Full paper

Calf Spleen Extractive Injection (CSEI), a small peptides enriched extraction, induces human hepatocellular carcinoma cell apoptosis via ROS/MAPKs dependent mitochondrial pathway

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A B S T R A C T

Calf Spleen Extractive Injection (CSEI), a small peptides enriched extraction, performs immunomodulatory activity on cancer patients suffering from radiotherapy or chemotherapy. The present study aims to investigate the anti-hepatocellular carcinoma effects of CSEI in cells and tumor-xenografted mouse models. In HepG2 and SMMC-7721 cells, CSEI reduced cell viability, enhanced apoptosis rate, caused reactive oxygen species (ROS) accumulation, inhibited migration ability, and induced caspases cascade and mitochondrial membrane potential dissipation. CSEI significantly inhibited HepG2-xenografted tumor growth in nude mice. In cell and animal experiments, CSEI increased the activations of pro-apoptotic proteins including caspase 8, caspase 9 and caspase 3; meanwhile, it suppressed the expressions of anti-apoptotic protein B-cell lymphoma 2 (Bcl-2) and anti-oxidation proteins, such as nuclear factor-erythroid 2 related factor 2 (Nrf2) and catalase (CAT). The enhanced phosphorylation of P38 and c-JunN-terminal kinase (JNK), and decreased phosphorylation of extra cellular signal-regulated protein kinase (ERKs) were observed in CSEI-treated cells and tumor tissues. CSEI-induced cell viability reduction was significantly attenuated by N-Acetyl-L-cysteine (a ROS inhibitor) pretreatment. All data demonstrated that the upregulated oxidative stress status and the altered mitogen-activated protein kinases (MAPKs) phosphorylation contributed to CSEI-driven mitochondrial dysfunction. Taken together, CSEI exactly induced apoptosis in human hepatocellular carcinoma cells via ROS/MAPKs dependent mitochondrial pathway.

1. Introduction

Hepatocellular carcinoma (HCC), the sixth common type of cancer, threatens humans’ life and health worldwide. Patients suffered from HCC are often diagnosed with severely impaired liver function at a late stage and die within 7–8 months after diagnosis (1). Although multiple therapeutic options have been applied in clinic, adverse effects including immune system disorders and liver dysfunction are noted in patients (2). Novel therapeutic agents with lower toxicity for HCC curing are urgently needed.

It has been reported that peptides, either synthetic or derived from natural products, show anti-hepatocellular carcinoma activities in animal models or clinical trials (3). A small molecular weight of polypeptide with low toxicity can readily penetrate the tumor cells without drug resistance (4). Bombesin, a 14-amino acid peptide originally isolated from the skin of the European fire-bellied toad (Bombina bombina), significantly inhibits the growth on SK-Hep-1-xenografted tumor in nude mice (5). Calf Spleen Extractive Injection (CSEI), a small peptides enriched extraction, has been used to ameliorate thrombocytopenia and leucopenia in cancer patients who underwent chemotherapy and radiotherapy in China (6, 7). Additionally, efficacious therapy of CSEI combined with
cyclosporine A was found in patients with chronic aplastic anemia from clinical observation suggested by the significantly increase of leukocyte and platelets in blood (8). In our group, CSEI has been proved to improve immune function in cyclophosphamide-induced immunosuppressed mice model via NF-kB signaling pathway (9). As a marketable drug, although occupational therapists have claimed that CSEI possesses anti-tumor activities, no studies systematically reported these effects.

Amount of anticancer agents can successfully induce cancer cell apoptosis, which is recognized as an orderly cellular autonomous death mediated by caspases cascade and mitochondrial dysfunction (10,11). The over-generation of reactive oxygen species (ROS) can modulate mitochondrial depolarization (12), and lead to cellular oxidative stress-induced apoptosis through c-Jun-N-terminal kinase (JNK), P38 and extra cellular signal-regulating kinases (ERKs) signaling pathways (13–15). Oxidation-reduction system is destroyed in tumor cells, and high levels of oxidative stress cause the oxidative modification of amino acid residues thereby inducing DNA mutations and cell apoptosis, therefore, antioxidant agents could interfere with carcinogenesis (16). In addition, nuclear factor-erythroid 2 related factor 2 (Nrf2) and catalase (CAT) are two important factors in apoptosis resistance associated with ROS level (17, 18). Nrf2 plays a critical role in cellular redox homeostasis by attenuating oxidative-stress-associated pathological phenomena (19), and shows mutual inhibition relationship between ROS (20).

