CPN-9 PROTECTS RABBIT NUCLEUS PULPOSUS CELLS FROM XANTHURENIC ACID-INDUCED CYTOTOXICITY BY ACTIVATING THE NRF2/ARE PATHWAY

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ABSTRACT

Xanthurenic acid as the metabolite of tryptophan can induce the cytotoxicity and damage of nucleus pulposus cells. In addition, it was reported that N-(4-(2-pyridyl)(1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide (CPN-9) played a pivotal role in oxidative stress-induced cell death by activating the Nrf2/ARE pathway. Therefore, it was of significance to investigate on mechanism of CPN-9 resisting xanthurenic acid-induced cytotoxicity. Cells used in this research were isolated from rabbit nucleus pulposus, analyzed by the growth curve and staining identifications. Hematoxylin eosin staining, toluidine blue, immunohistochemistry and immunofluorescent staining were performed to observe the cellular morphology. Furthermore, cell proliferative and apoptosis activities were detected by CCK-8 methods and AV-PI double-staining, respectively. Simultaneously, the expressions of Nrf2, ATF3, HO-1 and NQO1 were measured by western blot in this investigation. By analysis of growth curve plotting, cells isolated from nucleus pulposus growed in pattern of "S" in 1-5 generation. Staining observations revealed that nucleus pulposus cells were not only triangular, polygonal and short spindle shaped , but also cell nucleus was relatively large. The CCK-8 research demonstrated that xanthurenic acid had adverse effects on cell proliferative activity. Simultaneously, CPN-9(≤ 40 μM) resisted the impairments induced by xanthurenic acid and exerted protective effect on proliferative activity, consistent with the apoptosis results. Furtherly, the expressions of Nrf2, ATF3, HO-1 and NQO1 were elevated by injection of CPN-9. CPN-9 protects rabbit nucleus pulposus cells against xanthurenic acid-induced cytotoxicity by activating the Nrf2/ARE pathway.

***Keywords**: Rabbit nucleus pulposus cells, Xanthurenic acid, Cytotoxicity, CPN-9, Nrf2/ARE pathway.*

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Introduction

Intervertebral disc degeneration was known as the histopathological and physiological basis of spine degencrative disease, which resulted into lumbar disc herniation, spinal canal stenosis and lumbar spondylolisthesis[1]. In recent years, some investigators reported that 53%-73 % of intervertebral disc cells appeared to be apoptotic in aged and degenerated intervertebral disc tissues. Basically, intervertebral disc cells include nucleus pulposus cells (NPCs), annulus fibrosus cells and chondrocyte of the endplate, in which NPCs were considered to be the unique cells of intervertebral disc tissue that bear a striking effect on intervertebral disc degeneration[2-3].

Tryptophan which is one of essential amino acids provided from the diets, is the precursor of kynurenine, and it also could convert to niacin, CO2 and xanthurenic acid (XA)[4-5]. Some studies demonstrated that tryptophan played a pivotal role in process of immune response for infection, inflammation, immunoreaction and pregnancy reaction[6-7]. However, XA as the metabolite and the endogenous micromolecule produced from tryptophan possessed dreadful effects on regulating the immune function. Unceasing researches documented the association between XA and diabetes, in

which the metabolic disorders of tryptophan led to the increase of hydroxy kynurenine and XA[8-9]. It was demonstrated that the complexus of XA combining with insulin reduced biological effect and resulted into diabetes. Similarly, it was well known that the neuroregulation was controlled by the protein in disease model, furthermore once XA was covalently conjugated with the protein in cells, the cells would be damaged and resulted into death. Plus, the investigations documented Caspase-3 in primary cells activated by XA simulated the protein to bind with protein, leading to cell apoptosis and the occurance of disease. However details on relationship between XA and NPCs were not reported ever before.

The biological activities expressed through the thiazolyl derivatives of natural or complex compound were extensively justified. The results revealed that the inhibition of tumor cells proliferation (IC50) can reach the level of micromole remedied by the antineoplastic drug containing thiazolyl[10]. Thiazolyl derivatives received great attention from the domestic and overseas researchers, which possessed remarkable effects on anticancer, antibiosis, antiviral and anti-inflammatory. Recently, the predecessors focused on the novel compound based on acidylated 2-aminothiazole introduced into the potential active radicals in order to obtain the antineoplastic drugs.

