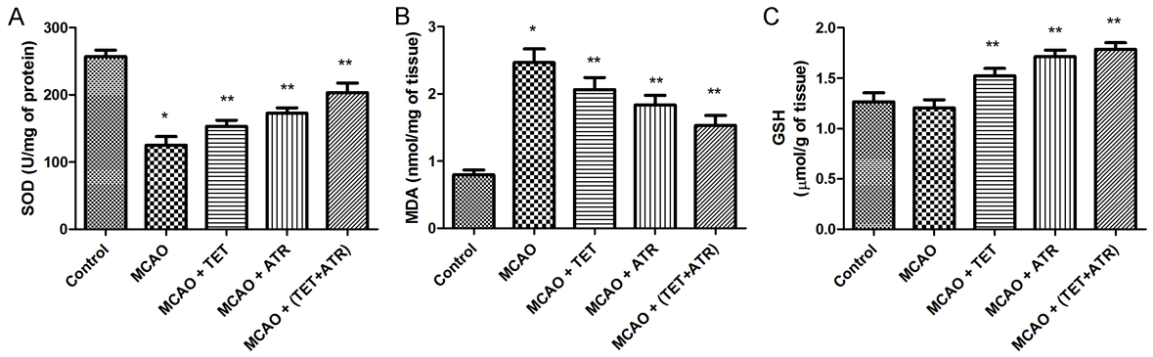

Figure 3. Effect on the level of oxidative stress mediators. (A) SOD, (B) MDA, (C) GSH. Results are conveyed as mean ± SD. *P < 0.05 vs control; and **P < 0.05 vs MCAO.

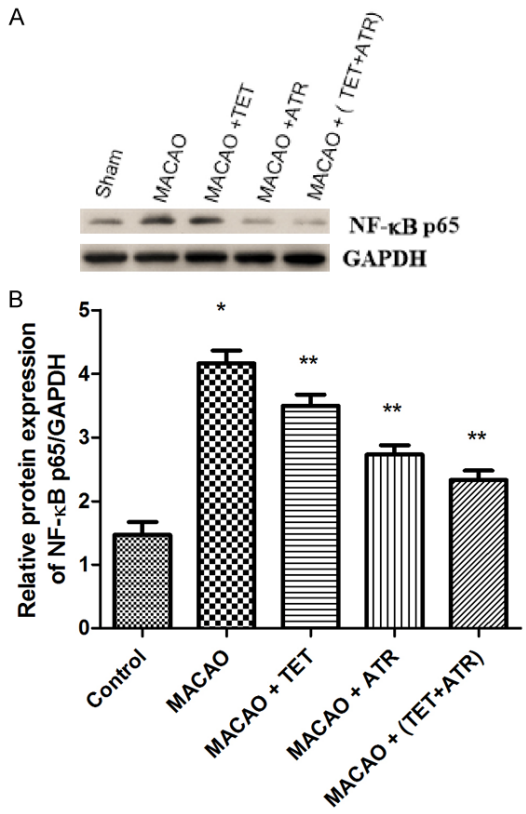

Figure 4. Effect various treatment on NF-κB expression in the tissue of the cerebral ischemia as determined by western blot analysis. A. Expression of NF-κB p65, (B) comparative protein expression level of NF-κB. Results are conveyed mean ± SD. *P < 0.05 vs control; and **P < 0.05 vs MCAO.

tissues. Therefore, next we intended to determine the effect of alone and combination of doses. It has been found that, the level of iNos and Cox-2 found to be significantly reduced in the case of the alone treatment of TET or ATR **Figure 2A** and **2B**. Whereas, in combination of TET and ATR the level of both the parameters has been significantly fallen. The observation was further confirmed with the help of western blot analysis, which shown that, the effect of combination dose is more effective in lowering the expression of iNOS and COX-2 in ischemic cerebral tissues, **Figure 2C** and **2D**.

Effect on the oxidative stress

Several studies emphasized the role of oxidative stress in the generation and progression of cerebral ischemia which leads to the damage of cerebral tissues. Thus, we have assessed the effect of treatment on the level of oxidative stress mediators. As shown in the **Figure 3A** and **3C**, the level of SOD and GSH was found to be significantly lowered in the case of MCAO treated mice than the control. Whereas, the level of MDA activity was reported to be significantly elevated in the MCAO treated rats **Figure 3B**. It was marked to note that, the level of SOD and GSH was significantly elevated in the case of TET and ATR treatment alone, with further significant improvement shown by the combination dose (i.e., TET+ATR). In the case of MDA activity, the level of MDA has been significantly elevated in the case of alone TET or ATR treatment with more pronounced inhibition in the case of combination of TET and ATR.