Collectively, we hypothesize that CSEI may display anti-cancer effects via oxidative stress related apoptotic pathway. To verify this hypothesis, in the present study, the anti-hepatocellular carcinoma activity of CSEI in HepG2 and SMMC-7721 cells is examined systematically, and ROS-related signalings are detected to further explore its underlying mechanisms.

2. Materials and methods

2.1. CSEI information

CSEI was supplied by JiLin AoDong Pharmaceutical Co., Ltd. in China, and is a marketable drug in China with the STATE MEDICAL PERMITMENT No. H22026121. CSEI is a small peptides enriched China, and is a marketable drug in China with the STATE MEDICAL.

2.2. Cell culture

Human HCC tumor cell lines HepG2 and SMMC-7721, and human embryonic kidney cell line HEK 293T obtained from Shanghai Cell Collection (Chinese Academy of Sciences, Beijing, China) were cultured in Dulbecco’s Modified Eagle Media (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 units/ml penicillin and 100 g/ml streptomycin under a humidified atmosphere containing 5%/95% CO2 air at 37 °C. Cell culture reagents were obtained from Gibco BRL (Grand Island, NY, USA).

2.3. MTT assay

HepG2, SMMC-7721 and HEK 293T were seeded into 96 well plates at density of 1 × 10^4 cells/well. After incubation with 0–1.5 mg/ml CSEI for 24 h, 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide (MTT; Sigma–Aldrich, USA) at a final concentration of 0.1 mg/ml was added to each well following an additional culture at 37 °C in darkness for 4 h. In the late incubation, culture medium was removed, and 100 µl of DMSO was added to dissolve formazan crystals within cells. The absorbance was measured using Synergy™4 Microplate Reader (BioTek Instruments, Winooski, VT) at a wavelength of 490 nm.

In another separate test, 5 mM of N-Acetyl-l-cysteine (NAC; a ROS inhibitor) was used to pretreat cells for 60 min. After co-incubation with CSEI for 24 h, cell viability was assessed.

2.4. Colony formation assays

HepG2 and SMMC-7721 cells were seeded into 6 well plate at density of 3 × 10^5 cells/well, and incubated with 0–1.5 mg/ml CSEI for continuous 7 days. Cells were incubated with 4% paraformaldehyde for 10 min and stained with 0.05% crystal violet for 60 min. The plate was washed three times with phosphate buffered saline (PBS), drained upside down on paper towels, and photographed. The experiment was repeated for six times.

2.5. Migration assay

HepG2 and SMMC-7721 cells were plated in 6 well plates, cultured to over 90% confluence, and then scraped with a syringe needle. After 24-h culture with CSEI (0, 0.35 and 0.70 mg/ml), the distances of migrating cells were used to evaluate the cell migratory ability. The width of the wound was expressed as a percentage of 0 mg/ml CSEI-treated cells.

2.6. Hoechst 33258 staining

Hoechst 33258, a blue fluorescent dye, could penetrate the cell membrane and specifically bind to DNA to show the form of chromatin; therefore, it is typically used to visualize the apoptotic nuclei (21). Cells were seeded at 3 × 10^5 cells/well in 6 well plates and incubated overnight. After treatment with CSEI for 24 h, cells were fixed with 4% paraformaldehyde for 10 min and then stained with Hoechst 33258 (1 µg/ml) in darkness for 15 min. The samples were examined via Nikon Eclipse TE 2000-S fluorescence microscope. The relative fluorescence intensity was analyzed by Image J software (National Institutes of Health, Bethesda, USA).

2.7. Assessment of cell apoptosis

HepG2 and SMMC-7721 cells were seeded into 6 well plates at 4 × 10^4 cells per well. After exposure to CSEI for 24 h, cells were washed with PBS and stained with propidium iodide (PI) and/or Annexin V at room temperature for 20 min. The intensity of fluorescence was analyzed utilizing Muse™ Cell Analyzer from Millipore (Billerica, MA) following manufacturer’s instructions.