In 2012, Takuya[1] firstly employed a novel ligand-based virtual screening system, identifying a novel small molecule CPN-9. This study mainly demonstrated that CPN-9 selectively resisted the oxidative stress-induced cell death through the Nrf2/ARE pathway. In addition, the documents referred that CPN-9 upregulated the expression of Nrf2 and the relevant regulatory factor including heme oxygenase-1(HO-1), NAD(P)H quinone oxidoreductase 1 (NQO1) and glutamate-cysteine ligase modifier subunit (GCLM), indicating that it was a novel Nrf2 activator.

In this study, we explored the protective functions of CPN-9 in alleviating the injuries from XA-induced cytotoxicity. Simultaneously, we evaluated the protective function of CPN-9 via the Nrf2/ARE pathway, therefore to provide a promising new therapeutic agent for intervertebral disc degenerative diseases.

Materials and methods

Animals

A total of four adult New Zealand white rabbits (female or male, 8 weeks old) were provided by Qingdao biotech Ltd.. The animals were maintained in a room under the specific pathogen free (SPF) conditions with controlled room temperature (22±1°C), a cycle of 12 h light/dark, free access to food and water. All animal procedures in this study were performed according to the regulation of the Ethics Committee for Animal Experimentation.

Isolation and primary culture of NPCs

Adult New Zealand white rabbits aged 8 weeks were injected intraperitoneally with chloral hydrate (Beyotime Institute of Biotechnology, China), and then executed through injection of 20 mL air into ear vein. Median incision was preformed along the spinous process, exposing the hypomere of thoracic vertebra and the whole lumbar vertebra. Then the whole lumbar vertebra were bluntly dissected, transferred to sterile operating platform, washed by stroke-physiological saline solution and immersed into PBS containing 1% penicillin-streptomycin (Beyotime Institute of Biotechnology, China). Nucleus pulposus tissues were obtained by sterilized curet and again infiltrated into PBS containing 1% penicillin-streptomycin. The obtained nucleus pulposus tissues were cut into pieces (1 mm×1 mm×1 mm) and digested with trypsin-EDTA at 37 °C for 20 min. The digestion were terminated by 10% FBS DMEM/F-12 culture (Beijing solarbio Ltd., China) and the mixtures were centrifuged at 1000 rpm for 5 min. The supernatant-free mixtures were digested by collagenase II (MP Biomedical, USA) at 37 °C for 60 min and added into the 10% FBS DMEM/F-12 culture to terminate the digestion reaction. After filtration with 200-mesh and centrifugation, the centrifugates without the supernatant were mixed with DMEM/F-12 culture containing with 10 % FBS and 1% penicillin-streptomycin. The obtained cells were adjusted to $3\sim5\times10^5$/mL and placed into 37 °C incubator containing 5% CO_2. The inverted microscope was used to observe the morphology of NPCs.

Passage cultivation

The NPCs were converged to 90% and washed 2 times with the preheated PBS. Then the mixtures without PBS were digested for 30~40s with 1 mL

trypsin-EDTA and observed by the microscope. Once the pseudopods extended by NPCs were in absence and the cells were shrinked into circles. DMEM/F-12 mediums containing 10% FBS were added in order to terminating the digestion. The cell suspensions were centrifuged, the centrifugates were mixed with DMEM/F-12 medium containing with 10% FBS and 1% penicillin-streptomycin with a cell intensity of 3~5×10^5/mL. Then cells were placed into 37 °C incubator containing 5% CO2. NPCs in second generation were cultured for 24 h to observe the adherence and cellular morphology through light microscope. When the NPCs reached approximately 80 % confluence and were used for the followed operation.

Growth curve plotting

To plot the growth curve, 10^5/mL 1-5 passage cells digested by 0.25 % trypsin and mixed with DMEM/F-12 medium were placed in 24 well culture plates for 7 days,three wells for each passage cells. And cells of each well were trypsinized daily and then the cells were lifted and counted. The growth curve was then plotted using the cell counting data.

Staining identification of NPCs

Hematoxylin eosin (HE) staining

NPCs were fixed in 4% paraformaldehyde for 24 h, washed with PBS for three times, stained with hematoxylin for 15 min, differentiated by 1% hydrochloride-ethanol and stained with eosin for another 15 min. The endochylema was differentiated to pink color and the H&E staining results were observed under the inverted microscope.

