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Original Article

Naringin Effectively Protects Cardiomyocytes Against Hypoxia/Reoxygenation Injury

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Abstract: This study was conducted to evaluate the protective effect of Naringin (NAR) on H9C2 cardiomyocytes in hypoxia/reoxygenation (HR) injury *in vitro* induced by the hypoxia chamber. In the study, H9C2 cells were grown under normal (control) and HR conditions. The viability, cardiolipin content and mitochondrial membrane potential of H9C2 cells in the experimental group were analyzed by using suitable kits. The obtained results show that the addition of NAR (16÷160 µM) significantly increased the survival rate of H9C2 cells under HR conditions. In particular, NAR showed remarkable efficiency in preserving mitochondrial function at concentrations of 80 µM and 160 µM. In HR-exposed H9C2 cell group, the cardiolipin content and mitochondrial membrane potential values of H9C2 cells decreased sharply (71.64±1.37% and 68.12±2.78%, $p<0.05$). Interestingly, mitochondrial cardiolipin contents signigicantly increased in H9C2 cells post-hypoxic treated with NAR at doses of 80 µM and 160 µM to 87.76±1.89% and 81.09±1.21%, respectively. Additionally, post-hypoxic supplementation of NAR at concentrations of 80 µM and 160 µM effectively increased mitochondrial membrane potential values. Overall, the obtained results are preliminary data on the effects of NAR in protecting mitochondria-targeted cardiomyocytes against HR injury.

Keywords: H9C2, cardiolipin, mitochondrial membrane potential.

1. Introduction

Hypoxia/reoxygenation (HR) injury is a myocardial tissue damage caused by revascularization to myocardial tissue after a period of time; this damage is ischemia or cessation of blood supply [1]. In fact, HR injury is a commonly used *in vitro* model of ischemia, which is useful to study the recovery processes following the hypoxic period. Previous work has shown that mitochondria play important roles in the pathophysiology of HR cardiomyopathy [2]. Therefore, the preservation of mitochondrial function is necessary to limit damage to cardiomyocytes and myocardium. In the last few decades, the screening and finding of natural active substances with high biological activity, few side effects as well as optimized conditions to improve cardiac function in HR have had remarkable achievements [2, 3]. Of

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those, Naringin (NAR) is a flavanone glycoside found in high concentrations in the peels of citrus fruits (genus citrus) and has been shown to potent biological and pharmacological activities [4]. Previous research demonstrated that NAR possessed the anti-inflammatory and antioxidant activities [5]. Pretreatment with NAR attenuated renal and myocardial ischemia/reperfusion injury in rats [6, 7]. Also, NAR pretreatment showed the potential to protect H9C2 cells from HR-induced apoptosis [8]. However, the mechanism underlying the post-hypoxic treatement of NAR on H9C2 cardiomyocytes remains poorly defined. Therefore, in this study, we investigated the effect of post-hypoxic treatment with NAR in HR-subjected H9C2 by analyzing the cell viability as well as the mitochondrial structure and function.

2. Materials and Methods

2.1. Materials

The main materials and equipment used in this study were H9C2 cell line (ATCC® -USA), Naringin (**$C_{27}H_{32}O_{14}$**; Molecular weight: 580.53g/mol; ≥ 95% HPLC; National Institute of Medical Materials, Vietnam), Dulbecco's Modified Eagle Medium 4,5g/l glucose (DMEM, Gibco, USA), Fetal bovine serum (FBS, Gibco, USA), Penicillin-Streptomycin (PS, Gibco, USA), Phosphate buffered saline (PBS, Gibco, USA), Cell Counting Kit-8 (CCK-8, Dojindo, Japan), Dimethyl Sulfoxide (DMSO, Sigma, USA), Inverted microscopy Axiovert (S100, Carl Zeiss, Germany), Tetramethylrhodamine ethyl ester (TMRE, excitation/emission: 535/570 nm, Invitrogen, USA), 10-N-nonyl acridine orange (NAO, excitation/emission: 495/519 nm, Invitrogen, USA); Culture dishes 90x20 mm (SPL, Korea), 96-well black, glass bottom plates (CAT. 33196, SPL), confocal dishes (CAT. 100350, SPL), CO_2 Incubator (Shellab, USA); ApoTome Fluorescence Microscope (Zeiss, Germany), and Microplate reader (Tristar, USA). The research was carried out at the Animal cell biotechnology laboratory, Life Science Research Center, Faculty of Biology, VNU University of Science.