2.8. Assessment of caspases activities

Cells treated with CSEI for 24 h were harvested using lysis buffer. After detecting the protein concentration by BCA Protein Assay Kit (Millipore, Billerica, MA), the activities of caspase 3, 8 and 9 were examined according to the protocols of assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China).

2.9. Assessment of intracellular ROS levels

2’-7’-Dichlorodihydrofluorescein diacetate (DCFH-DA, Sigma–Aldrich, USA) staining was used to objectively reflect the intracellular ROS level. Cells treated with CSEI for 24 h were suspended
and incubated with 10 μM of DCFH-DA at 37 °C for 15 min in darkness. After washing with PBS three times, the changes of intracellular ROS levels were examined via Nikon Eclipse TE 2000-S fluorescence microscope. Green fluorescence intensity is on behalf of intracellular ROS accumulation.

2.10. Assessment of mitochondrial membrane potential (MMP)

5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1; Calbiochem, USA) was used to detect MMP changes. In normal cells, JC-1 aggregates to form a polymer in the mitochondrial matrix, and the polymer emits intense red fluorescence. In unhealthy cells, JC-1 exists only in the form of a monomer in the cytoplasm, and it emits green fluorescence (22). HepG2 and SMMC-7721 cells were seeded into 6 well plates at 3 × 10⁵ cells per well and treated with CSEI for 24 h. After incubation with 2 μM of JC-1 at 37 °C for 15 min in darkness, the changes of fluorescent color in the mitochondria were analyzed by fluorescent microscopy via Nikon Eclipse TE 2000-S fluorescence microscope. The relative red and green fluorescence intensity was analyzed by Image J software (National Institutes of Health, Bethesda, USA).

2.11. HepG2-xenografted tumor model

5-week-old Male BALB/c nude mice (Chares River Experimental Animal Technical Co., Ltd., Beijing, China) were housed in groups of two in clear plastic cages and maintained on a 12 h light/dark cycle at 23 ± 1 °C with water and food available ad libitum. The experimental animal protocol was approved by the Animal Ethics Committee of Jilin University. A total number of 5 × 10⁶ cells/100 μl was subcutaneously implanted below the right back near hind leg. 3 days after the cell injection, palpable tumor size was measured with a caliper. Nude mice were randomly divided into two groups (n = 6 each). Mice were intraperitoneally

Fig. 1. CSEI induced cytotoxicity in hepatocellular carcinoma cells and showed little effect on HEK 293T cells. CSEI dose-dependently reduced cell viability in HepG2 and SMMC-7721 (A), but not in HEK 293T (B) after 24-h treatment (n = 6). (C) CSEI inhibited the migration ability of hepatocellular carcinoma cells determined via a wound healing assay (10X; Scale bar: 100 μm) (n = 6). Data are expressed as the means ± S.D. *P < 0.05, **P < 0.01 and ***P < 0.001 versus 0 mg/ml of CSEI-treated cells. (D) CSEI suppressed hepatocellular carcinoma cells proliferation detecting via crystal violet staining after 7-day treatment (n = 6).
injected with saline solution (0 mg/kg CSEI) and 1.75 mg/kg of CSEI every other day, and the body weight and tumor size were recorded at the same time. Tumor size was calculated using the equation length (mm) × (width (mm))^2 × 0.5. After 14-day treatment, the tumors were harvested and stored at −80 °C for western blot assay.