Toluidine blue staining

NPCs were fixed with 4% paraformaldehyde for 20 min, washed with PBS for three times, perfused with toluidine blue for 15-20 min, washed twice by PBS for 15-20 min. The results of toluidine blue staining were observed and recorded by the inverted microscope.

Immunohistochemistry staining

NPCs were fixed with 4% paraformaldehyde for 20 min, washed 3 times with PBS, perfused in 0.1 % Triton X-100 for 15 min, washed again with PBS followed by incubating with 3% H_2O_2 in ambient temperature for 5 min. After washing 3 times with PBS, NPCs were incubated with Collagen II antibody(1:200, Sigma) overnight at 4°C. Then, the mixtures were incubated with PV-6002 goat anti mouse IgG/HRP polyclonal antibody(1:200, Sigma) in 37°C for 20 min. Finally, the solutions washed by PBS were injected with DAB to observe the color. Finally, the results were observed under the inverted microscope.

Immunofluorescent staining

The cell climbing coverslips were fixed with 4 % formaldehyde for 20 min, washed with PBS and perfused in 0.1 % Triton X-100 for 15 min. After washing 3 times with PBS, cells were incubated with DSA for 30 min and then incubated with Collagen II antibody (1:200) overnight. After washing with PBS, cells were incubated with fluorescein isothiocyanate-labeled mouse anti-rabbit antibody in 37 °C for 30 min and washed thrice with PBS. Then cells were counterstained with DAPI in dark for 30 min before cells were sealed with fluorescence quenching mounting medium. The results were observed and recorded by fluorescence microscope (Olympus, Japan).

Experimental groups

Four groups in this study were designed to analyze the effects of CPN-9 on XA-induced cytotoxicity, including negative control group (CN, only treated with the DMEM/F-12 medium), XA group (received the DMEM/F-12 medium with 50 mM XA), CPN-9 group (treated with the DMEM/F-12 medium with 40 μM CPN-9) and XA+CPN-9 group (incubated with DMEM/F-12 medium containing 50 mM XA and 40 μM CPN-9). Cells in each group were intervened for 6 h, while XA+CPN-9 group was received 50 mM XA before intervention. In addition, seven groups in this study were used to analyze the protective mechanism of CPN-9, including CN group, XA group (50 mM XA), XA+CPN-9 group (50 mM XA and 40 μM CPN-9), XA+SF group (50 mM XA and 5 μM sulforaphane), XA+ATRA group (50 mM XA and 10μM all-trans retinoic acid), XA+CPN-9+SF group (50 mM XA, 40 μM CPN-9 and 5 μM SF), XA+CPN-9+ATRA group (50 mM XA,40 μM CPN-9 and 10 μM ATRA). Cells in each group were intervened for 4 h.

Proliferative activity assay

NPCs were incubated with CPN-9 in different concentration of 0, 10, 20, 30, 40, 50 and 60 μM to determine the optimum concentration. 100 μL NPCs (1.0×10^5/mL) in second generation were

inoculated into 96-well plate, 6 holes of each concentration and incubated in incubator containing with 5% CO2 at 37 °C until the cells reached up to approximately 80%. Then CPN-9 solution with corresponding concentration was added into 96-well plate and incubated for another 6 h. Then, 10 μL CCK-8 solution was added in dark for incubation of 2 h. Finally, the OD values were measured at 450 nm through Microplate Reader (Spectra Max M2, Molecular Devices, USA). The NPCs in control was given mediumwithout FBS only.

Apoptosis assay

5×10^5 NPCs obtained from the third generation were washed three times with PBS, incubated with DMEM/F-12 medium without FBS for 12 h followed by incubating with the respective medium (XA, CPN-9 and mixed solution of XA and CPN-9) for 6 h, respectively. Then, the cells were trypsinized, centrifuged and stained with Annexin V FITC and propidium iodide (PI). Finally, cells were quantified with flow cytometry using a BD Accuri C6 Flow Cytometer (BD Biosciences, USA).