Effect on NF-κB activation

It has been well documented that, NF-κB found to exhibit key role in the inflammation of cerebral damage. Therefore, in this study, the effect of TET and ATR was examine on the level of NF-κB p65 expression in the nuclei matter of cerebral ischemic tissues. It has been shown

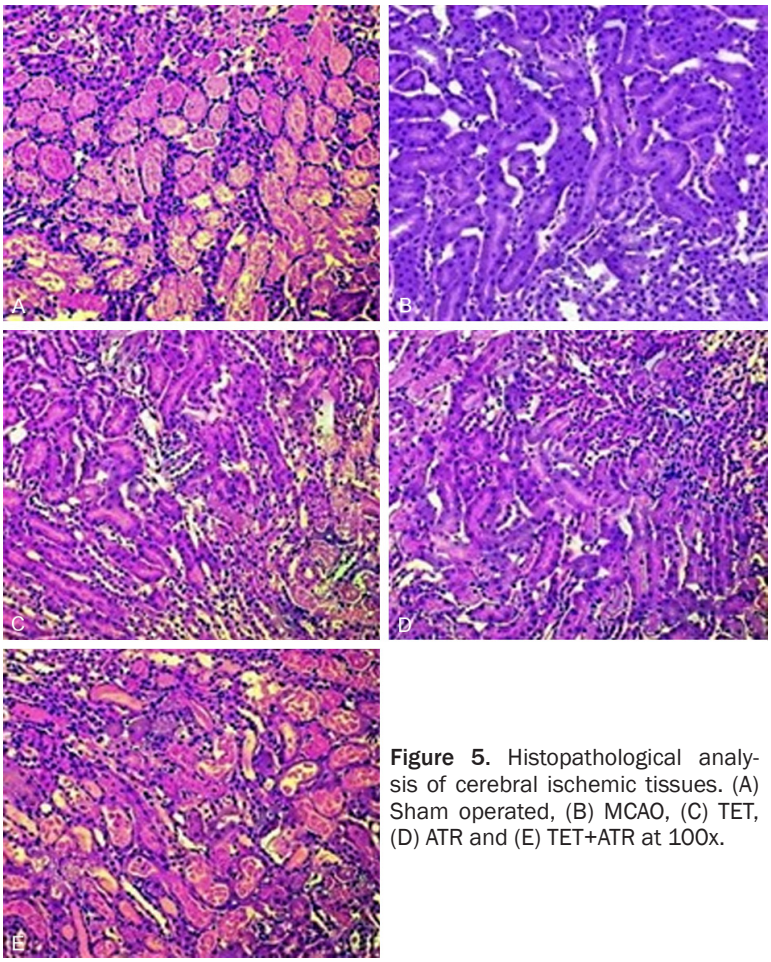

Figure 5. Histopathological analysis of cerebral ischemic tissues. (A) Sham operated, (B) MCAO, (C) TET, (D) ATR and (E) TET+ATR at 100x.

by the western blot analysis, the level of NF-κB p65 was found to significantly elevated in the MCAO group as compared to sham. Whereas, in the case of TET and ATR treatment alone, a significant decrease in the expression of NF-κB p65 were observed. Whereas, more pronounced inhibition was revealed in the case of TET and ATR combination dose, **Figure 4A** and **4B**.

Histopathological analysis

As show in **Figure 5**, the sham operated group did not shown any histological alteration (**Figure 5A**), whereas, the MCAO group marked lesion in the brain tissue (**Figure 5B**). The MCAO group showed significant meningeal and neuropathological changes confirmed by pyknotic and shrinkage of nucleus with broadened pericellular spaces. These changes has been reversed by the TET and ATR, with more significant reversal of microstructural changes in TET+ATR dose **Figure 5C-E**.