2.2. Methods

2.2.1. Cell Culture and Hypoxia-Reoxygenation (HR) In Vitro Model

H9C2 cells were maintained culture dishes (90x20 nm) containing DMEM, 10% FBS, and 100 μg/ml of PS at 37 °C with 5% CO_2. Culture medium was changed every 2-3 days. For HR *in vitro* model, H9C2 cells were further transferred to either 96-well black plate, glass bottom or a confocal dish at density of 5.10^3 cells/well at 37 °C, 5% CO_2. After 24 h, the cells were then subjected to hypoxic condition and treatments as described in a previous publication [9] and the experimental cells were divided into different groups as follows: The normal control group: H9C2 cells were continously cultured under normal condition (DMEM, 10% FBS, 1% PS, 37 °C and 5% CO_2 for 48 h; the HR group: H9C2 cells were cultured in serum-free low-glucose DMEM at 37 °C, 95% N_2, 5% CO_2, and 2% O_2 for 6 h. Then, the old medium was removed and the H9C2 cells were then transferred to normal condition (DMEM, 10% FBS, 1% PS, 37 °C and 5% CO_2) for reoxygenation for 24 h. At the time of reoxygenation (stimulating the reoxygenation stage), with the further treatments (i, ii):

i) HR group: the reoxygenation stage normal culture medium contained DMEM, 10% FBS, 1% PS;

ii) NAR groups: the reoxygenation stage medium contained DMEM, 10% FBS, 1% PS, DMSO 0,1%, and NAR at doses of 16, 32, 80, 160, 320 μM as previous study [10]. NAR stocks were prepared in DMSO and the final concentration of DMSO in cultured medium was about 0,1%.

At the end of the experiment, the viability of cells was determined by CCK-8; cardiolipin content and mitochondrial membrane potential were measured indirectly through fluorescent indicators NAO and TMRE.

2.2.2. Measurement of Cell Viability

The cell viability was determined by using CCK-8. After being subjected to HR and treatment, H9C2 cell groups were further incubated for 1-4 h with CCK-8 as previously described [9]. For each group, an absorbance value indicating cell viability was measured at 450 nm using a microplate reader. The alive cell number in each well was expressed as a value relative to the normal control well. Experiments were repeated 4 times.

2.2.3. Measurement of Mitochondrial Cardiolipin and Mitochondrial Membrane Potential

Mitochondrial cardiolipin and mitochondrial membrane potential were indirectly assessed as in a previously discribed [9]. H9C2 cells were seeded in confocal dishes and subjected to HR model and treatments. After being subjected to different conditions, the cells were stained with either NAO (0.1 µM, ex/em: 495/519 nm) or TMRE (1 µM, ex/em: 535/570 nm) at 37 °C for 30 min at room temperature to detect changes in cardiolipin content or mitochondrial membrane potential, respectively. After being washed with PBS, NAO- or TMRE-stained cells were captured using the ApoTome and the images were then reconstructed from individual tiles (X:6, Y:9) using ZEN Blue 2.5 software (Carl Zeiss). The total fluorescence intensities were expressed as percentage value relatives to the normal control. Experiments were performed for 3-4 times.

2.2.4. Statistical Analysis

Origin 8.5 software was chosen to analyse data. Data are presented as means ± standard deviation (SD). Differences between the two groups were evaluated by ANOVA and Turkey test. A p-value $\leq$ 0.05 was considered to be significant.

3. Results and Discussion

3.1. NAR Exerts Weak Cytotoxic Effect on H9C2 Cells

H9C2 cells were cultured in normal condition for 24 h and were then added NAR at dose of 16÷3200 µM to culture media for 48 h. The effects of NAR on the viability of H9C2 cells assessed by using CCK-8 are shown in Figure 1.