Western blot

The total proteins were obtained by the BCA Protein Assay Reagent Kit (Pierce, USA). Protein samples (30 μg) were separated on SDS-PAGE gel electrophoresis and transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories, Hercules, CA, USA). Membranes were blocked with 5 % skim milk for 1 h and incubated overnight at 4 °C with anti-Nrf2 antibody (1:1000; Abcam), anti-ATF3 antibody(1:500; Abcam), anti-HO-1 antibody(1:1000; Abcam), anti-NQO-1 antibody(1:500; Abcam). The membrane was washed with TBST (TBS and 1 mL/L Tween 20) three times and incubated for 1 h at room temperature with HRP-conjugated secondary antibody goat anti rabbit IgG (1:2000, Abcam). The membranes were washed as described above. The film was developed in the enhanced chemiluminescence (ECL) in dark room. The expression of protein samples was then scanned and quantified by the Image J software. β-tubulin was used as an endogenous control.

Statistical analysis

Data are presented as the mean ± SD. Statistical differences were examined by one-way ANOVA followed by Dunnett's method for multiple comparisons between groups (SPSS 19.0). p values < 0.05 was considered to be statistically significant.

Results

Isolation, culture and characterization of NPCs

As shown in Fig. 1A, we could observe intuitively the shape of lumbar vertebra obtained from rabbit. Primary NPCs were obtained through digestion with trypsin-EDTA and inoculated in culture dish. The morphology of NPCs was observed under the light microscope at different points of day 0, 8, 14 and 21 (Fig. 1B-E). As shown in Fig. 1B, we observed that the cells are sphere-shaped, different in size and suspended in culture medium at the beginning of inoculation. On the day 8, the photos revealed that cells with a great deal of interconnected pseudopods absolutely attached on microtextured surface (Fig. 1C). On the day 14, substrate precipitation was visible around the cells which most of them were converged (Fig. 1D). Futhermore, the cells reached confluence of more than 85% on day 21 (Fig. 1E).

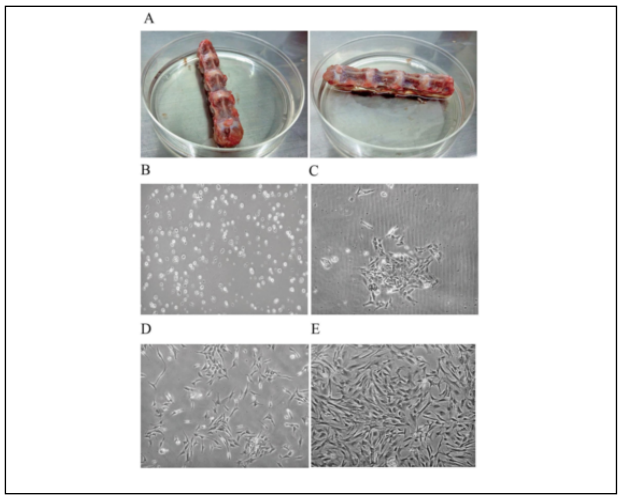

Fig. 1: Schematics of morphology of normal NPCs. (A) The photo of rabbit lumbar vertebra isolated in aseptic condition. (B-E) Primary NPCs after inoculation at day 0, 8, 14 and 21 under light microscopy (magnification 40×).

Growth curve of 1 to 5 generation of NPCs

In Fig. 2, we observed the growth curve of NPCs exhibiting a typical "S" shape in different generation of NPCs over time. The growth profile revealed that cells showed a slow-growing tendency on day 1 and 2, rapid proliferation at day 3 to 6 , while the growth of cells entered into lag phase from day 7.

Simultaneously, the results also revealed that the proliferation activity of 1, 2 and 3 generation of NPCs was much better than that of 4 and 5 generation cells. Generation 4 NPCs regressed and entered in transitory stage. And proliferation activity of generation 5 NPCs was significantly declined and the cellular morphology was changed from polygon-shaped to fusiform-shaped.