2.12. Western blot

Cells were planted into 6 well plates at 4 × 10^5 cells per well and treated with doses of CSEI for 24 h. Cells were harvested, washed with cold PBS twice and lysed with RIPA buffer (Sigma–Aldrich, USA) containing 1% protease inhibitor cocktail (Sigma–Aldrich, USA) and 2% PMSF (Phenylmethanesulfonyl fluoride) (Sigma–Aldrich, USA). Similarly, tumors were lysed in the same protocol mentioned above. The protein concentration was measured by BCA Protein Assay Kit (Millipore, Billerica, MA) for three times repeated. 40 μg of proteins were separated using a 10%–12% SDS-PAGE gel and transferred electrophoretically onto PVDF membranes. The transferred membranes were blocked in 5% bull serum albumin (BSA) for 4 h, and then blotted with the following primary antibodies at 4 °C overnight at dilution of 1: 1000: phosphor (P)-ERKs (ab47339), total (T)-ERKs (ab17942), P-JNK (ab192200), T-JNK (ab1798461), P-P38 (ab31828), T-P38 (ab31828), cleaved poly (ADP-ribose) polymerase (cleaved-PARP) (ab32064), poly (ADP-ribose) polymerase (PARP) (ab32138), Bcl-2 (ab32124), cleaved-caspase 3 (ab2302), cleaved-caspase 8

Fig. 2. CSEI induced apoptosis in hepatocellular carcinoma cells after 24-h treatment. (A) CSEI induced nucleus apoptotic morphologic alteration in HepG2 and SMMC-7721 cells examined by Hoechst 33258 staining (200X; Scale bar: 100 μm). Numerical data were demonstrated by the average optical density (n = 6). (B) CSEI enhanced hepatocellular carcinoma cell apoptosis rate analyzed via flow cytometry. Numerical data were demonstrated by the sum of early and late apoptosis rates (n = 6); (C) CSEI enhanced the activation of caspase 3, 8 and 9 in HepG2 and SMMC-7721 cells (n = 6). Data are expressed as the means ± S.D. *P < 0.05, **P < 0.01 and ***P < 0.001 versus 0 mg/ml of CSEI-treated cells.
(ab181580), cleaved-caspase 9 (ab2014), Nrf2 (ab62352), CAT (ab52477) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (ab8245) purchased from Abcam (Cambridge, MA), followed by treatment with horseradish peroxidase-conjugated secondary antibodies diluted 1:2000 (Santa Cruz, USA). Chemiluminescence was detected using ECL detection kits (Millipore, USA) and imaging system (Biospectrum 600). The intensity of the bands was quantified by scanning densitometry using software Image J (National Institutes of Health, Bethesda, USA).

2.13. Statistical analysis

All of the experiments were carried out in sextuplicate. All values were expressed as mean ± S.D. A one-way analysis of variance (ANOVA) was used to detect statistical significance followed by post-hoc multiple comparisons (Dunn's test) using SPSS 16.0 software (IBM corporation, Armonk, USA). The value of P < 0.05 was considered to be significant.

3. Results

3.1. CSEI causing cell damage in hepatocellular carcinoma cells

CSEI dose-dependently reduced the cell viability of HepG2 and SMMC-7721 cells (P < 0.001; Fig. 1A), but showed no significant effects on HEK 293T cells after 24-h treatment (P > 0.05; Fig. 1B). The 24-h IC50 of CSEI in HepG2 and SMMC-7721 were approximately 0.70 mg/ml and 0.66 mg/ml respectively. A wound healing assay was performed to observe the inhibitory effect of CSEI on the migration ability of hepatocellular carcinoma cells. Compared with non-treated cells, whose wound areas were almost healed, the migration ability was strongly inhibited by CSEI (P < 0.01; Fig. 1C).

Fig. 3. CSEI caused mitochondrial dysfunction in hepatocellular carcinoma cells after 24-h treatment. (A) CSEI induced the dissipation of MMP detected by JC-1 staining (20X; Scale bar: 100 μm). Numerical data were expressed as the ratio of red to green fluorescence intensity (n = 6). CSEI significantly enhanced the expressions of cleaved-caspase 3, 8, 9, and cleaved-PARP, and evidently reduced the levels of Bcl-2 in HepG2 (B) and SMMC-7721 (C) cells. Quantification data of protein expressions were normalized by corresponding GAPDH (n = 6). Data are expressed as the means ± S.D. *P < 0.05, **P < 0.01 and ***P < 0.001 versus 0 mg/ml of CSEI-treated cells.
The robust inhibitory on HepG2 and SMMC-7721 cell colony formation were apparent at 0.5 mg/ml, and from 1.0 to 1.5 mg/ml of CSEI, the clonogenic ability was completely blocked (Fig. 1D).