Previous research demostrated that NAR effects on cell viability in a dose-dependent manner [10, 11] and cell types [11, 12] via modulating different pathways. NAR inhibits proliferation and induces cell apoptosis of thyroid cancer cell [12]. NAR also protected H9C2 cells against damage caused by high glucose [10]. In consistence with previous study [10], the obtained results show that at concentration of 16÷3200 µM, NAR showed weak cytotoxic effect on H9C2 cell with IC_{50} value of NAR was about 1,174.24 µM (Figure 1). We chose a concentration range of 16÷320 µM to further investigate the effect of NAR on the viability of H9C2 cells under HR conditions targeting mitochondrial function.

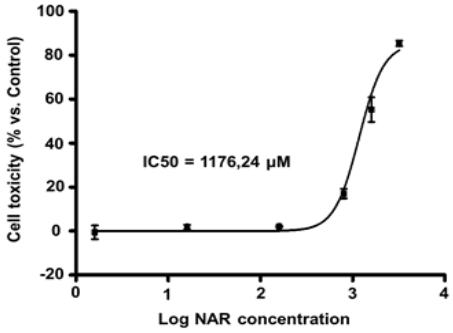

Figure 1. The toxicity of Naringin on H9C2 cells. Naringin (NAR) concentration (16÷3200 µM).

3.2. NAR Reduces H9C2 Cell Death in HR Injury

The effects of NAR at different concentrations on the viability of H9C2 cells under HR conditions are presented in Figure 2 and Figure 3.

The representative image of H9C2 cells shown in Figure 2 demonstrated that the number of dead cells in HR group (Figure 2B) is much higher than that in the normal control group (Figure 2A). Cell detachment, death and floating in the culture medium were clearly observed in HR-subjected cell group. Treatment

with NAR increased the density of adhesive cells subjected to HR conditions (Figures 2C, D). The morphology result is quite similar to the H9C2 cell viability result acquired by CCK-8 (Figure 3).

Under normal condition, NAR has no protective effects on H9C2 cells (Figure 3A). However, it exerts strong protective effects in HR-stimulated conditions (Figure 3B).

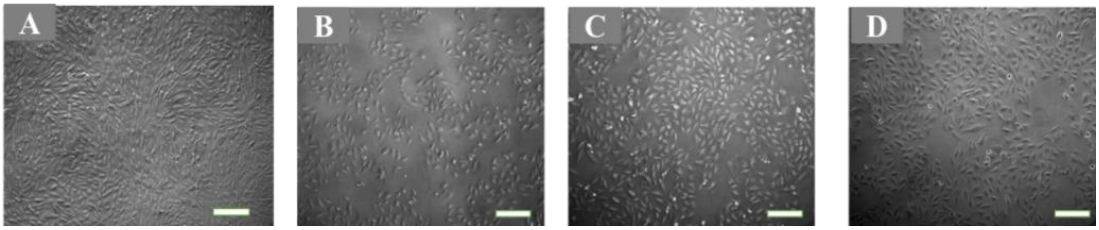

Figure 2. Images of H9C2 cells under different conditions.
A: H9C2 cells were cultured in normal condition; B: H9C2 cells were cultured in hypoxia/reoxygenation (HR) condition; C: H9C2 cells were cultured in HR condition and subjected to post-hypoxic treatment with 80 µM NAR; D: H9C2 cells were cultured in HR condition and subjected to post-hypoxic treatment with 160 µM NAR; 5X magnification; scale bar: 5 µm.