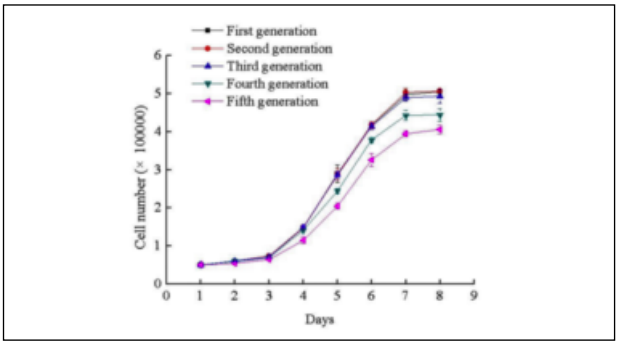

Fig. 2: Growth curve of rabbit different generation NPCs. The cell density of NPCs inoculated was 3~5×10^5/mL. The NPCs were digested by 1 mL trypsin-EDTA daily and counted. Data were mean ± SD.

Staining observation of NPCs

Morphology of NPCs was observed by HE staining, toluidine blue, immunohistochemistry and immunofluorescent staining. Toluidine blue staining result indicated that the aggregated protein polysaccharide were blue, in which the colors of the area closed to the cell nucleus become darker (Fig. 3A).

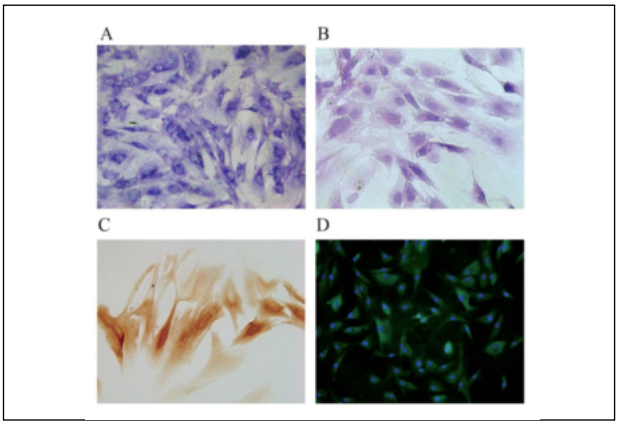

Fig. 3: Morphology observation of NPCs. (A) Toluidine blue staining (200×). (B) HE staining (200×). (C) Immunohistochemistry staining (200×). (D) Immunofluorescent staining (200×).

As seen from Fig. 3B, HE staining showed that NPCs were polygon and spindle shaped, in which karyons located in center or amesiality of cell wall were purple and cytoplasm was light purple (Fig. 3B). CollagenII were stained and observed by immunohistochemistry and immunofluorescent staining, exhibiting yellowish-brown color (Fig. 3C) and green-fluorescent visualized (Fig. 3D), respectively. All the pictures listed demonstrated that the color got darker nearby the cell nucleus than cytoplasm. And the morphology of NPCs was polygon and spindle, consistent with the photos taken under light microscopy.

Effect of CPN-9 on proliferative activity

CPN-9 medium solution in different concentration (0, 10, 20, 30, 40, 50 and 60 μM) were prepared to analyze the effect of CPN-9 on proliferative activity (Fig. 4). The CCK-8 result demonstrated that the proliferative activities of NPCs in different concentrations were not significantly changed with the concentration changed from 0 to 40 μM (100.92, 100.43, 100.97, 92.09, 89.81, respectively). While the concentration of CPN-9 was equal or higher than 50 μM, proliferative activity of NPCs was significantly suppressed ($P < 0.05$ or $P < 0.001$). From the experimental result, we chose the 40 μM as the favorable concentration.

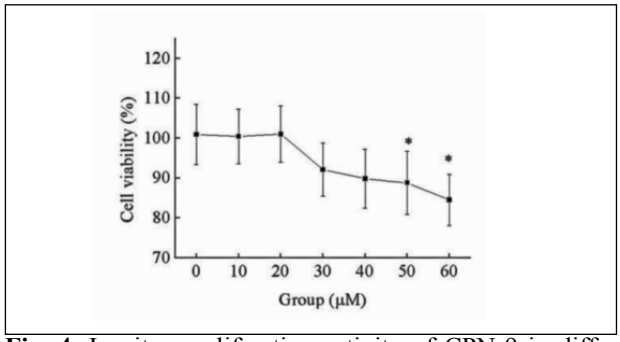

Fig. 4: In vitro proliferative activity of CPN-9 in different concentration of 0, 10, 20, 30, 40, 50 and 60 μM against NPCs following 6 incubation. The proliferation activity was measured by CCK-8 aasay. Data are mean ± SD (n=6). *P<0.05 vs. 0 μM of CPN-9.