3.2. CSEI inducing apoptosis in hepatocellular carcinoma cells

Hoechst 33342 staining corroborated that CSEI at doses of 0.35 and 0.7 mg/ml strikingly induced nucleus apoptosis in hepatocellular carcinoma cells indicated by the enhanced blue fluorescence intensity ($P < 0.01$; Fig. 2A). Exposure to CSEI for 24 h significantly increased the early and late apoptosis rate in HepG2 and SMMC-7721 cells in a dose-dependent manner, and reached the maximum of 23.97% and 15.53% respectively ($P < 0.001$; Fig. 2B). The activation of caspase 3, 8 or 9 is considered as typical events in mitochondrial apoptosis (23). CSEI enhanced 14.1%, 26.2% and 9.1% activities of caspase 3, 8 and 9 in HepG2 cells, respectively ($P < 0.05$; Fig. 2C). In SMMC-7721 cells, 32.2%, 6.3% and 22.3% enhancement of caspase 3, 8, 9 activities were observed in CSEI-treated cells ($P < 0.01$; Fig. 2C).

3.3. CSEI causing apoptotic alteration on mitochondrial function

Mitochondrial function is one of the factors responsible for cell apoptosis. 24-h CSEI incubation significantly caused MMP loss in hepatocellular carcinoma cells indicated by the augmented green fluorescence (JC-1 monomers) and the reduction in red fluorescence (JC-1 aggregates) ($P < 0.01$; Fig. 3A). In CSEI-treated cells, the expressions of cleaved-caspase 3, 8, 9, which are active forms inducing cell apoptosis, as well as cleaved-PARP, were enhanced significantly; conversely, the expression of Bcl-2 was suppressed in HepG2 and SMMC-7721 cells respectively ($P < 0.05$; Fig. 3B,C).

3.4. ROS accumulation being responsible for CSEI-induced hepatocellular carcinoma cell apoptosis

CSEI caused significant intracellular ROS accumulation indicated by the enhanced green fluorescence in HepG2 and SMMC-7721 cells ($P < 0.05$; Fig. 4A). Nrf2 and CAT, two oxidative stress markers, which could reduce the production of ROS, were selected...
to characterize the effects of CSEI on oxidative status of hepatocellular carcinoma cells. CSEI significantly reduced the expressions of Nrf2 and CAT in hepatocellular carcinoma cells (Fig. 4B). Further data showed that the reduced cell viability caused by CSEI was strongly abolished via pre-treatment with 1 mM of NAC in HepG2 and SMMC-7721 cells (P < 0.01; Fig. 4C) for 60 min. Data indicated that CSEI-mediated hepatocellular carcinoma cell apoptosis may be associated with intracellular ROS accumulation.

3.5. CSEI regulating MAPKs activation in hepatocellular carcinoma cells

The activation of mitogen-activated protein kinases (MAPKs) is involved in cell proliferation, differentiation, inflammation and apoptosis (24). CSEI dose-dependently suppressed the phosphorylation of ERKs, and enhanced the activities of JNK and P38 in HepG2 and SMMC-7721 cells after 24-h treatment (P < 0.05; Fig. 5).

3.6. CSEI inhibiting HepG2-xenografted tumor growth in nude mice

14-day CSEI treatment at 1.75 mg/kg strikingly suppressed the HepG2-xenografted tumor growth in nude mice without influencing their body weights, suggesting that CSEI is an effective agent without aggressive side-effects (Fig. 6A, B and D). Compared with non-treated mice, 29.6% tumor size was decreased in CSEI-treated mice at the end of the treatment (P < 0.01; Fig. 6C). CSEI enhanced the expressions of cleaved caspase 3 and cleaved-PARP, conversely, suppressed the levels of Nrf2 and CAT (P < 0.05; Fig. 6E). Furthermore, in tumor tissues, CSEI resulted in a 30% reduction of P-ERKs level, and 30% and 90% enhancement of P-JNK and P-P38 expression indicating that MAPKs may play important roles during CSEI-mediated anti-hepatocellular carcinoma (P < 0.01; Fig. 6F).