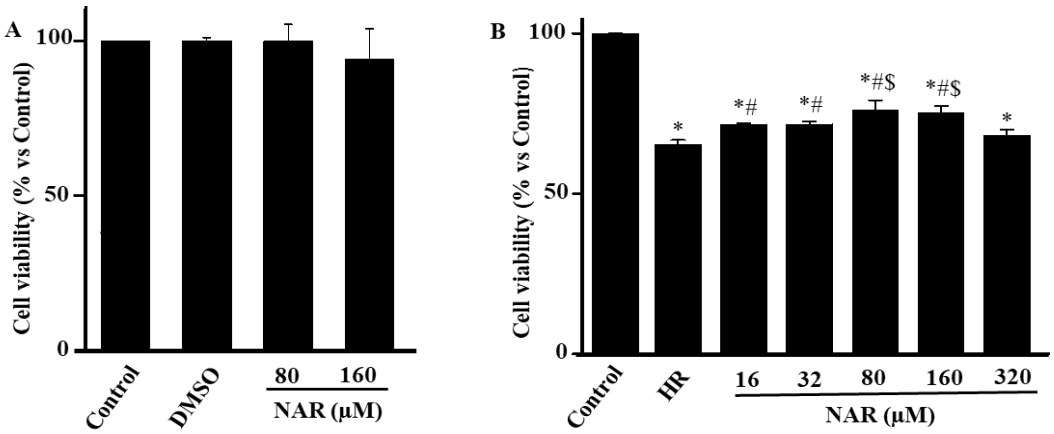

Figure 3. Cell viability of H9C2 under different conditions.
A: H9C2 cells cultured in normoxia condition; B: H9C2 cells subjected to hypoxia/reoxygenation (HR) model and treatment with Naringin (NAR). Control: H9C2 cells were cultured in normal condition (normoxia); DMSO: H9C2 cells were cultured in normoxia condition plus DMSO 0.1%; HR: H9C2 cells were cultured in HR condition; NAR: H9C2 cells were cultured in normoxia or conditions of post-hypoxic treatment with NAR at doses of 16, 32, 80, 160 and 320 µM; *p<0,05 vs. control, $^{\#}$p<0,05 vs. HR, $^{\$}$p<0,05 vs. NAR at dose of 320 µM; n=4.

The obtained results showed that survival rate in HR-exposed cells was significant lower (65.50±3.75%) than those in control group ($p<0.05$, Figure 3B). Post-hypoxic treatment with NAR at dose of 16÷160 µM markedly increased the cellular survival rate compared to those in HR group ($p<0.05$). NAR treatments at doses of 80 µM and 160 µM showed highly cellular protective effect with cell alive percentage (% of control) were about 76.42±2.90 and 75.32±2.25%, respectively. These results are quite similar to previous report [8]. Pretreatment with NAR increased the survival rate of H9C2 cells againt HR injury through inhibition of oxidative free radical production. The effects of NAR resemble a NAR derivative, Naringenin. Previous studies proved that Naringenin was able to protect cells against HR injury [8, 13]. Also, Naringenin significantly reduced the cell viability of A431 cells with a concomitant increase in nuclear condensation and DNA fragmentation in a dose

dependent manner [11]. The findings in this study further confirmed the effect of NAR on ischemic-reperfusion injury models *in vivo* [5, 14]. In these researches, NAR treatment reduced infarct size compared to the non-treated group. Moreover, NAR was reported to show its protective role through inhibition of inflammatory response [15], oxidative stress [16], and preservation of mitochondrial structure [17]. Here, the cell viability in cell group treated with NAR at doses of 80 µM and 160 µM was not significantly different ($p>0.05$). Based on this result, we decided to choose NAR treatment at these two doses for further evaluation.

3.3. Naringin Ameliorates Mitochondrial Dysfunction in HR-subjected H9C2 Cells

Mitochondria are powerhouse of cells and play important roles in cellular biological process. In this study, the effects of NAR on mitochondria of H9C2 cells were assessed via mitochondrial cardiolipin contents and mitochondrial membrane potential indexes.

3.3.1. Naringin Preserves the Mitochondrial Cardiolipin Content

Cardiolipin is a characteristic phospholipid of the mitochondrial inner membrane and plays an important role in the structure and functioning of the respiratory chain [18]. Cardiolipin was reported to be involved in the apoptosis of animal cells through interaction with several lethal proteins [19]. In HR pathophysiology, decrease in content impaired electron transport chain activity, subsequently disrupting mitochondrial function and leading to cell death [20]. Therefore, maintaining stable levels of cardiolipin is important to limit mitochondrial and cellular damage in cardiomyopathy [21]. In this study, the changes in cardiolipin content among cell groups were determined by using NAO fluorescence assay (Figure 4).