CPN-9 protected the NPCs against XA-induced cytotoxicity

As shown in Fig. 5, the levels of proliferative activity in XA group was significantly lower than CN group (69.98% vs. 100%), indicating that XA significantly suppressed the proliferative activity ($P < 0.001$). In CPN-9 group, the viability (85.43%) of NPCs was not remarkably changed as compared to CN group ($P < 0.001$), However, after pre-treatment of 50 nm XA, the administration of CPN-9 could ameliorate cytotoxicity caused by XA with a significant difference ($P < 0.001$).

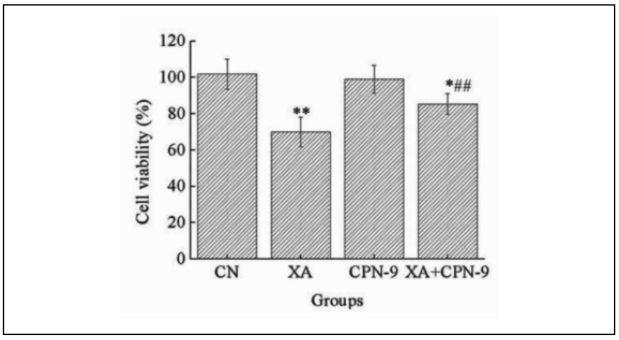

Fig. 5: CPN-9 alleviated proliferation activity of NPCs against XA-induced cytotoxicity detected by CCK-8 aasay. The concentrations of XA and CPN-9 were 50 mM and 40 μM. Data are mean ± SD (n=6). *P < 0.05 indicates statistically significant difference from control group; #P < 0.05 and ##P < 0.01 indicate statistically significant difference from XA group.

CPN-9 prevented cell apoptosis caused by XA

The rate of cell apoptosis was demonstrated by flow cytometry in Fig. 6. The results suggested that there was no significance in CPN-9 group (10.15%) and control group (9.31%, P > 0.05), XA-treated NPCs exhibited a higher rate of apoptosis (17.7%) in comparision with that in CN group, while the CPN-9 administration in XA+CPN-9 group could dramaticlly improve the cell apoptosis induced by XA (11.6% vs. 17.7%, P < 0.05), suggeating its' protective effect against XA-induced cytotoxicity.

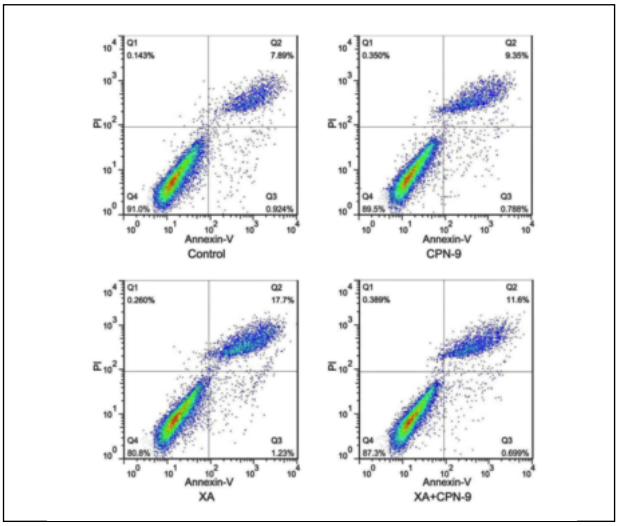

Fig. 6: Effects of CPN-9 on XA-induced apoptosis activity detected by flow cytometry with Annexin V-FITC/PI staining following incubation of 6 h. The concentrations of XA and CPN-9 were 50 mM and 40 μM. Data are mean ± SD. The experiments were performed in triplicate. *P < 0.05, **P < 0.01 vs. NC group; #P < 0.05, ##P < 0.01 vs. XA+CPN-9 group.

CPN-9-mediated cytoprotection regulated by Nrf2/ARE pathway

As shown in Fig. 7A, the expressions of Nrf2, ATF3, HO-1 and NQO1 were time-dependent elevation with the treatment of CPN-9 at 0, 2 and 4 h. At 4 h, the levels of the above proteins was highest expression content. After 4 h, the above proteins expression decreased. Therefore, we considered 4 h as the optimum treatment time of CPN-9. In addition, we also explored the protective mechanism of CPN-9 measured through western blot. From the Fig. 7B, we found that after induction of XA, addition of SF improved the cell viability NPCs in XA+SF group, while ATRA led to the lower cell viability than XA group. Similar results could be found in the XA+CPN-9, XA+CPN-9+SF and XA+CPN-9+ATRA. In addition, western blot results suggested that SF could increase the level of Nrf2, ATF3, HO-1 and NQO1, instead, ATRA down-regulated the expression of these proteins.