Discussion

The cerebral ischemia/reperfusion (I/R) injury is a very critical condition where brain observe stroke due to insufficient supply of the blood. It causes brain to be deprived of oxygen, and hinder the normal function of the brain. During this injury, a generous amount of free radical has been generated and compromise the natural defense mechanism of the brain [1, 2]. Under the normal circumstances, the generated free radical has been easily neutralised by the endogenous antioxidants and thereby preserves the brain. But during the stroke, the endogenous anti-oxidant system is not able to counteract the generated free radical and leads to the release of excitatory amino acid. It further potentiate the expression of specific genes which increases the lipid peroxidation and DNA oxidation which ultimately resulting in neuronal apoptosis. The SOD, glutathione peroxidase, and catalase are considered as natural anti-oxidant defense system of the body. The SOD and glutathione are occurred in the cytosol and mitochondria causes reduction of superoxide anion to H_2O_2 and H_2O. Whereas, the catalase (CAT) is responsible for removal of high level of H_2O_2. Thus, agents improving the level of above mentioned endogenous antioxidant defense system have shown beneficial effect on the cerebral ischemic injury [11, 12].

It has been reported that, tetrandrine (TET) can able to inhibit the lipid peroxidation in the rat liver mitochondrial fractions. It prevent the generation of ROS induced by complex ADP/Fe^{2+}, thereby decreasing the rate of lipid peroxidation of mitochondrial membranes [5, 13]. Whereas, the atorvastatin belonging to the family of -statins also showed excellent antioxidant effect. It cause reduction in E-selectin expressionand ameliorate free radicalinjury [9].

Thus, in the present study we have observed that, both the drugs are effective in improving the anti-oxidant status of the cerebral tissues by modifying the level of SOD, CAT and GSH. It was significant to note that, when both TET and ATR was used in the combination, the level of improvement of the endogenous oxidant system is found to be more as compared to alone treated group and significantly enhanced as compared to MCAO group.

Various studies have accumulated the effect of pro-inflammatory cytokines, comprising TNF-α and IL-1β, which found to be over-expressed in ischemic region induced byimmune cells, showed to markedly influence the progression of inflammatory responses in the brain. It has been found that during the ischemic/reperfusion injury, the concentration of TNF-α and IL-1β were found to be augmented in the cerebral tissues of the experimental animal model [14]. It has been further confirmed by a study, which showed that administration of TNF-α during ischemic brain injury causesamplification of the injury, showed by amplifiedtissue destruction and neuralshortfalls [15]. Thus, the effect of TET and ATR was determined on the level of these inflammatory mediators. Results of the study showed that, combined dose of TET and ATR were more found to be effective in reducing the level of TNF-α and IL-1β than the individual treatment. This has suggested that, the cerebral protection of the drugs may be because of the combined anti-inflammatory response. The similar pattern of inhibition was observed in the case of MPO which serve as a separate indicator of tissue permeation of inflammatory cells marker enzyme. It was found to be increase in response of I/R injury, and its activity is directly correlates with the neutrophil infiltration into the brain tissues. Thus its inhibition offers selective advantage for preserving the integrity of the brain in response of damage.

The role of iNos in the cerebral ischemia is widely studied and found that, it plays a significant role in progression of the cerebral damage. Whereas, the expression of COX-2, was also found to be aberrantly activated in the cerebral ischemic injury. Together with this, both iNos and Cox-2 found significant role in the advancement of the damage of cerebral tissues [16]. Thus, in the present study, we have found that, both TET and ATR showed more effectivesuppression of iNos and Cox-2 expression, as compared to individual treated group. The level of attenuation was found to be more significant when compared with MCAO treated group where, the level of iNos and Cox-2 are highly expressed.

It has been reported that, NF-κB, a dimeric protein belongs to the family of transcription factor plays a key role in the synthesis of various pro-inflammatory mediators, such as, transcription of various genes including TNF-α gene. It is widely believed that NF-κB is exists in the sequestered form in cytoplasm by specific IκB proteins. Upon stimulation of various mediators such as pro-inflammatory cytokines, it will migrates into the nucleus, where it attaches to the DNA of interest and recruits large collection of genes and initiate inflammatory response. Thus, blockade of NF-κB causes selective inhibition of inflammatory response in the cerebral damage and provide beneficial effect [17-19]. Thus, in the present study, we have found that, TET and ATR alone found to be significantly inhibit the expression of I/R induced NF-κB p65. Whereas, the effect of inhibition was found more prominent when both the drugs are used in combination. These results showed a conclusive evidence that, both TET and ATR are effective in reduction of cerebral damage followed by the ischemic injury possibly by inhibiting NF-κB and various pro-inflammatory response.

Conclusion

As a concluding remark, it has been substantiated that, the combination of tetrandrine and atorvastatin are found to be more effective than alone for the protection of brain tissues against the cerebral ischemic injury via inhibition of NF-κB and other pro-inflammatory mediators together with antioxidant activity.

Disclosure of conflict of interest

None.

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