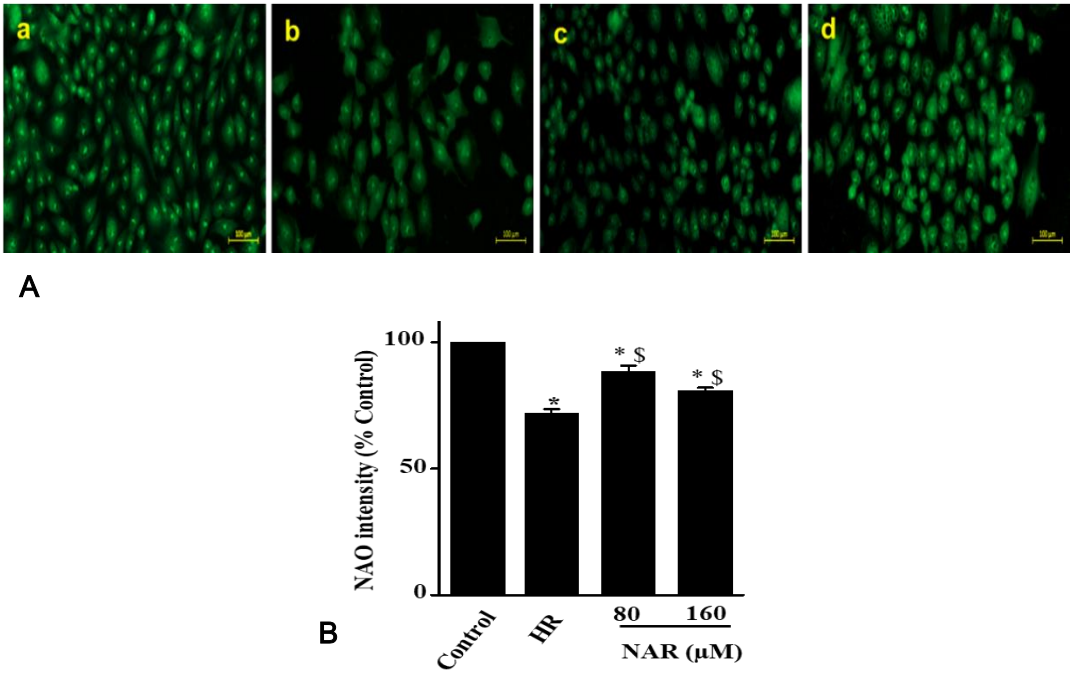

Figure 4. NAO intensity in H9C2 cells.
A: Representative images of NAO-stained H9C2 cells under normal control (a), hypoxia/reoxygenation (HR, b), post-hypoxic treatment with Naringin (NAR) at dose of 80 µM (c), 160 µM (d) conditions; B: NAO intensity in different conditions. *$p<0.05$ vs. control, $^{\$}p<0.05$ vs. HR, scale bar = 100 µm, n = 4.

The results in Figure 4 show that HR group has the lowest NAO intensity, remaining only about 71.64±1.37% of control. Interestingly, NAO fluorescence intensities of cell groups treated with NAR at doses of 80 µM and 160 µM were highly increased and were about 87.76±1.89 and 81.09±1.21% (of 100% control), respectively. This shows that NAR at concentrations of 80 and 160 µM has the ability to preserve mitochondrial cardiolipin in HR-induced injury; thereby, increasing the efficiency of the electron transport chain. This was consistent with previous study [20]. Besides, the data in Figure 4 also show that post-hypoxic treatment with NAR at dose of 80 µM had higher value of the NAO fluorescence intensity than those in cell group treated with NAR at concentration of 160 µM. However, this difference was not statistically significant ($p>0.05$). This result is quite consistent with the change in survival of H9C2 cells (Figure 3).

3.3.2. Naringin Preserves Mitochondrial Membrane Potential

Mitochondrial membrane potential is an important parameter for assessing mitochondrial function. The collapse of membrane potential might lead to mitochondrial malfunction and cell death [22]. In this study, mitochondrial membrane potential index was indirectly assessed by measuring TMRE fluorescence intensity (Figure 5).