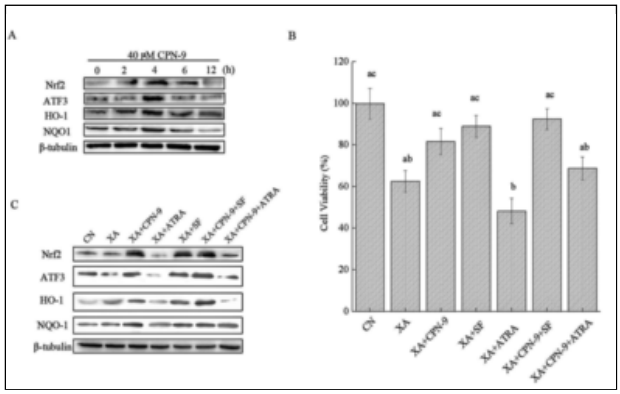

Fig. 7: Effects of CPN-9 on XA-induced cytotoxicity activated by Nrf2/ARE pathway. (**A**) Expression of Nrf2, AFT3, HO-1 and NQO1 protein in NPCs treated with 40 μM at different time point (0, 2, 4, 6 and 12 h) measured by western blot. (**B**) Cell viability treated with different administration. The concentrations of XA, CPN-9, SF and ATRA were 50 mM, 40 μM, 5 μM and 10 μM. (**C**) Expression of Nrf2, AFT3, HO-1 and NQO1 protein in NPCs in seven groups. β-tubulin was used as the endogenous control. Data are mean ± SD. a,b,c Values not sharing a superscript letter are significantly different, P < 0.05.

Discussions

Essentially, NPCs similar to chondrocyte can produce plentiful proteoglycan and collagenII, enabling to maintain the biological and mechanical characteristics of intervertebral disc[12]. Therefore, intervertebral disc degeneration is greatly associated with deficiency of NPCs, suggesting that the structure and viability of NPCs are essential factors for protecting intervertebral disc, whose identification is of great significance.

Biochemistry changes of NPCs include cell apoptosis, degradation of extracellular matrix and decrement of protein polysaccharide and collagen II[13]. Abundant collagenII and proteoglycan exist in extracellular matrix of normal NPCs. In our study, HE staining, toluidine blue, immunohistochemistry and immunofluorescent staining were performed based on the special binding of macromolecule glycoprotein and core protein to observe the cellular morphology. The results demonstrated that NPCs were polygon and spindle.

XA as a close analog of kynurenic acid involving kynurenine pathway is generally considered as the ultima compound of a dead-end branch with no functional role. However, the predecessors previously revealed that the application of XA in schizophrenia model provided its role for the mechanism of psychotic behavior, possibly through the interaction with metabotropic glutamate receptor(s)[14]. In this investigation, we examined the effects of XA-induced cytotoxicity on NPCs, demonstrating that XA suppressed the proliferative activity and promoted apoptosis of NPCs. Consistent with several researchers, it was documented that the involvement of kynurenine pathway metabolites containing XA resulted into auto-immune diseases[8]. Furthermore, Caspase-3 in primary cells activated by XA simulated the combination between proteins, leading to cell apoptosis and the occurance of diasease[15]. Plus, the Haruki revealed that the reduction of serotonin, dopamine and tetrahydrobiopterin was appeared by upregulating XA, indicating that XA release exerted unfavorable effects on the pathophysiological processes concerning kynurenine pathway metabolites[16]. In concise, we provided that XA can damage the NPCs, resulting into the intervertebral disc degeneration.