4. Discussion

Recently, the incidence and mortality of cancer continue to rise, especially for the rapid development of hepatocellular carcinoma in Asia and Africa (25). Due to multi-target effects and low toxicity, the multi-component drug has become a research focus. CSEI, widely used as an adjuvant therapy agent, alleviates chemotherapy-induced adverse reactions, especially improves immune ability (26,27). In cyclophosphamide-induced immunosuppressed mice model, we have successfully confirmed the immunomodulatory activity of CSEI. Encouragingly, in our present study, the anti-hepatocellular carcinoma activities of CSEI in human HCC tumor cells and HepG2-xenografted tumor mouse model were confirmed, and the roles of ROS-dependent mitochondrial pathway during this effect were further explored.

CSEI enhanced the dissipation of MMP in hepatocellular carcinoma cells. Bcl-2, locating in outer mitochondrial membrane, regulates nucleic mitochondrial membrane permeability and was reduced by CSEI (28). Bcl-2 expression is known as a hallmark to detect whether the cell damage related with mitochondria function (29). Following with MMP reducing, mitochondrial permeability transition pores (PTP) are opened, which further leads to pro-apoptotic molecules release; consequently, caspase 3 and PARP are activated (30). As an apoptotic biomarker, cleaved-PARP expression was enhanced by CSEI, which may be regulated by caspase 3 (31). Mitochondria and caspases engage in a self-amplifying pathway of mutual activation (32). As reported, cleaved-caspase 8 induces cleavage and activation of Bid acting on the intrinsic pathway (33). Cytochrome c released from mitochondria activates caspase 9 via binding to apoptotic protease activating factor 1 (Apaf-1) (34,35). Through proteolytic cleavage, caspase 3 is activated by caspase 8 and caspase 9, and then it cleaves vital cellular proteins or other caspases causing dissection of DNA molecules and leading to apoptosis (36).

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On the other hand, it has demonstrated in amount of studies that oxidative stress induces apoptosis through DNA damage and mitochondrial dysfunction (38,39). ROS accumulation contributes to abnormal changes in mitochondrial membrane permeability (40). As a positive feedback loop between intracellular ROS and mitochondria, during mitochondria-dependent apoptosis, a large amount of ROS is released into cytoplasm (41). NAC, a common ROS inhibitor, strongly abolished CSEI-reduced cell viability, which confirmed the central role of ROS. NAC regulates CAT and Nrf2 levels (42). Therefore, the increased ROS levels and decreased Nrf2 and CAT expression in hepatic carcinoma cells may be responsible for CSEI-caused apoptosis.

Moreover, ROS accumulation contributes to MAPKs activation (44,45). ROS inhibits JNK phosphatase and inactivates other potential inhibitory factors to promote tumor cell apoptosis (46). ERKs and JNK regulate antioxidant response element (ARE) to influence antioxidases expressions. Nrf2 could be phosphorylated by P38 inducing strengthen binding with Kelch-like ECH-associated protein.
corresponding GAPDH and/or total protein expressions, respectively. Data are expressed as mean ± S.D. Growth curves of HepG2 xenografted tumors in nude mice. (A) Tumor volumes were measured every other day. (B) Mean (±S.D.) body weight of 0 and 1.75 mg/kg of CSEI-treated mice. (C and D) CSEI significantly enhanced the expressions of cleaved-caspase 3, cleaved-PARP, P-JNK and P-P38, and evidently reduced the expressions of Nrf2, CAT and P-ERKs in tumor tissues. Quantification data of protein expressions were normalized by corresponding GAPDH and/or total protein expressions, respectively. Data are expressed as mean ± S.D. (n = 6) and analyzed using a one-way ANOVA. *P < 0.05, **P < 0.01, and ***P < 0.001 versus 0 mg/ml of CSEI-treated mice.

**Conflict of interest**

The authors indicated no potential conflicts of interest.

**Ethical approval**

The experimental animal protocol was approved by the Animal Ethics Committee of Jilin University.

**Authors contribution**

DW and LT conceived and designed the experiments. DJ, WL, XZ, XW and MZ performed the experiments. CL, GC and YZ analyzed the data. DJ wrote the manuscript. LT and DW revised the paper.

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**Appendix A. Supplementary data**

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jphs.2016.08.006.

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