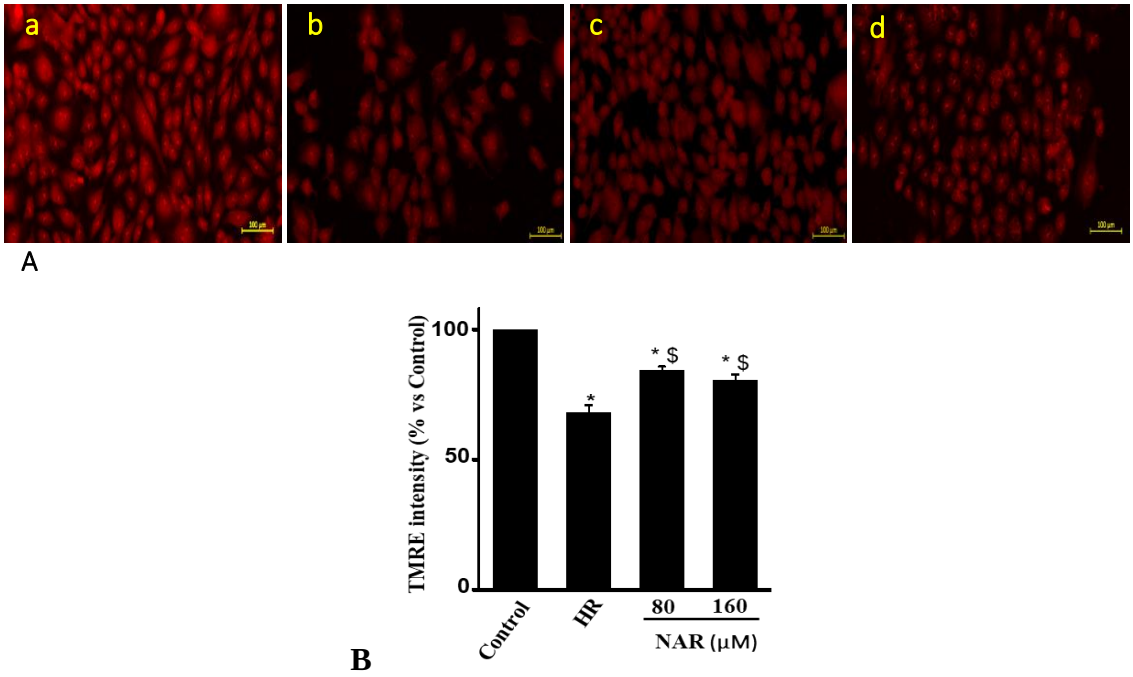

Figure 5. TMRE intensity in H9C2 cells.
A: Representative images of TMRE-stained H9C2 cells under normal control (a), hypoxia/reoxygenation (HR, b), post-hypoxic treatment with Naringin (NAR) at dose of 80 µM (c), 160 µM (d) conditions; B: TMRE intensity in different conditions. *$p<0.05$ vs. control, $^{\$}p<0.05$ vs. HR, scale bar = 100 µm, n = 3÷4.

Figure 5A shows the representative images of TMRE-stained H9C2 cells captured in different groups. In this assay, the redder density shows the higher mitochondrial membrane potential. The results show that HR sharply reduced mitochondrial membrane potential, therefore markedly inducing mitochondrial malfunction. In HR group, the number of red cells is smaller than those in other groups (Figure 5A). Percentage of TMRE fluorescence intensity in HR was 68.12±2.78 (of 100% control, Figure 5B). The cell groups

supplemented with NAR at concentrations of 80 µM and 160 µM had the brighter red signal as well as a higher TMRE fluorescence intensity (84.32±1.49% and 80.60±2.19% respectively) compared to those in HR group ($p<0.05$). The results suggest the ability of NAR in preserving mitochondrial membrane potential under HR condition. It is reported that stabilizing mitochondrial membrane potential reduces mitochondrial dysfunction and increases cell viability [23]. This result is quite consistent with last study [8]. Pretreatment with NAR at concentration of 10-40 µg/ml might preserve the mitochondrial membrane potential against HR, thereby limiting programmed cell death. The mitochondrial membrane potential value is relatively consistent with both the cell survival rate (Figure 2, 3) and the mitochondrial membrane cardiolipin content (Figure 4). Interestingly, post-hypoxic treatment of NAR at doses 80 µM and 160 µM had no significant difference. The results indicate that the most effective dose of NAR against HR damage was approximately 80 µM.

Thus, the results show that NAR has the ability to protect H9C2 rat cardiomyocytes through targeting mitochondria. However, the detail cytoprotective mechanism of NAR on HR-related molecules is still unknown and should be elucidated in further study.

4. Conclusion

The study documented that post-hypoxic treatment with NAR significantly increased the viability of disease-modified H9C2 cells through preserving mitochondrial structure and funtion. The new role of NAR may be potential for attenuating HR damage.

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