Based on the immune function of XA which was characterized as the metabolite and the endogenous micromolecule produced from tryptophan, we explored the protective function of CPN-9 on cytotoxicity induced by XA. Appropriate concentration was indispensable for CPN-9 (40 μM) for disease therapy. Our results illustrated that CPN-9 protected the NPCs against XA-induced cytotoxicity including proliferative activity and cell apoptosis. It was firstly demonstrated by Takuya that both in vitro and in vivo CPN-9 as a perfect inducer of antioxidant and detoxifying enzymes selectively suppressed oxidative stress-induced cell death[8]. Similarly, the cell-type-independent manner of intervertebral disc degeneration including lower proliferative activity and cell apoptosis induced by XA can be prevented by CPN-9 in our investigation.

It is well known that cell injuries and death were associated with changes of oxidative stress. Kelch-like ECH-associated protein-1(Keap1)-NF-E2-antioxidant response element (Keap 1-Nrf2-ARE) was defined as the transduction pathway to defense oxidative stress[17]. Previously, the investigators have pointed that CPN-9 was a novel Nrf2 activator, suggesting that administration of CPN-9 against XA-induced cytotoxicity was an effective therapeutic method to prevent cell apoptosis. In addition, several researches have documented that oxidative stress modified cysteine residues of Keap1 led to Nrf2 release and its nuclear translocation, resulting into the upregulation of ARE-regulated cytoprotective genes[18].

In our research, we evaluated and revealed the effect of CPN-9 on XA-induced cytotoxicity by the Nrf2-ARE mediated pathway, indicating that CPN-9 upregulated the expression of Nrf2, ATF3, HO-1 and NQO1. In normal physiological organism Nrf2 is distributed in cytoplasm and binded with Keap1. In this investigation, we found that CPN-9 activated Nrf2, resulting into the isolation of Keap1-Nrf2 and nucleus transfer. Nucleus transfer promoted Nrf2 to bind with ARE and induced the transcription of relevant genes including HO-1 and NQO1, thus preventing the inflammation. In addition, Nrf2 as a kind of transcription factor of leucine zipper plays a pivotal role in oxidative stress and inflammation[15]. Plus, HO-1 is an exceeding anti-inflammatory enzyme mediated by Nfr2 in Keap 1-Nfr2-ARE pathway, in which HO-1 can prevent the oxidative stress induced by XA. Therefore the expression of the relevant stress regulatory factors including Nrf2, ATF3, HO-1 and NQO1 was enhanced by injection of CPN-9. The protective effect of CPN-9 through regulation of Nrf2/ARE pathway was furtherly proved by introduction of the activator SF and the inhibitor ATRA into CPN-9 protective system on XA-induced cytotoxicity.

Taken together, the administration of XA in rabbit nucleus pulposus supports a role for this compound in the mechanism of cytotoxic behavior, possibly through the interaction with protein. Notably, CPN-9 alleviated the impairments of NPCs induced by XA, indicating that the regulation of Nrf2/ARE pathway might be a promising therapeutic target for curing intervertebral disc degeneration.

Findings from another research suggested that the activating the Nrf2-ARE signaling pathway is known to protect against cell death induced by oxidative stress and oxidative stress was implicated in degenerative diseases except for intervertebral disc degeneration[19], thus application of CPN-9 could also be a therapeutic agent for intervertebral disc degeneration.

Conclusions

In conclusion, NPCs were well isolated, obtained and identified, providing a favorable basis for this research in our investigation. Furtherly, we demonstrated that CPN-9 protected against XA-induced cytotoxicity via activation of the Nrf2/ARE pathway, indicating that CPN-9 exerted the fascinating effects on protective functions for intervertebral disc. Simultaneously, our data revealed that activating of the Nrf2/ARE pathway might provide a potential drug target for curing intervertebral disc degeneration. Therefore, our investigation provided proof-of-concept support for unrevaling the efficacy of CPN-9, providing a promising new therapeutic agent for intervertebral disc degenerative diseases.

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Abbreviation
CPN-9: N-(4-(2-pyridyl)(1,3-thiazol-2-yl))-2-(2,4,6-trimethylphenoxy) acetamide; GCLM: glutamate-cysteine ligase modifier subunit; HO-1: heme oxygenase-1; IC50: inhibition of tumor cells proliferation; Keap 1-Nrf2-ARE: Kelch-like ECH- associated protein-1(Keap1)-NF-E2-antioxidant response element; NPCs: nucleus pulposus cells; NQO1: NAD(P)H quinone oxidoreductase 1; XA: xanthurenic acid

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